Pristine aquatic habitats – a relic of the past?

Habitat analysis of the freshwater pearl mussel *Margaritifera margaritifera* (L.) and its community

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von
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aus
Friedberg

im Jahr
2024

Das Promotionsgesuch wurde eingereicht am

05.11.2024

Die Arbeit wurde angeleitet von

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Unterschrift

Summary

Pristine aquatic habitats are increasingly at risk of becoming relics of the past, as anthropogenic impacts continuously degrade freshwater ecosystems. While comprehensive nature conservation efforts have helped to improve the condition of many bodies of water, they have not yet succeeded in sufficiently protecting the particularly sensitive species. This is exemplified by the constitution of the freshwater pearl mussel *Margaritifera margaritifera*, which serves as an indicator species of oligotrophic freshwater habitats and is currently one of the most endangered species. While the direct impacts of pollution and overexploitation are pervasive in the discourse of pearl mussel protection, a significant aspect of habitat quality is the availability of suitable food resources and the biotic composition of its habitats. This thesis advances beyond the conventional approaches to species conservation by examining the habitat of *M. margaritifera* and its ecological community through a comprehensive, multifaceted approach.

After providing a **general introduction** to the scope of this thesis, the **first chapter** introduces the freshwater pearl mussel *M. margaritifera* and presents an overview of its conservation status and threats, its biology and ecology, and the current state of knowledge about its habitat and diet.

By analysing historical maps from 1850 onwards, the **second chapter** analyses the alterations in land utilisation over the past 170 years and their potential consequences for the habitats of the freshwater pearl mussel. It becomes evident that landscape alterations, including the straightening of watercourses, the reduction of wetlands, and the loss of small-scale land use, have been prevalent across the study area, including remote regions where biodiversity conservation efforts are underway. These changes illustrate the extensive impact of land-use practices on freshwater ecosystems.

Further investigation of the indicator species *Myriophyllum alterniflorum* reveals in the **third chapter** that the interaction between sediment composition, flow velocity, shading, and water quality, plays a critical role in the species' survival. Multiple stressors acting simultaneously exacerbate threats to its existence.

The habitat of *M. margaritifera* and its associated community is examined in the **fourth chapter** using environmental DNA (eDNA) and joint species distribution models (jSDM). This demonstrates that species assemblages either positively or negatively associate with the

freshwater pearl mussel. Additionally, the extent of agricultural land in the catchment has a negative influence on the pearl mussel-associated community. However, biotic interactions have a greater impact on community composition than environmental or spatial variables.

The **fifth chapter** examines the impact of traditional meadow irrigation practices on the habitat of *M. margaritifera*. It finds that meadow irrigation not only correlates with the mussel's historical distribution but also enhances water and sediment oxygenation, as well as calcium and organic content of detritus, which in turn facilitates enhanced juvenile mussel growth and survival.

Finally, a **general discussion** is held on the significance of these results for applied nature conservation. Collectively, they emphasise the intricate nature of conserving pristine aquatic habitats, underscoring the necessity for comprehensive conservation strategies. Environmental metabarcoding can be a part of such strategies, as it represents a promising avenue for advancing ecological understanding, provided that its inherent limitations in practical applications are carefully considered.

Acknowledgements

The successful completion of this thesis was supported by invaluable contributions of numerous individuals. I extend my sincerest gratitude to each of them for their support and assistance.

I would like to express my gratitude to Peter Poschlod, who initiated the project and provided me with a framework within which I was able to work freely. This encompasses the colleagues in his department, who were instrumental in providing assistance, counsel, and a productive exchange that propelled the process forward. First and foremost among them is Christoph Reisch who was consistently available to address organisational and strategic challenges. The work on the historical maps was significantly facilitated by Sabine Fischer, who spearheaded the digitisation with remarkable dedication. A big thank you also goes to Max Pichler, whose method of data analysis enabled the results from the eDNA experiment to be obtained. The analysis of the soil samples and the laboratory infrastructure were overseen by Irina Weinberger, while Joachim Rewitzer helped with the detritus analyses.

I would also like to thank Wolfgang Degelmann (BN Hof) for providing the Huschermühle as a base for fieldwork and a centre for meetings and exchanges. Many thanks also to his employees Daniel Höllering and Michal Blahar who were responsible for the meadow watering and for pearl mussel bioindication. The network of the Huschermühle also connected me with Ondrej Spisar, who greatly facilitated the selection of the sampling sites and the sampling itself. He also established the contact to other specialists who oversee the sampled freshwater pearl mussel populations. These include Jonas Ständer (Naturpark Fichtelgebirge), Marco Denic (LPV Passau), Bohumil Dort and Sarah Höfler (Büro Blattfisch).

Measurements of open water and interstitial, as well as analyses of sediment input within the meadow irrigation experiment were conducted with the dedicated assistance of Rebecca Höß (TUM). Many thanks for this!

Last but not least, my family and friends have supported me through good and bad times and have been a source of support and motivation. My parents always encouraged me to go my own way and supported me on every path. I often drew new energy and creativity from my relationship with my husband Martin. My son Anton was always a great distraction, but also ensured that I (almost) never lost my good humour.

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Abbreviations

AAS	*atomic absorbtion spectrometer*
AI	*artificial intelligence*
anova	*analysis of variance*
COI	*cytochrome c oxidase 1*
CAL	*calcium-acetate-lactate buffer solution*
eDNA	*environmental DNA*
EGB	*european green belt*
EIV	Ellenberg indicator values
FFH	*fauna flora habitats directive*
FPM	*freshwater pearl mussel*
GPU	*graphical processing unit*
IM	*irrigation meadow*
ITS2-primer	*primer pair for detection of vascular plants*
JSDM	*Joined Species Distribution Model*
LOI	*loss in ignition*
MZB	*macrozoobenthos*
MZB-I	*MZB primer pair mINTCOI1F nad HCO2198 by Leray at al. (2013)*
MZB-II	*MZB primer pair fwhF2 (Vamos et al., 2017) and EPTDr2n (Leese et al., 2021)*
NBS	*nature based solutions*
NGS	*next generation sequencing*
NH_3	*ammonia*
NH_4^+	*ammonium*
NMDS	nonmetric multidimensional scaling
OTU	*operational taxonomic unit*
POM	*particulate organic matter*
PUFA	*polyunsaturated fatty acids*
RFU	*Relative Fluorescence Unit*
RL	*red list of endangered species*
sjSDM	scalable joined Species Distribution Model
WFD	*European water framework directive*

Declaration of manuscripts

Manuscripts included in this thesis were submitted with the thesis author as lead author:

Chapter three:

Sattler, J., Poschlod, P., 2023. Habitat requirements of *Myriophyllum alterniflorum* DC. in river stands of the Upper Palatinate Forest, Bavaria. Aquatic Botany 188, 103680.

Chapter five first part:

Sattler, J., Fischer, S., Poschlod, P., 2023a. Meadows and mussels – The relationship between historical irrigation systems and the habitat of the freshwater pearl mussel *Margaritifera margaritifera* (L.). Limnologica 98, 125994.

General introduction

‘The last 50 years have without doubt seen the most rapid transformation of the human relationship with the natural world in the history of humankind” (Steffen et al., 2004).

Throughout history, humanity has consistently been dependent on nature, and thus has been subject to its intrinsic forces. This has always been the basis of the relationship between humanity and nature. In awareness of the question whether both can be separated from another or not (e.g. Bourdeau, 2004), this anthropocentric perspective guides the following paragraph as it is thought to have mostly influenced our present perspective on the world.

Concurrently, human pursuit of economic growth and prosperity has enabled an increasing degree of control over natural processes. The application of innovation and the dedication to progress have enabled a more efficient and more productive utilisation of land and enhanced protection against the forces of nature. Until the 19th century, these developments were slow and extensive enough to allow a co-evolution of species and ecosystems, resulting in the creation of cultural landscapes that even promoted biodiversity (Poschlod, 2017). Since the second half of the 20th century, however, there has been a notable acceleration in the pace of human development. From the advent of industrialisation to the agricultural revolution, and continuing with population growth, mechanisation and the digital revolution, humans have markedly increased their impact on nature in a relatively short period of time. This process was designated „The Great Acceleration“ (IGBP, 2015). It reflects humanity’s growing ability to harness and alter natural processes, driven by technological advancements, economic growth, and the pursuit of progress.

Today, even the most remote ecosystems are impacted by human activities, including nutrient and pollutant deposition, radioactive contamination or the introduction of genetically modified organisms. Global climate change exacerbates these pressures, altering ecosystems and disrupting the delicate balance that once characterised the Holocene epoch (Rockström et al., 2009). This rapid transformation of Earth’s systems marks the advent of the Anthropocene - a conceptual framework, designed to capture the era of dominant human influence on the planet (Steffen et al., 2011). In this era, the prevalence of environmental crises, including habitat destruction, biodiversity loss, and ecosystem degradation, has become increasingly apparent (IPBES, 2019).

Aquatic ecosystems, particularly those of freshwater habitats, are among the most susceptible to the adverse effects of these crises. The deterioration of water quality, overexploitation of water bodies, flow regulation in rivers and streams and the introduction of alien species have been identified as factors contributing to the alarming decline in freshwater biodiversity (Strayer and Dudgeon, 2010). While the implementation of the EU Water Framework Directive (WFD) has led to a general improvement in water quality across Europe, the development has primarily been a shift from poor to medium status. Despite this overall improvement, high-quality water bodies have increasingly been downgraded to good or medium status, thereby leaving them at constant risk (EEA, 2018). This trend highlights the necessity for targeted conservation efforts to safeguard vulnerable, high-quality habitats. Among the species most affected by this development is the freshwater pearl mussel *Mararitifera margaritifera*, a species which is indicative for healthy oligotrophic waters. Once widespread across Europe, its populations have drastically declined due to habitat degradation, overexploitation, pollution, and alterations in watercourses caused by agricultural runoff, deforestation, and dam construction (Geist, 2010). Additionally, the mussel's complex life cycle, which requires specific host fish such as the salmonid species *Salmo salar* and *Salmo trutta* for its larval stage, has been severely disrupted by declining fish populations (Araujo and Ramos, 2000). The species is frequently considered a keystone species in freshwater ecosystems due to its role in water filtration and sediment stabilisation. With a lifespan exceeding 100 years, the mussel serves as a natural biofilter, markedly enhancing water quality by filtering vast quantities of water, which benefits other aquatic species (Lopes-Lima et al., 2017). Currently, the freshwater pearl mussel is facing critical threats, and its survival depends on the restoration and protection of pristine aquatic habitats (Geist and Auerswald, 2007).

In this context, technological advances, that have once facilitated human impact on nature, also present new opportunities for the protection and restoration of the natural environment (Montana et al., 2023). The emergence of novel techniques, including environmental DNA (eDNA) analysis, metagenomics and artificial intelligence (AI), enables more accurate monitoring and management of ecosystems, providing tools to counteract the biodiversity crisis (Baird and Hajibabaei, 2012). The possibility of environmental metabarcoding to genetically discover whole species assemblages from environmental samples, for instance, can be a chance for novel approaches in nature conservation. A comprehensive understanding of a species community can facilitate its conservation, as it elucidates the intricate ecological interactions that influence species survival (Zipkin et al., 2010).

Community theory postulates, that species are interconnected through a variety of relationships, including predation, competition, and mutualism, which collectively influence ecosystem dynamics (Leibold et al., 2004). By using DNA sequencing to identify species within environmental samples, including those that are otherwise difficult to detect, such as fungi and microorganisms, environmental metabarcoding has revolutionised our ability to uncover these interactions (Taberlet et al., 2012). This technique allows researchers to explore the roles of these often-overlooked organisms in ecological networks. It can, for instance, reveal the presence and diversity of fungal species involved in nutrient cycling or symbiotic relationships, and microorganisms that influence habitat quality or pathogen dynamics (Baird and Hajibabaei, 2012). By providing a comprehensive snapshot of community composition, metabarcoding helps to identify previously unknown interactions and dependencies, offering deeper insights into the ecological roles of all community members and forming more effective conservation strategies (Bush et al., 2019).

In many ways, humans are now faced with a moral responsibility to determine not only the future of individual species but the broader integrity of ecosystems. For species like *M. margaritifera*, which depend on highly specific environmental conditions, such interventions may be the only path to survival (Geist and Auerswald, 2007). This species, emblematic of the broader degradation of freshwater habitats, highlights the urgent need for sustainable management practices that strike a balance between human development and ecological preservation.

While novel technologies hold promises for conservation, they also empower humans to exert even greater control over ecosystems. In this context, humanity faces a dual challenge: on the one hand, the need for human intervention to mitigate the effects of past degradation; on the other hand, the necessity to minimise harmful human influences on the processes of nature in order to enable species and ecosystems to exist sustainably in the modern world (Barnosky et al., 2012). This duality gives rise to the question of the role of pristineness in the context of nature conservation. Does pristine nature still exist? Can it be achieved for certain habitats, or has human activity long since become an integral part of many ecosystems? Ultimately, the capacity of the natural world to support our continued existence as a species is contingent upon our capacity to act and manage in ways that ensure the preservation of nature as the foundation of our living.

This dissertation examines the habitat of the freshwater pearl mussel from a variety of perspectives and approaches.

Following an introduction to the target species, *M. margaritifera* in chapter one, the second chapter examines the alterations in land utilisation over the past 250 years and their potential consequences for the habitats of the freshwater pearl mussel, in addition to their contemporary management.

Subsequently, the third chapter presents an examination of the habitat parameters of the habitat indicator *Myriophyllum alterniflorum,* the alternate water-milfoil, in streams located in the upper Palatinate region of Bavaria..

The fourth chapter will analyse the habitat of the freshwater pearl mussel and its community with the application of environmental metabarcoding (eDNA) and scalable joint species distribution models (sjSDM).

Finally, the potential of traditional meadow irrigation, by means of utilising an old land use concept that combines the economic viability of agriculture with species conservation, is tested for freshwater pearl mussel habitat preservation in present days.

By combining traditional ecological approaches with cutting-edge technologies, this study aims to provide a comprehensive understanding of the current state of freshwater pearl mussel habitats and explore the potential for sustainable management practices that can safeguard these ecosystems for the future.

1 The freshwater pearl mussel *M. margaritifera* L.

The freshwater pearl mussel *Margaritifera margaritifera* has been present in Bavaria for more than 12 000 years. Following the colonisation of inland waters by brown trout and salmon, the species arrived and established dense, stable and highly abundant populations. It widely found habitat in softwater brooks and rivers on bedrock with a Holarctic distribution. In Europe, its range is confined to the north-western regions, extending from north-west Portugal and Spain, across France and Great Britain, to Belgium, Germany, the Czech Republic and Austria. It also occurs in the Baltic states, Scandinavia, and Russia (Araujo and Ramos, 2000). Given its dependence on near pristine waters, the species' niche throughout Europe has narrowed drastically in times, where a high ecological status is seldom attained (EEA, 2018). The species was first identified and studied by humans due to the presence of pearls in a small proportion of individuals, at a rate of one in 70,000. Nevertheless, due to the species' high abundance, pearl fishing was profitable, leading to the establishment of a governmentally controlled pearl fishing industry. This exerted considerable pressure on mussel populations, yet the distributions remained relatively stable until the beginning of the 20th century. However, the significant alterations to landscapes and natural ecosystems imposed by humans throughout the Anthropocene have placed the species at risk and caused the extinction of approximately 90% of its populations. Currently, the freshwater pearl mussel is among the most endangered freshwater organisms globally (Geist, 2005). Despite the implementation of robust conservation measures, successful autonomous reproduction remains a rarity. Consequently, the vast majority of these populations are overaged, comprising individuals that represent a relic of a time when our landscapes were differently constituted.

1.1.1 Species description

M. margaritifera is one of the largest known species of freshwater mollusc. The typical kidney-shaped shell is covered by a dark brown to black periostracum (Figure 1), which serves to protect the calcareous shell from corrosion in the slightly acidic waters in which *M. margaritifera* typically resides. However, marks of corrosion often appear on old whirls of the dorsal edge or apex (Moorkens, 1999). The species can attain a length of up to 150 mm, with an increase in length observed in a south-to-north gradient. Annually, the shell grows from the dorsal edge, resulting in a ringed structure that reflects the age of the individual. The two principal and lateral teeth on the left side and one principal and lateral tooth on the right side built the so-called “schizodont” hinge.

The anterior region, located in the shorter part of the shell, contains the white foot aperture, which can extend to the length of the mussel shell and contains the gonad at its dorsal part. The anterior labial palps of the foot serve to select food particles that have been pre-filtered by the gills. The posterior part, which is located in the longer region of the shell, contains the water interchange apertures breathing. In this part, the edge of the mantle is thickened by rows of papillae (Araujo and Ramos, 2000).

Figure 1: Adult individual of *M. margaritifera*. Photo: J. Sattler

1.1.2 Ecology and Life cycle

Young mussels reach sexual maturity at the age of 12 to 15 years. They reproduce unisexually, with the males releasing their sperm into the surrounding water and the downstream females taking it up with their respiratory water (Figure 2, step 1, Young, 1984). Females then temporarily reorganise their gills into breeding chambers where the fertilised eggs develop into the glochidia, the mussel larvae. This process takes two to three months and is accelerated by warm temperatures. When fully developed, they reach a size of 0.04 to 0.07 µm. Up to four million glochidia may be released into the open water per female and reproductive cycle (Bauer, 1987). At this point, the presence of a suitable host fish to accidentally inhale the glochidia is vital, as the small mussel larvae can only survive for a few days on their own (Figure 2, steps 2 and 3). *M. margaritifera* is a specialist parasite because the metamorphosis from larval stage to juvenile mussel requires a parasitic phase on the gills of the salmonid species *Salmo salar,* Atlantic Salmon, or *S. trutta,* the Brown Trout (Young, 1991; Geist, 2005). Infection of other fish or previously infected individuals is prevented by the host immune response (Boon et al., 2019). As the oligotrophic waters in which *M. margaritfera* lives are naturally low in fish density, the density of the host fish population is also low making this step even more critical. The duration of the parasitic phase is temperature dependant. It can either last for 20 to 60 days, or from seven to nine months (Araujo and Ramos, 2000). During the summer months of the following year, the juvenile mussels reach a size of 0.5 - 0.7 mm. They leave their hosts and burrow into the sediment, where they remain for four to seven years (Figure 2, steps 5 and 6)). At this stage, they are highly dependent on a well-perfused, oxygen-rich interstitial space. Buried in the interstitial, they use their flexible foot to graze on small particles of detritus. When juvenile mussels appear at the sediment surface, they switch to filter feeding of small organic particles such as detritus, bacteria or phytoplankton (Figure 2, step 7). They reach sexual maturity at an age of 12 - 15 years (Figure 2, step 8). Despite this long juvenile period, *M. margaritifera* is highly reproductive throughout its life span of up to 100 years. Adult freshwater pearl mussels are highly fecund regardless of age and there is no post-reproductive period. The dioecious reproduction relies on high population densities to ensure a sufficient concentration of sperm in the water to fertilise the downstream females. This is often not the case today. Under such conditions, females can become hermaphrodites and switch to self-fertilisation. In this way, a female can produce more than 200 million glochidia during her lifetime (Bauer, 1987). This high overproduction is necessary due to the high mortality of glochidia and early juvenile stages. According to Bauer (1989), one billion glochidia are needed to generate ten sexually mature mussels, which makes up for two reproductive offspring per life span of one reproductive female. However, these estimates must be rather conservative, as this would mean that one female would only be able to produce two sexually mature offspring, which would be just enough for reproduction, but not for proliferation. The life span of *M. margaritifera* is inversely related to growth rates and increases from south to north (San Miguel et al., 2004). In the northern parts of the distribution range, it reaches more than 100 years, with the oldest individual found in Sweden being 280 years old (Degerman et al., 2013). The combination of high fertility and long life span is a speciality in the animal kingdom. In general, high fertility is associated with high energy costs, which reduces lifespan. *M. margaritifera* compensates for this by adjusting the number of glochidia per female to the current supply of resources. Thus, the higher the individual body weight prior to the reproductive phase, the higher the number of glochidia released (Bauer, 1989).

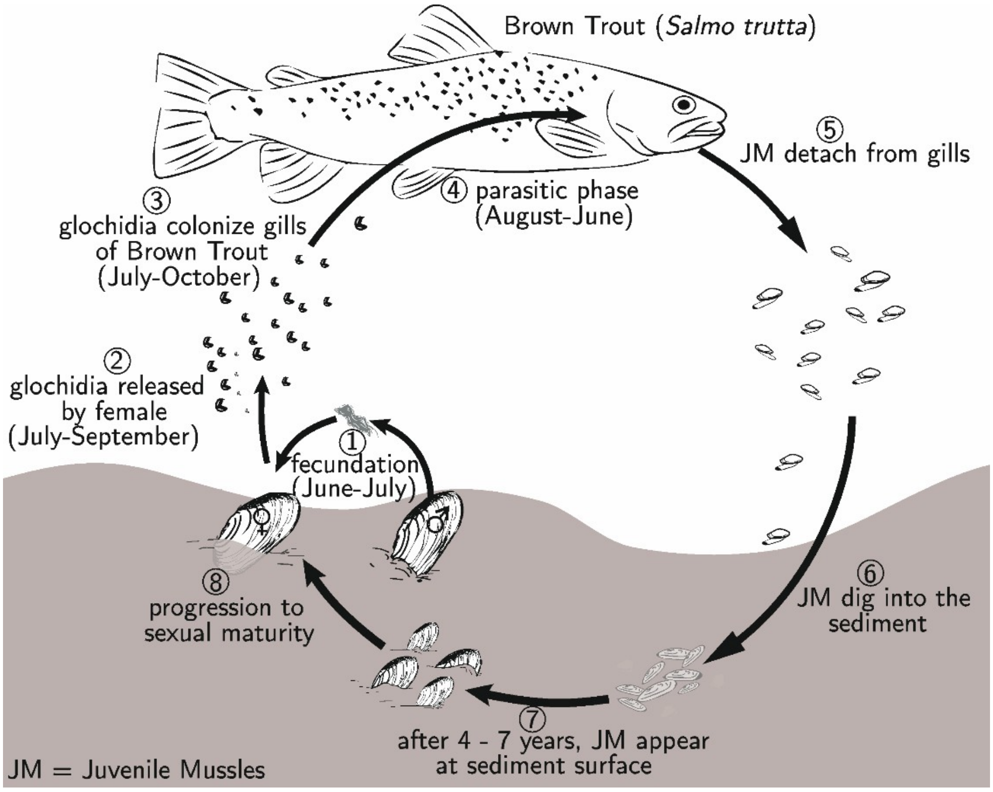

Figure 2: Reproduction cycle of *M. margaritifera* in 8 steps, adapted from Boon et al. (2019).

1.1.3 Distribution, habitat and conservation

The freshwater pearl mussel has a holarctic distribution. It occurs from the northeastern coast of the USA and eastern Canada to the west, central and northern Europe, and from Siberia to Japan (Jungbluth, 1985). It is restricted to regions on bedrock with low nutrient and lime content.

As a stenoeceous species, *M. margaritifera* colonises the relatively fast flowing upper and middle reaches of oligotrophic rivers with lime deficiency (Jungbluth et al., 1985). These have pH values between 6.2 and 7.5, i.e. around or slightly below neutral (Boon et al., 2019). Temperatures range between 0 and 20 °C, and although short periods of up to 28 °C can be survived (Araujo and Ramos, 2000), prolonged high temperatures are considered a limiting factor as they can lead to oxygen depletion (Boon et al., 2019). The composition and nature of the sediment is particularly important for juvenile mussels. It should be composed of gravel and sand that is well aerated to provide a high oxygen supply at depths of up to 20 cm, where the mussels reside.

Despite its highly specialised life history and life cycle, *M. margaritifera* was once widespread and common. Since the mid-20th century, a sharp decline in populations has been reported throughout its range, and the species is now listed as critically endangered or near extinction in the Red Data Books of many countries (IUCN, 2022). Since the turn of the millennium, it has been listed in Annexes II and V of the European Habitats Directive, following the loss of 90 % of central European populations. Strongly altered environmental conditions reduce the fecundity of *M. margaritifera* and increase mortality, especially in the juvenile stage. As a result, many populations have a high average age, with the youngest individuals being 30 to 40 years old (Geist, 2005). The Bavarian pearl mussel populations contain 2.2 % juveniles (Stöckl et al., 2020). The great loss of *M. margaritifera* during the last century has been attributed mainly to anthropogenic influences. Araujo and Ramos (2000) list eutrophication, river regulation, drainage, sewage disposal, dredging, farms, new agricultural land use, loss of forests and natural river banks, water pollution, acidification, pesticides and introduced exotic fish species, as well as other factors affecting host fish populations. The direct, sometimes single event, factors leading to mussel mortality, such as dredging, poisoning or mussel extraction for pearl harvesting, are ultimate and irreversible, but as they are mostly non-structural, they are easier to deal with through restrictions on areas where pearl mussels occur. The indirect factors leading to habitat degradation may be less obvious because they are slower to take effect and unfavourable conditions may be tolerated to some extent by adult individuals. These factors are often associated with structural changes that have occurred throughout the period of modernisation and technification. For example, eutrophication, pesticide deposition and siltation are closely linked to conventional agriculture and land consolidation, which have removed most of the buffering structures such as riparian trees, edges or shrubs that could trap some deposition. Also, river regulation like canalisation and dams change the flow regime and sediment composition and hinder the migration of host fish. It further modifies the floodplain, reducing its capacity for water retention and nutrient supply to aquatic fauna.

The supply with appropriate food is another factor related to habitat quality for *M. margaritifera*. This has been named a major factor for the poor constitutions of habitats observed even in sites, where anthropogenic impacts are relatively low (Hruska, 1999). *M. margaritifera* feeds from small floating particles originating from riparian wetlands. In order for such particles to reach the mussel's habitat, a land water connection is thought to be required, where natural flat river banks give way to alluvial meadows during high floods and to return the water stored in the meadow to the river during droughts (Simonab et al., 2015).

1.1.4 The diet of *M. margaritifera*

Throughout its life cycle, *M. margaritifera* exhibits a variety of feeding modes. During the parasitic stage, glochidia parasitise the gill epithelia of salmonid hosts, where they undergo encystment and host-mediated nourishment (Wächtler et al., 2001). In the post-parasitic stage, the mussels take up nutritious particles and bacteria from biofilms on surfaces or water via their foot. This pedal feeding mechanism is subsequently refined into a filter feeding apparatus, whereby filtration occurs through the gills (Schartum et al., 2017). The pedal feeding mode permits a greater degree of selection, as it encompasses both active foraging and uptake. When filter feeding, primary selection occurs only by particle size. The excretion of ingested unsuitable particles represents a more energy-consuming process than primary selection (Gee, 1991). A variety of potential food sources have been discussed in the literature. It is generally accepted that fine particulate organic matter (FPOM), with a size range of 30 to 40 µm, is consumed (Bauer and Wächtler, 2001; Geist, 2005). According to Hruska (1999), these are mainly derived from species of *Poaceae* growing in the riparian zone and being washed into the aquatic system during rain events or via subsurface flow. Particles, composed of phytoplankton, zooplankton, living or dead cells of algae, dead cells of decomposed plant material, animal cells and sediments are further discussed in the literature (Gum et al., 2011; Eybe et al., 2013). In contrast to other species of freshwater mussel, *M. margaritifera* does not exhibit a preference for consuming living algae cells (Bauer and Wächtler, 2001). In captive breeding, juveniles of *M. margaritifera* are typically fed a combination of commercially available algae, such as commercially used shell fish diet, detritus collected from natural habitats, and animal cells, which are predominantly composed of crushed *Chironomids* (Gum et al., 2011). Algae are assumed to contribute to the pearl mussel diet, especially by providing polyunsaturated fatty acids (PUFA) which facilitate growth (Grunicke et al., 2022). Growth rates are of great importance for survival during the initial post-parasitic phase. However, consistent elevated levels are also associated with a reduction in overall lifespan (Araujo and Ramos, 2000). In feeding experiments, a pure algal diet was observed to induce the best growth rates, but also resulted in higher mortalities when compared to mixtures of algae and detritus. The presence of detritus led to a reduction in the concentration of ammonia and nitrites in the water, which were oxidised to more tolerable nitrates, thereby reducing the toxicity of the medium. The addition of crushed red blood worms, as proposed by M. Lange (Gum et al., 2011) , did not result in any discernible impact on growth or survival rate (Eybe et al., 2013). Despite the higher nutritional value of algae in comparison to detrital particles, feeding experiments demonstrated that juvenile mussels consistently select food patches containing detritus, irrespective of the presence of other nutritious components (Gramstad, 2014). Adult mussels demonstrated a preference for certain algal cells over others. They rejected *Micractinium* and *Cyclotella* cells in favour of *Microcysts,* yet they did not distinguish between algae and detritus (Baker and Levinton, 2003). Based on these findings, it can be inferred that growth rate is not the primary factor influencing the health and survival of pearl mussels.

M. margaritifera is endemic to granitic areas with low calcium rates (Araujo and Ramos, 2000). An elevated concentration of calcium in the water was found to be associated with an increased mortality rate among both adult and juvenile specimens. Concurrently, calcium is a vital component in the formation of the mussel shell. It is therefore necessary that it is present in an organic, soluble form as calcium carbonate ($CaCO_3$, Norbury, 2015). The transition from elementary calcium to calcium carbonate is mediated by submersed macrophytes or roots of certain *Poaceae* that are in contact with the water (Simon et al., 2017). Calcium carbonate exhibits adhesive properties with respect to sediment particles that constitute the detrital composition. It is therefore hypothesised that *M. margaritifera* requires calcium in the form of detrital food in order to construct the thick, distinctive shell that enables it to withstand the relatively acidic conditions prevalent in pearl mussel streams (Hruska, 1999). Stable isotope analysis lends support to this hypothesis, indicating that terrestrial FPOM serves as the primary food source, rather than autochthonous detritus (Brauns et al., 2021). However, Grunicke et al. (2022) propose that the quality of detrital food is enhanced in streams when it undergoes further processing by macroinvertebrates and when biofilms of bacteria, fungi and algae are formed. These are important synthesisers of PUFAs, which mediate growth. It can be reasonably deduced that a diet comprising a combination of riparian and stream detritus, along with algae, bacteria, fungi and sediments, represents an optimal nutritional profile for *M. margaritifera.* This necessitates an interconnectivity between terrestrial and aquatic ecosystems, characterised by a landscape comprising an array of wetlands and a well-structured stream morphology and bed.

2 Land use since 1850 and impacts on pristine aquatic habitats

2.1 Introduction

Land use has played a crucial role in shaping European cultural landscapes for thousands of years. From the agricultural practices of ancient civilisations to the sophisticated farming techniques of the Middle Ages, human activities have continuously modified the natural environment (Poschlod, 2017). These modifications have created diverse habitats that support a wide range of species, making cultural landscapes essential for biodiversity. However, the Industrial Revolution marked a significant turning point in land use patterns. Rapid technological advances and the expansion of industrial activities led to profound changes in the landscape. Land consolidation, mechanisation of agriculture, and urbanisation intensified land use, often at the expense of natural habitats. The widespread use of synthetic fertilisers and pesticides further altered ecosystems, reducing habitat heterogeneity and leading to a decline in biodiversity. The shift from small-scale, diverse farming systems to large-scale monocultures resulted in habitat loss and fragmentation, posing a serious threat to wildlife (Newbold et al., 2019). According to the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES, 2019), land use change is one of the main drivers of biodiversity loss, alongside direct exploitation of organisms, climate change, pollution, and distribution of invasive species. The report estimates that land use change, including agriculture, forestry, and urbanisation, is responsible for about 30 % of the total global biodiversity loss. Freshwater ecosystems, including wetlands, have lost 85 % of their area since 1700 due to drainage and land reclamation for agriculture and development (Fluet-Chouinard et al., 2023).

However, the extent of these effects is not consistent across all regions. In addition to nature reserves and national parks, restricted military zones are typically established with reduced human access. The exclusion of industrial and agricultural activities facilitates the conservation of natural habitats and ecosystems. Consequently, they can play a significant role in nature conservation, often serving as unintended sanctuaries for biodiversity (Lawrence et al., 2015). The Iron Curtain zone, which divided Eastern and Western Europe for over three decades, provides an illustrative case study of such a phenomenon. The area is now designated the European Green-Belt (EGB) and has become a *de facto* nature reserve, helping to conserve a variety of habitats and species. Additionally, it represents a unique corridor of preserved landscapes (Zmelik et al., 2011). Many species that became endangered during the 20th century were able to find refuge in this area, which is why it plays an important role in species protection today. The freshwater pearl mussel *M. margaritifera,* which was once widely distributed, now inhabits the area of the former Iron Curtain and is considered to be one of the most endangered freshwater organisms in the world. Approximately 90 % of pearl mussel populations have become extinct throughout the 20th century (Geist, 2010). The intensified pressures exerted by human activities on freshwater ecosystems encompassing alterations to watercourses, such as channelling and straightening, as well as land consolidation measures that have resulted in the destruction of buffering structures and an increased influx of sediments, nutrients and pesticides (Hunsaker and Hughes, 2002). Furthermore, the reduction of wetlands, which play a pivotal role in water retention, filtration and the provision of nutrients for freshwater organisms (Fluet-Chouinard et al., 2023), has contributed to the deterioration of these ecosystems.

The border areas between the German state of Bavaria and the Czech region of Bohemia were subject to disparate land use pressures as a consequence of their location within the former Iron Curtain zone. The establishment of the military zone on the Czech side of the border resulted in a reduction in the intensity of land use practices in comparison to areas situated further inside the country. There, agricultural and industrial activities were more pronounced. Besides, in Western Europe, thus on the Bavarian side of the border, land consolidation was driven by technical advances which is in contrast to Eastern Europe, where the regime implemented a policy of land collectivisation, transferring ownership from private individuals to large, state-managed farms known as kolkhozes. This unique historical context provides an opportunity to investigate the effects of land use on ecosystems.

This study analyses land use change in the area surrounding the EGB between Bavaria and Bohemia, examining five time layers since the year 1850. The history of land use is reconstructed in six plots along the Bavarian-Bohemian border, distributed equally between the two countries, and one reference plot located further inside each country.

It is hypothesised that the freshwater pearl mussel has profited from the geopolitical history of the study area, with the potential for adverse effects from human activities to be less pronounced in the border near regions. These effects are expected to be (1) the intensification of anthropogenic impact in general and alongside riverbanks in particular; (2) the straightening of watercourses, leading to reduced river sinuosity; (3) the increase in the size of individual arable fields and (4) the decrease in the area of wetlands.

2.2 Material and methods

The historical landscape analysis was conducted in eight squares of 4 x 4 km or 16 km^2 each, distributed in a way that include two triplets of squares along the Bavarian-Bohemian border and two reference squares located further within the countries (Figure 3). The northern triplet (plots 4, 5 and 6) is situated in close proximity to the border triangle of Bavaria, Saxony and Bohemia. In this area, several relict or reintroduced populations of the freshwater pearl mussel are managed by the pearl mussel hatchery Huschermühle of the non-governmental organisation (NGO) Bund Naturschutz Hof. The southern triplet (plots 1, 2 and 3) is situated in the Pfreimd valley, including parts of the Upper Palatinate and the Czech district of Budweis/České Budějovice. Some historical records of *M. margaritifera* exist for the Pfreimd river, although no confirmation has been found in the last ten years. The two reference sites were selected to represent similar landscape structures. The Bavarian reference site is situated in the vicinity of the town of Pfreimd and is drained by the river Pfreimd, while the Bohemian reference site is located to the south of the town of Kladruby and is drained by the river Úhlavka.

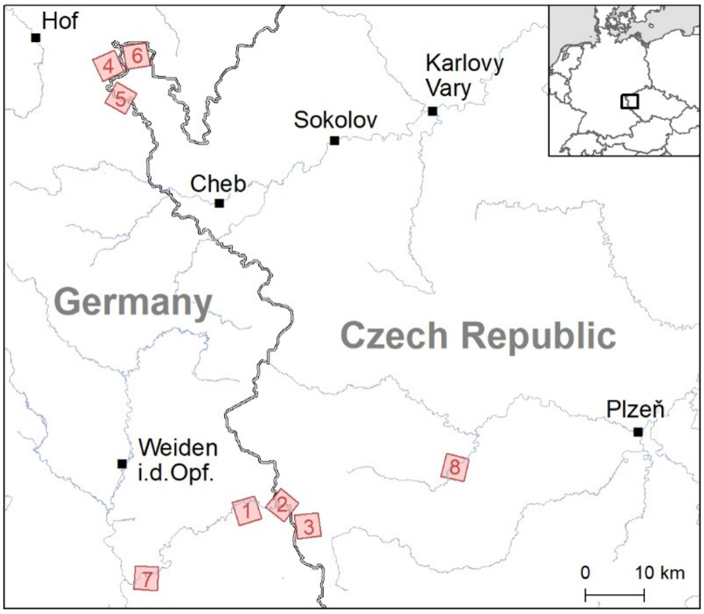

Figure 3: Overview of the project area – eight red coloured squares measuring 4x4 km, on which the historical landscape analysis was carried out. Map resource: Bavarian Surveying Administration, ESRI 2010.

2.2.1.1 Landscape character and historical background

The two regions under examination along the border exhibit historical parallels, having been subject to the influence of the former Iron Curtain, which divided the area into East and West for a period exceeding 30 years. The border, delineated in 1920 following the conclusion of the First World War, did not function as a rigid barrier until the 1950s. It was not until the beginning of the Cold War that a de facto division was established. On the Bohemian side, a significant proportion of the human population was expelled, and numerous settlements were destroyed. The landscape within the exclusion zone developed largely without human intervention. The use of agricultural land was taken away from many private owners, consolidated, and transferred to the large-scale cultivation of collective farms (Simonab et al., 2015). In contrast, on the Bavarian side, a new era of intensification began, as the use of artificial fertilisers, modern machinery, and a further period of land consolidation reshaped the landscape and brought about changes in biodiversity (Poschlod, 2017). Nevertheless, the two study areas differ in terms of their history.

History of the southern observation area: Plots 1-3

The southern observation area has historically exhibited low population density. The lack of natural resources and the relatively infertile soil meant that the population remained relatively small, which in turn limited industrial development. A special feature of this part of the project region is the so-called Pfrentschweiher, which was created in the late Middle Ages. The damming of the Pfreimd River near the present-day Bavarian-Czech border began in 1362 to create what was then the largest reservoir in Bavaria, covering 450 hectares. It was used for fish farming, irrigation of the surrounding land and to power the many mills downstream of the reservoir. By 1840, however, the reservoir was emptied due to a lack of economic viability. Subsequently, Bavaria's first meadow cultivation school was established on the site, which over the next 50 years promoted the systematic drainage, improvement and fertilisation of the area. One of the hydraulic engineering measures was the diversion and merging of two streams, the Netschbach and the Katarinabach, which further dried out the surrounding floodplain forests. From the beginning of the 20th century until after the Second World War, peat was extracted from the bog. In the meadows, occasional irrigation increased the yield, but the flora and fauna no longer corresponded to that of a wetland (Ibel, 1997). After the 1960s, the bog was broken up and spruce trees were planted in addition to large reforestations. In 1987, the existing 10 hectare nature reserve was extended to 170 hectares and a management plan included reversing the previous afforestation and the rewetting of an area of about 30 hectares. A mosaic of different biotopes has been created in the immediate vicinity, with species-rich meadows, dams, ditches, ponds, wetlands and woodlands adjacent to each other, to provide habitats for a variety of species (Knipping, 1990).

2.2.1.2 History of the northern area – Plots 4-6

The settlement history of the area around the border triangle between Bavaria, Saxony and the Czech Republic is much more intensive than that of the southern study area. The first records of settlement in this area date back to the 12th century. Although the area did not offer a pleasant climate for living and farming, it was rich in natural resources such as tin. Therefore, the population has never lived exclusively from agriculture, but also from a variety of crafts and trades. The streams of this land were exploited not only for their tin content, but also for the presence of the freshwater pearl mussel. This was discovered in the 1730s and subsequently, pearl fishing was declared a princely prerogative, which meant that pearl mussel stocks were exploited for economic reasons until the mid-20th century. In the 20th century, as industrialisation progressed, pearl fishing became less profitable and the last official fishing took place in the 1950s (Zeh, 1987). The town of Asch, which lays within study plot 6, in particular, played a pioneering role in industrialisation, which led to renewed immigration to the area and made it one of the most populous regions of the Austro-Hungarian Monarchy. In the period between the 1850s and the beginning of the First World War, the region was extremely successful, highly developed and inhabited by more than 21 thousand people. An advanced infrastructure reflected this prosperity (Alberti, 1938). The political developments after the Second World War finally marked a turning point in the development of the landscape, dividing it into East and West and leaving the Eastern part under decolonisation and public exclusion. Although the restricted military zone has been open to the public since 1989, the region remains sparsely populated.

2.2.2 Map sources and digitisation

Map sources and aerial photographs from five time periods, from the mid-19th century to the present day, were digitised for each of the eight squares, covering a total area of 128 km^2, using the ArcMap 10.8.2 geographic information system (ESRI, 2020). For each 4 x 4 km square, map sources and, after the mid-20th century, aerial photographs were collected to cover five time periods – A: mid-19th century (1836-1852), B: first third of the 20th century

(1911-1936), C: mid-20th century (1936-1954), D: last third of the 20th century (1970-1988), E: present (2017-2022, see Table 1). The type and origin of the map material used is shown in Table 1, references and copyrights are given in Table A 1 in the appendix. The legends of the individual map sources were extracted and converted into a generalised 'master legend', which makes the landscape structures comparable in all quarters and time periods. This is provided as supplementary material. All sources were georeferenced and digitised under their respective legends using the UTM zone 33 coordinate system. All original information was retained, allowing the master legend to be reorganised throughout the process. Finally, all categories were translated into the master legend, on the basis of which new comparable maps were produced and the changes in the coverage of the different land use categories were calculated.

Table 1: Resolution and year of the map sources used for each time slice (A - E) and plot (1 - 8) in each region. For authors of each source see appendix Table A 1.

time period	A	B	C		D		E	
source	map	map	map	photo	map	photo	DOP	field
resolution	1:5.000	1:50.000	1:25.000	1:42.000	1:25.000	1:14.000	20 cm	mapping
year of map resource in Bavarian plots								
1	1836	1911/13	1936/36	1945	1970	1970	2019	2021
2	1836	1919	1936/43	1947	1976	1983	2017	2021
4	1841	1921	1948	1948	1970	1970	2017	2020
5	1852	1919	1943	1950/45	1970	1970	2019	2020
7	1852	1919	1954	1964/45	1985	1983	2019	2022
year of map resource in Bohemian plots								
2	1838	1924/25	1952/53		1983		2019	
3	1838	1924/25	1952/53	1947	1988	1987	2017	2021
4	1852	1926			1987	1987	2019	
6	1841	1926	1948	1948	1987	1987	2017	2020
8	1938	1936	1953/54	1949/57	1949/57	1987	2017	2022

To obtain information on river length and morphology, running waters were digitised from the maps as line segments. Starting with time layer A, the longest continuous runoff was digitised as one segment and shorter lines as confluents. The following time layers were adapted to time layer A to create river sections that were comparable between the time layers.

2.2.3 Data analysis

The quantities of land use change were calculated in MS Excel at different levels. Each square was treated separately and the developments on both sides of the border of the northern and southern triplets of squares were compared with each other and with the two reference squares of each country. The development of the arable land structure was analysed in terms of the dimensions of individual fields. To this purpose, box plots were generated in R using the ggplot2 package (Hadley, 2016). Due to non-normal distribution, which was tested with Shapiro-Wilk test, the means of each time layer and area were compared in a Wilcoxon rank sum test. To reflect the development of running waters, the total river sinuosity was calculated by determining the ratio of the length of a river section to the length of the straight line between the start and end points (see Horacio, 2014). The development of river sinuosity over the time layers A, C and E was visualised by box plots according to the arable fields, as described above. Significance of changes was calculated with Kruskal-Wallis test and p-values for each transition were obtained from post-hoc Dunn-test with Bonferroni correction. To show the qualitative transition of wetlands, Sankey diagrams were generated using the r-package networkD3 (Allaire et al., 2022). The categories of wet open land (wet meadow, swamp, peatland or peat extraction), wet forest, standing and flowing water were used for these analyses. Finally, a buffer of 100 m to both sides of running waters was created for each time layer and the development of the land use categories forest and grassland within this buffer was analysed.

2.3 Results

Maps, illustrating the transition of land use in all eight observation squares are shown in Appendix Figure A 1 – A 8. Due to the low resolution of the B-layer map resources, some structures are always missing in the digitised maps from the 1920s. This must be taken into account when interpreting the map series. Despite the uniqueness of each observation square in terms of land use structures, some general trends become clear.

1. Disappearance of small-scale structures:

Maps from the mid-19th century depict a rural landscape characterised by small-scale agriculture across all observation squares. The open land was constituted by a multitude of small parcels, separated by an array of hedges or unpaved field paths. In the Bavarian squares (Figures A 1, 2, 4 5 and 7), this small-scale character became progressively larger. In the Bohemian squares (Figures A 2, 3, 4, 6 and 8), it almost completely disappeared between time periods C and D and arable land was largely converted into meadows or pastures.

2. Increase of settlements

In general, the study area is rather sparsely populated. However, the extent of settlements increased in every observation square during the study period (Figure 4b and 5b). This increase was continuous in the Bavarian squares, while in the Bohemian squares it stagnated or even decreased between the 1950s and the 1980s. The largest increase in settlements was that of the town of Asch in square number 6 (Figure A 6). Transport infrastructure increased in the Bavarian squares along with the increase in settlements. Contrary, in Bohemian squares they overall decreased despite the growth of settlements (Figure 4a and 5a).

3. Greatest extent of wetlands in the 1950s

Open wetlands and alluvial forests reached their maximum extent in the middle of the 20th century throughout the study area and decreased continuously thereafter (Figure 4i and 5i). These wetlands are mostly located along watercourses (Figures A1 – A6). Over time, they have partly been converted into wet forest, which has increased over the same period as the wetland declined (Figures 4f and 5f).

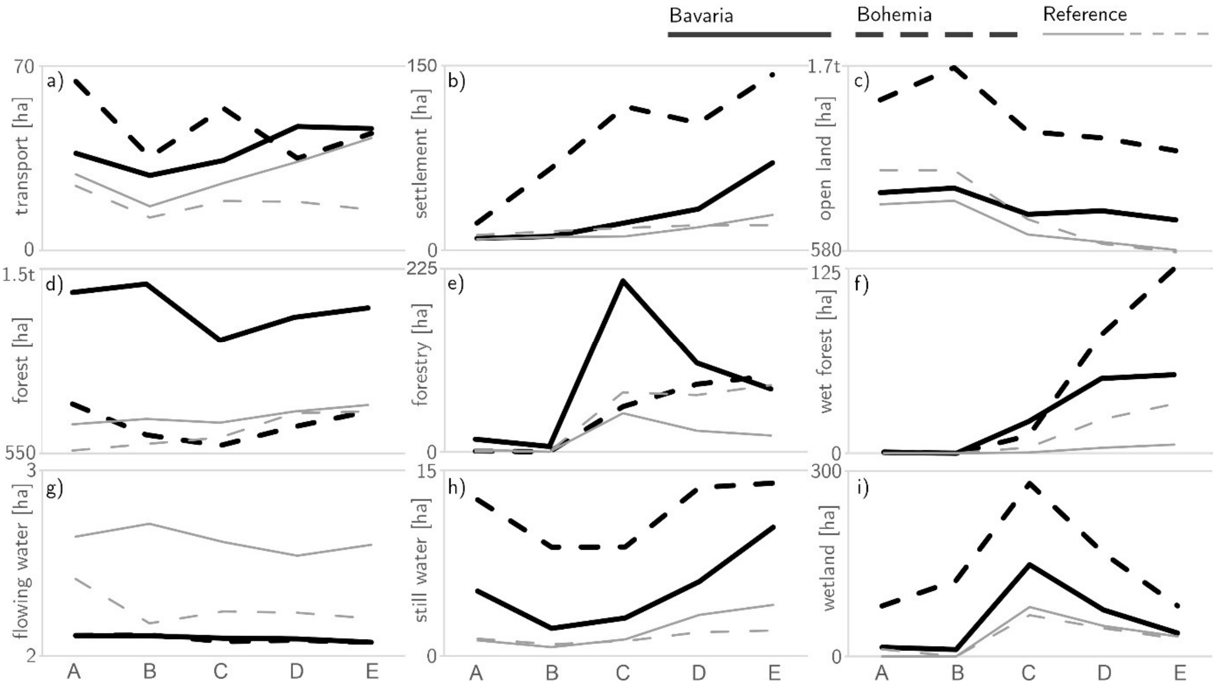

Figure 4: Transition of land use types in the northern observation triplet over the five time layers A: ± 1850, B: ± 1920, C: ± 1950, D: ± 1980 and E: ± 2020. Areas on the Bavarian and on the Bohemian side of the border are compared.

The development of the other types of land use was not as uniform. Open land decreased slightly throughout the study area. In the southern part on the Bohemian side, this decrease was most pronounced and occurred between time layers B and C (Figures 4c and 5c). Forest and forestry developed in opposite directions. The lowest forest cover was everywhere in the middle of the 20th century, when forestry reached its maximum. Afterwards, with the exception of the Bohemian side in the north, forestry decreased and forest cover increased again (Figures 4d, e and 5d, e). The area covered by flowing waters decreased continuously on the Bavarian side. On the Bohemian side, it decreased in the south and in the reference between the first two time layers and remained constant thereafter, whereas in the north it alternately increased and decreased, but the steep decline between time layers B and C led to an overall decrease (Figures 4g and 5g). Still water increased in the north and in the reference of both countries but decreased in the south (Figures 4e and 5e).

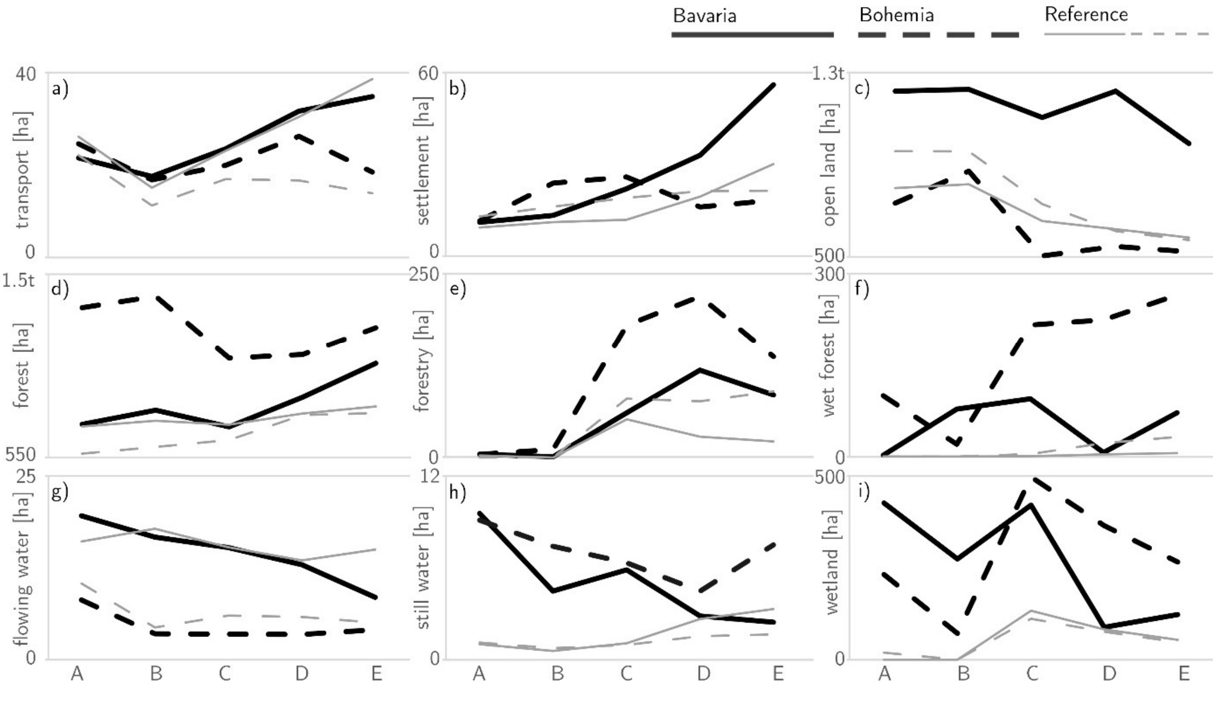

Figure 5: Transition of land use types in the southern observation triplet over the five time layers A: ± 1850, B: ± 1920, C: ± 1950, D: ± 1980 and E: ± 2020. Areas on the Bavarian and on the Bohemian side of the border are compared.

In the following sections, the developments that are closely linked to the quality of freshwater habitats are analysed in more detail.

2.3.1 Land use along river banks

Within an area of 100 m on both sides of watercourses, forest cover has increased in all sites since the middle of the 19th century. In the reference squares, the increase commenced subsequent to time period B (1920s) in both countries, with a more pronounced trend in the Bohemian reference square (Figure 6e and f). In the border areas, the increase commenced after time period C in the northern and southern regions, with a more pronounced increase observed in the northern regions on both sides of the border (Figure 6 a - d). Conversely, grassland coverage exhibited a general decline across the study area, although with greater differences in the north than in the south. On the Bohemian side of the border, however, there has been a slight increase in grassland extent since the mid-twentieth century (time period C).

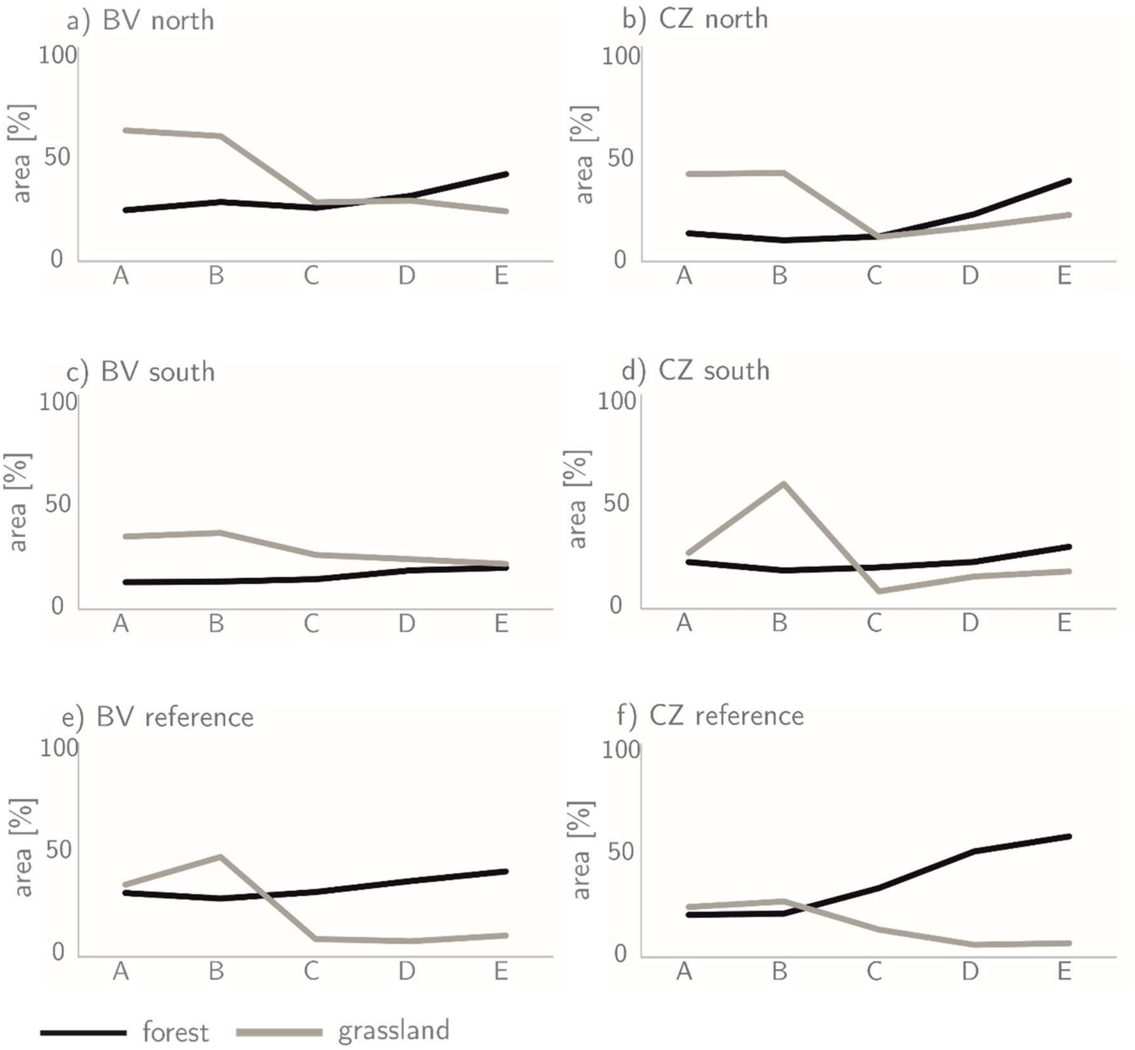

Figure 6: Percentage of forest and grassland cover within 100 m along watercourses in the five time periods A (± 1850), B (± 1920), C (± 1950), D(± 1980) and E (± 2020) in the northern and southern parts of the study area on the Bohemian (BH) and Bavarian (BV) sides of the border and in the reference squares of both regions.

2.3.2 Morphology of running waters

River sinuosity has decreased throughout the study area since the mid-19th century (Figure 7). However, this development was not always uniform in its intensity. In the southern part of the study area, the changes were significant on the Bohemian side of the border, but not on the Bavarian (Figure 7a). The Bohemian plot 3 showed a significant decrease when comparing time layer A and E (Figure 7c). Summing up all river sections in the southern part on the Bohemian side, also the decrease between time layer A and C was significant. In the northern part of the study area, significant decreases in river sinuosity were observed on both sides of the border when comparing time layer A with C and with E. While in the cross border plot 4 and the Bohemian plot 6 sinuosity decreased mostly before the mid-20th century (time layer A to C), decreases in the Bavarian plot 5 were marginally significant when comparing time layer C and E. The reference plots showed marginal decreases of medians and means of river sinuosity, which however were not significant (Figure 7b).

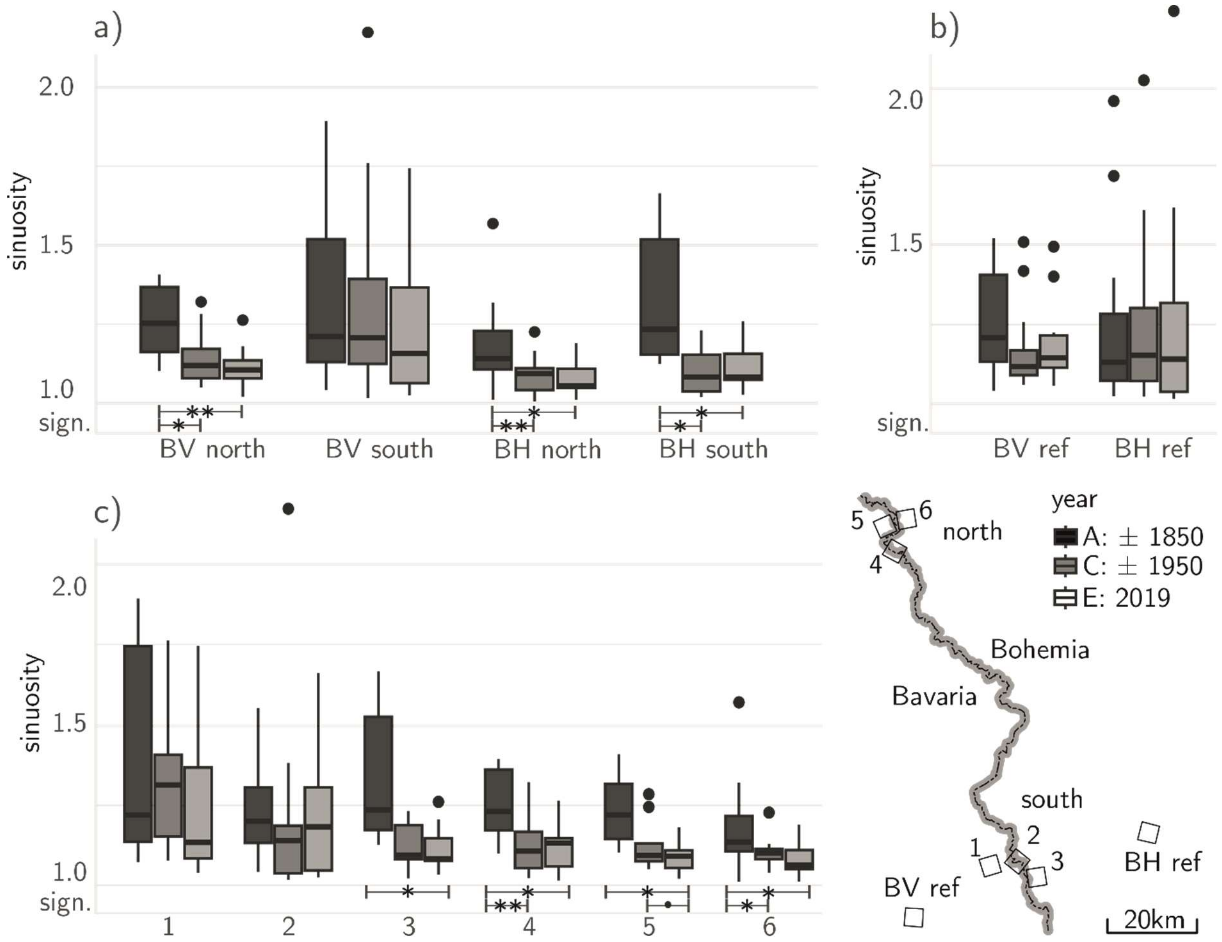

Figure 7: Changes in river sinuosity over the years A: ± 1850, C: ± 1950 and E: ± 2020 compared between a) the northern and southern regions, c) single plots and b) the reference squares. Exact years of map resources are found in the appendix Table A 1, p-values and differences in means and medians are given in Table A 2. Levels of significance are $p \leq 0.01$ = highly significant: **, $p \leq 0.05$ = significant: * and $p \leq 0.1$ = marginally significant: •.

2.3.3 Area of arable land

The extent of connected arable land increased in most of the plots. In the northern area, this increase was significant on both sides of the border (BV and BH north, Figure 8a). Looking at the individual plots 4 – 6, which make up the northern area, it becomes clear, that this development can be traced back to the Bavarian plot 5 and the cross border plot 4, whereas the Bohemian plot 6 has increased in the period A-C and decreased again in the period C-E (Figure 8c), although not significantly. In the southern area, a significant increase was observed for the Bavarian side, which goes back to the strong increase in plot 1. For the Bohemian side, the development is similar to that of plot 6, reflecting an increase in arable field size in the period A-C and a decrease in the period C-E. Reference squares also reflected an increase in arable field size significant for the whole time span on the Bavarian side. On

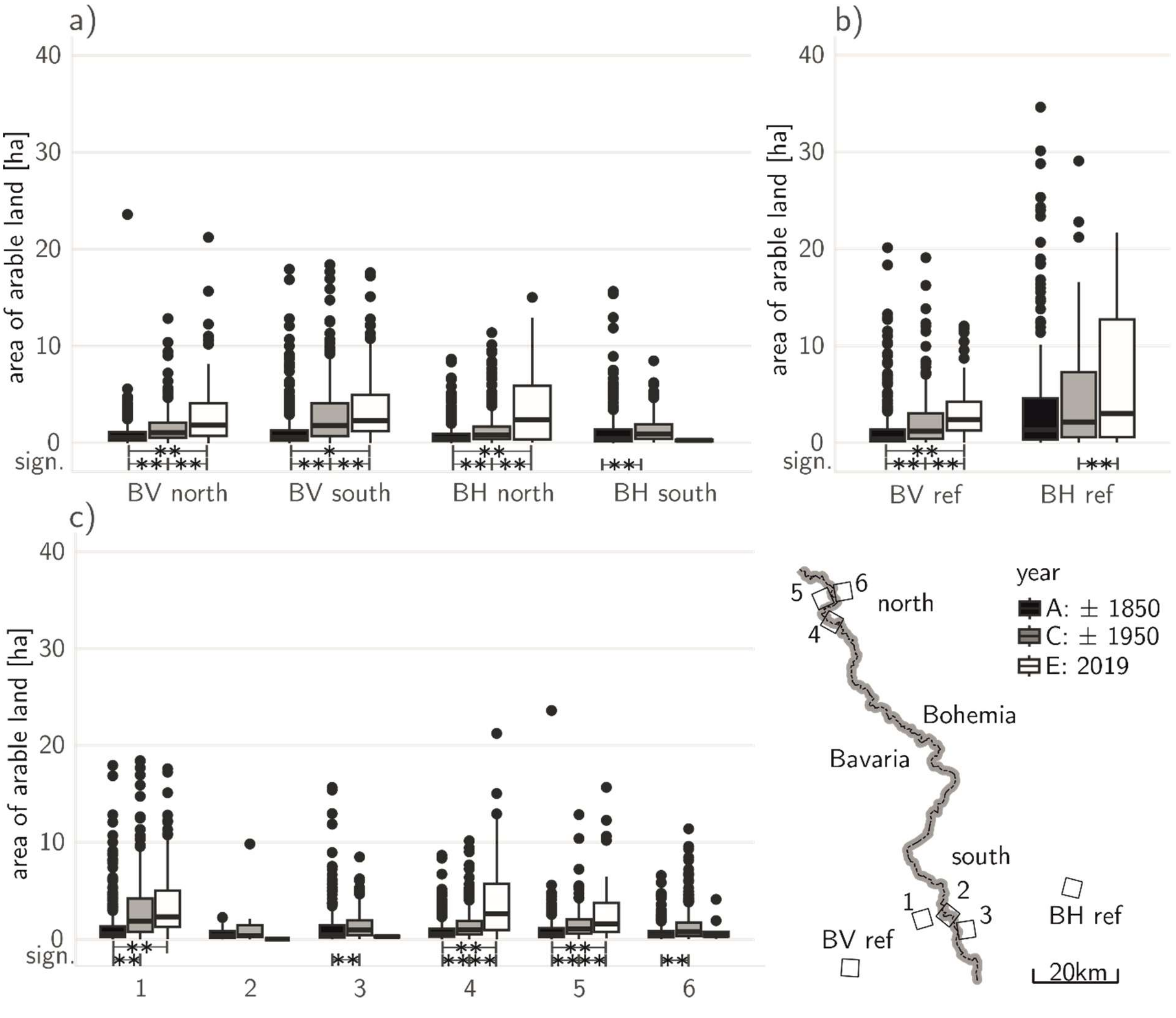

Figure 8: Alteration in size of connected arable fields over the years A: ± 1850, C: ± 1950 and E: ± 2020. compared between a) the northern and southern regions, c) single plots and b) the reference squares. Exact years of map resources are found in the appendix Table A 1, p-values from Wilcoxon rank sum test are found in the appendix Table A 3. Levels of significance are p ≤ 0.01 = highly significant: **, p ≤ 0.05 = significant: *.

the Bohemian side only the increase between time-spans A and E was significant (Figure 8b). The area of connected arable land increased in most of the plots. In the northern area (plots 4 - 6), this increase was significant on both sides of the border (BV and BH north, Figure 8a) This development was most evident in the Bavarian plot 5 and in the cross-border plot 4, whereas the Bohemian plot 6 exhibited a notable increase during period A-C, followed by a subsequent decline during period C-E (Figure 8c), although not statistically significant. In the southern area, a significant increase was observed for the Bavarian side, which can be attributed to the strong increase in plot 1. In contrast, on the Bohemian side the development was comparable to that of plot 6, indicating an expansion in arable field size in the period A-C and a contraction in period C-E. The reference squares also reflected an increase in arable field size which was significant for the whole time span in the Bavarian plot and between time spans A and E in the Bohemian plot (Figure 8b).

2.3.4 Wetlands

The transition of the wet landscape structures of the study area over the study period is illustrated in (Figure 9). In the northern and southern observation triplets, the transition from wet landscape structures to drier ones, including open land, arable land and forest, was more pronounced on the Bavarian side of the border. In the northern area, the conversion of wet open land to woodland and open land, which includes the land use types arable land, grassland, pasture, heathland and fallow land, has been a gradual process. A smaller proportion of the area was converted into alluvial forest, which subsequently developed into dry forest. In particular, between time layers C and D, wetlands were also transformed into settlement or transportation structures. The transformation of standing and flowing water into wetlands was primarily observed during the initial time period, up to the mid-twentieth century, and remained relatively consistent thereafter. These developments are comparable on both sides of the border, with the exception of the pronounced increase in dry open land between time layers D and E, which is discernible solely on the Bavarian side.

In the southern triplet, on both sides of the border, a larger proportion of former wetland was transformed into dry forest until the mid-twentieth century. Subsequently, the proportion of dry forest remained relatively stable. The proportion of wetland converted to dry open land was comparable to that observed in the northern triplet, but had already occurred between time layers C and D and remained constant thereafter. On the Bavarian side, some of the former wetlands were also converted into arable land until the 1950s. This proportion had largely increased by the 1980s and was developed from wet and dry open land. In the current layer, it has fallen back to the level observed in the 1950s.

In the reference square on the Bavarian side, the dynamic interplay between flowing and stagnant water shaped the evolving wet landscape structures in time layer A. These underwent a continuous transformation, giving rise to settlement and transport structures on the one hand, and to wet or dry open land on the other. However, some of these structures were also transformed into flowing water bodies. In the Bohemian reference area, approximately 55 % of the wetlands were open wetlands in the 1850s. These subsequently developed into forest or open land, which then largely became forested. Some of the open wetlands were also continuously transformed into settlements or transport structures, although the majority of these were developed from running waters. The former area of watercourses was reduced, and this land was developed into woodland and settlements.

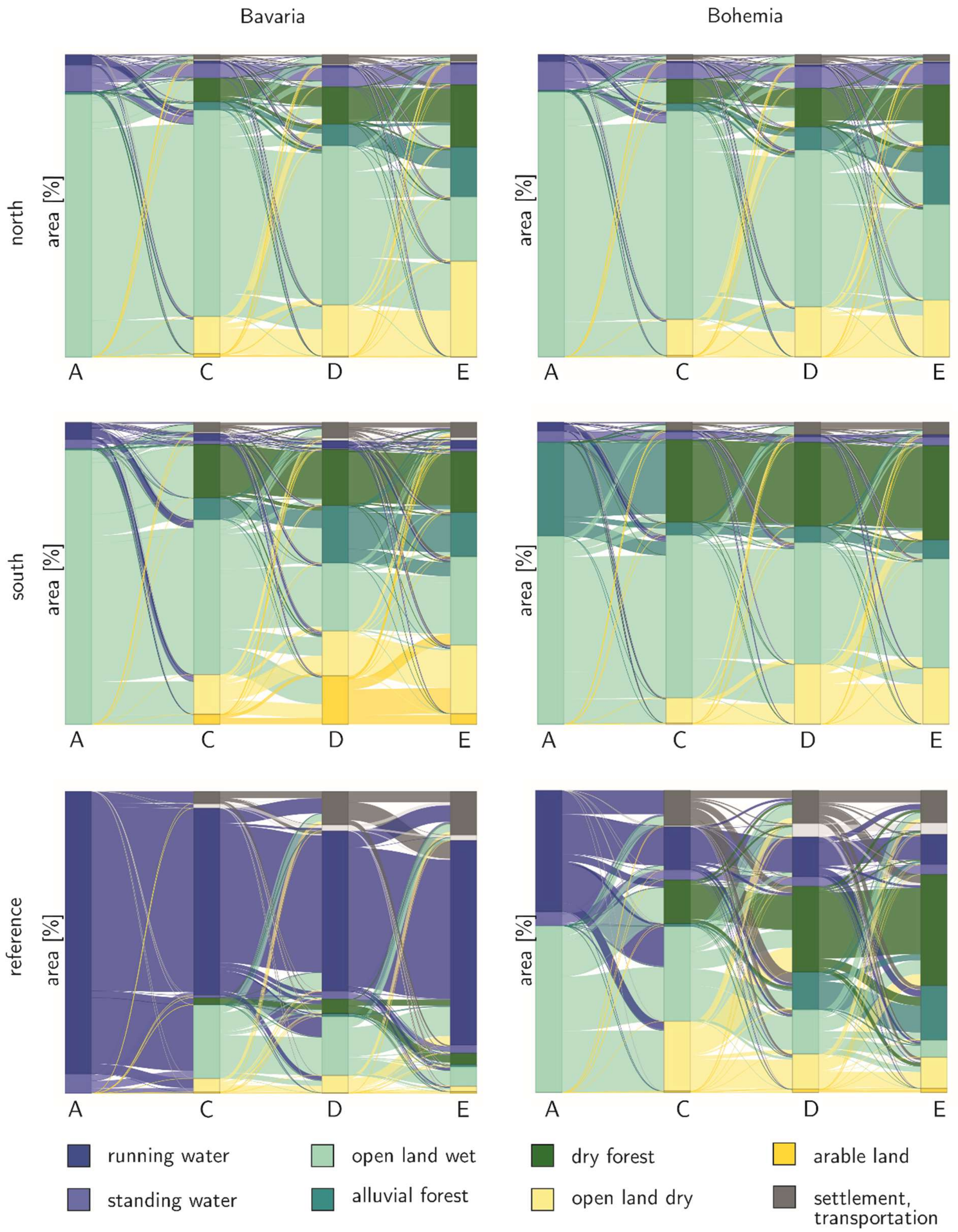

Figure 9: Development of wet landscape structures (wetlands, alluvial forests, standing and running waters) from time period A (± 1850) through the following time periods C (± 1950) and D (± 1980) to the present time E (± 2020) in comparison between the two countries (Bavaria and Bohemia) in the northern and southern part of the study area and in the reference squares.

2.4 Discussion

The structural landscape analysis revealed a degradation of habitat quality indicators throughout the study area. Significant increases in cropland sizes and settlement structure, combined with decreases of watercourse area and river sinuosity, conversion of wetlands to forest and open land, loss of small-scale buffer structures and grasslands around watercourses, mediate the influx of fine sediments, nutrients and pesticides (Allan et al., 1997) and reduce natural food sources for *M. margaritifera* (Hruska, 1999). Conversely, the total area of alluvial forest has increased, as well as the total area of wet open land in parts of the study area and forest cover around watercourses. This, together with the conversion of large arable fields to grassland on the Bohemian side, are developments that may support the quality of pristine aquatic habitats and provide a differentiated picture of the influence of land use history on aquatic ecosystems in the study area.

2.4.1 Forest and grassland cover along river banks

A decline in the percent cover of grasslands has been observed along the river banks, within a buffer of 100 m, throughout the study area. This decline is particularly pronounced between the early and mid-20th century (time layer B and C). Prior to the implementation of systematic land drainage, grassland cultivation was the sole viable land use strategy in proximity to rivers. This is due to the fact, that groundwater tables can be managed effectively in these areas, and that periodic flooding has no adverse impact on yield (Strobel and Hölzel, 1994). Extensively managed grasslands contribute to the health and functioning of aquatic ecosystems by filtering sediments and pollutants, retaining water, and providing detritus for filter feeders such as the freshwater pearl mussel. The decrease of riparian grasslands was named a major contribution factor to the degradation of pearl mussel habitats in the Czech Republic (Hruska, 1999). Such a decrease was most pronounced between time layers B and C. In the northern region of the study area on the Bavarian side, it correlates with the distribution of *M. margaritifera* populations, where it should be of concern for conservation approaches.

The extent of forest cover showed a net increase throughout the study area. The increase was more pronounced in the northern region than in the southern region, with the greatest increase observed in the Bohemian reference plot. Riparian forests play a pivotal role in maintaining the health of aquatic ecosystems in Central Europe. They regulate water temperature through shading, which is essential for many species (Johnson and Jones, 2000) and contribute organic matter and nutrients, thereby supporting aquatic food webs and enhancing biodiversity (Sweeney and Newbold, 2014). It is therefore encouraging to observe an increase in forest cover in the vicinity of waters inhabited by pearl mussels. However, the implementation of intensive forestry practices, such as the establishment of coniferous monocultures, has the potential to increase levels of sedimentation, nutrient runoff, evapotranspiration and acidification, thereby affecting water quality and disrupting habitats (Boyle et al., 1999). The extent of forestry increased throughout the study area, reaching its maximum in the 1950s (in the northern part) and in the 1980s (in the southern part). As observed by Simonab et al. (2015), the proportion of spruce monocultures increased throughout the Czech Republic, thereby endangering pristine aquatic habitats. Consequently, a qualitative analysis of the riparian forests is essential for an accurate evaluation of the riparian zone. However, this analysis was not conducted in the present study.

2.4.2 River sinuosity

River sinuosity has decreased throughout the study area. Contrary to our assumption, however, the decrease has been greater near the boundary than in the reference sites and greater in the northern part, where the freshwater pearl mussel is still present, than in the southern part where it has become extinct. River regulation has largely altered river morphology since the 19th century, resulting in homogenised flow regimes and reduced habitat diversity (Kemp et al., 1999). This affects pristine aquatic habitats such as those of the freshwater pearl mussel, which are characterised by a variety of flow velocities, sediment depositions, and stable riverbeds (Geist and Auerswald, 2007). As stream alteration can be a driver of mussel extinction, it was expected to be lower in the parts, where the mussel was still present. However, the results of this study indicated that the greatest decreases in river sinuosity occurred in squares 4, 5 and 6, where mussel populations remain present. The large decreases in river sinuosity mostly occurred before the 1950s, and thus occurred independently of the geopolitical history of the study area and within the time period of the first massive die-off documented for freshwater mussels in the Czech Republic (Simonab et al., 2015). According to Simonab et al. (2015), subsequent changes in land use, such as sewage pollution, intensified agriculture or drainage of wetlands, caused cumulative effects that prevented reproduction and survival of juvenile mussels, leading to the widespread collapse of the population structure of *M. margaritiefera.* Therefore, the decrease in river sinuosity may have weakened the population structure throughout the study area, but the reduced anthropogenic impact during the Cold War may have saved the remaining populations from extinction. Certainly, the present populations in the three border triangle of Bavaria, Saxony and Bohemia are not stable and its conservation was due to great efforts by nature conservation, which took over in the 1980s after the military exclusion zone was lifted (Stöckl et al., 2020). The finding, that sinuosity has declined particularly sharply in this area, suggests that stream morphology should be given greater consideration in conservation measures for freshwater pearl mussel populations.

2.4.3 Size of individual arable fields

The size of individual arable fields has increased throughout the study area, resembling the common pattern of land use change in industrialised areas in the 20th century (Orians and Lack (1992), Baessler and Klotz (2006)). The expansion of arable fields is associated with a loss of biodiversity for a number of reasons. It leads to habitat loss and fragmentation through the removal of ecotones such as hedgerows and field margins (Tscharntke et al., 2005). In addition, larger arable fields tend to be associated with more intensive farming practices, such as increased use of fertilisers and pesticides, and an increased risk of run-off, which carries pollutants, nutrients and sediments into nearby water bodies and adversely affects aquatic ecosystems (Schürings et al., 2024). However, this strong expansion of arable field size occurred mainly on the Bavarian side of the border, while on the Bohemian side there was a decline between the mid-20th century and today. The digitised maps (Appendix Figures A1 - A8) show, that this decrease is largely due to the conversion of arable land into grassland or pasture. Grasslands are generally more favourable for stream biodiversity than arable land Their cultivation requires fewer chemical inputs. Further, their continuous vegetation cover stabilises the soil and reduces erosion, which reduces the amount of sediment runoff into streams, while also acting as a natural buffer, filtering run-off and trapping sediment and excess nutrients before they reach streams (Kupiec et al., 2021). Thus, the disappearance of small-scaled structure since the mid-19th century represents a loss of landscape heterogeneity across the study area, which correlates with a loss of biodiversity (Hass et al., 2018). With respect to aquatic ecosystems, the impact of this development is greater, where it is associated with large arable fields. In areas where large arable fields were converted into grasslands, the buffer functions of margins and hedges may have been partially replaced. This conversion was a phenomenon for the Bohemian study area. It also occurred in the reference area, but was more pronounced in the border region, where it may have helped pearl mussel populations to outlast until conservation management began.

2.4.4 Area and transition of wetlands

The quantitative analysis of landscape structures in the study area showed, that wetlands were most extensive in the 1950s. After that, the overall extent decreased. However, in the north and in the references, the present extent is still larger than in the 1850s. In the south, this was also the case on the Bohemian side, but not on the Bavarian side, where the present extent is far below that of the 19th century. The overall expansion of wetlands in the mid-20th century is surprising, as it is generally reported that global wetland loss has been ongoing for at least the last three centuries (Davidson, 2014) and that improved drainage techniques have been used since the 19th century and intensified in the course of the modern agricultural revolution (Valipour et al., 2020). Furthermore, population growth and urbanisation, as well as agricultural expansion, are developments observed alongside the wetland expansion in this study, but are usually correlated with wetland loss (Mitsch, 2015). At the same time, the construction of the military zone along the former Iron Curtain has led to a reduction in population pressure and changes in agricultural practices, such as the abandonment of marginal farmland. These developments have allowed wetlands in Central Europe to recover or be restored in areas, where rural depopulation has led to the renaturalisation of landscapes (Poschlod, 2017). However, the observed expansion of wetlands was not limited to sites within the former military zone.

The mapping of dynamic landscapes, such as some wetland categories, is a challenge for cartography, as it is necessary to decide what condition should be recorded on the map. Before land drainage was pushed to its maximum in the middle of the 20th century due to political subsidisation, high groundwater levels, especially around streams, were common ground for cultivated meadows (Fluet-Chouinard et al., 2023). Consequently, the perception of wetlands at that particular point in time was distinct. It seems probable that cartographers only included areas of permanent wetlands on their maps, while regions that were alternately wet or cultivated despite their wet conditions were not included. To provide additional context and detail for the digitisation, aerial photographs were consulted in this study, which enabled the incorporation of all kinds of wet grounds into the digitised maps. However, these were only available from the 1950s onwards. It can thus be posited that the observed caesura in the expansion of the wetlands is attributable to the heterogeneity of the map sources between the time slices A to B and C to E. Consequently, the development within the last three time periods is comparable and reflects an ongoing decline of wetlands throughout the study area.

The most significant driver of wetland loss globally is the conversion of these areas into cropland (Fluet-Chouinard et al., 2023). In the present study, however, the conversion of wetlands into arable fields was of minor importance. In the southern part of the study area, a larger proportion of former wetlands were utilised as agricultural land during the 1980s and subsequently converted into open land. Conversely, the continuous conversion of wetlands into either open land (incorporating grassland, pasture, heath and wasteland) or forest was observed. In comparison to the conversion of wetlands into arable land, these types of land use have a lesser impact on aquatic ecosystems in terms of biodiversity, hydrology and water quality. Grasslands typically exhibit reduced surface runoff and erosion compared to arable land and require lower inputs of fertilisers and pesticides. However, a certain degree of drainage is also required, which reduces the capacity of the landscape to retain water. Furthermore, they are less effective than wetlands in slowing down water flow and filtering sediments, which still can result in increased sediment loads in adjacent water bodies (Mitsch, 2015).

Dry forests, especially if they consist of coniferous monocultures, have been observed to elevate evapotranspiration rates, which can diminish water availability in streams and rivers, particularly during periods of drought (Ellison et al., 2012). The transition from wetlands to dry forests can thus result in diminished base flows in proximate aquatic systems, which may impact species that depend on constant water levels. The third principal factor contributing to the loss of global wetlands was urban expansion (Fluet-Chouinard et al., 2023), which was also discernible in this study area, though to a greater extent only in the reference plots and predominantly between the 1950s and 1980s. Wetlands perform a pivotal function in sustaining aquatic ecosystems, offering indispensable ecological services such as water filtration, flood control, and habitat connectivity (Zedler and Kercher, 2005). In particular for the freshwater pearl mussel, wetlands are of significance due to their impact on water quality, the general health of the river systems in which the mussels reside, and the provision of wetland detritus for their diet (Hruska, 1999). Consequently, the observed conversions of wetlands into alternative land use structures can be regarded as a degradation of *M. margaritifera* habitats.

2.5 Conclusions

It was hypothesised that the freshwater pearl mussel has benefited from the geopolitical history of the study region; specifically, that habitat quality indicators are better conserved in border areas where they helped the species to persist. However, the study revealed, that the examined factors were present in all parts of the study area. The reduction of river sinuosity occurred largely before the construction of the military zone. The loss of small-scaled buffering structures was even pushed during communist times which resulted in largely increased sizes of arable fields. These, however, were less intensively managed in Bohemia. The loss and transition of wetlands and the reduction of open land in the surrounding of watercourses were present in all parts of the study area. It may therefore be concluded that the observed developments of land use have contributed to habitat degradation also in the restriction zones, but that the reduced human impact in this area prevented full extinction of the species at least in the northern study area. In order to ensure the long-term survival of remaining pearl mussel populations in the project region, it is essential to consider the potential impact of the proposed landscape developments.

3 Habitat requirements of *Myriophyllum alterniflorum* DC. in river stands of the Upper Palatinate Forest, Bavaria

This chapter literally quotes an article authored by Julia Sattler and Peter Poschlod, published in June 2023 in the journal Aquatic Botany 188, doi: 10.1016/j.aquabot.2023.103680.

Abstract

Biodiversity loss is, at present, one of the most severe global environmental issues. However, more demanding species are put at greater risk than generalists. Knowledge of the habitat requirements of threatened species is essential for defining the levels at which nature conservation efforts should prospectively operate. This study describes the habitat niche of the macrophyte species Myriophyllum alterniflorum DC., based on its occurrence along environmental gradients of sediments, flow velocity, turbidity, depth and shade on five brooks of the Upper Palatinate Forest, Bavaria. To examine the transregional validity of our results, we first carried out an analysis on the Pfreimd River and defined the outcomes as a reference. We then defined a transfer system for the other four brooks with M. alterniflorum occurrences, repeated the analysis and compared the results. We found a general pattern of preferred habitat conditions in combination with a regional aspect influencing the species distribution in each brook. We, suggest a broader formulation of habitat characteristics when aiming to describe a transregional pattern. Instead of preferences, we defined local extinction criteria for this species, namely: i) sediment with saprobic fraction, ii) stagnation of flow velocity, iii) strong turbidity iv) shading $>$ 75% and v) water depth $>$ 100 cm. Furthermore, we found that eutrophication is less effective under high-flow conditions. With its high demands on water quality, M. alterniflorum overlaps with the requirements of other endangered species. Therefore, conservation efforts should focus on promoting high-quality habitats, which include sustainable land management approaches at the catchment level.

3.1 Introduction

Freshwater ecosystems are among the most endangered ecosystems in the world (Grabowski et al., 2020). There is a great variety of scientific studies dealing with freshwater biodiversity (Grabowski et al., 2020; Tickner et al., 2020). Most of these focus on the fauna, while the flora is often considered in the context of its role as an ecosystem engineer, e.g. providing of habitats, regulating nutrient cycling, stabilising sediments, controlling oxygen or alkalinity (Sand-Jensen and Pedersen, 1999; Thomaz, 2021) or for its bioindication potential (Haury et al., 2006; Szpakowska et al., 2021). Macrophytes live in extraordinarily close interaction with the environment. Being sessile, they are directly exposed to the local, chemical and physical conditions of the surrounding water. Consequently, we can observe a high turnover in macrophyte communities when environmental conditions change rapidly (García-Girón et al., 2018).

The use of macrophytes as indicators of water quality has been increasingly promoted since the 1970s, when several indices for water quality assessment using macrophytes were published (Robach et al., 1996; Haury et al., 2006), and was widely accepted after the introduction of the European Water Framework Directive (WFD). Fundamentally, however, it must be kept in mind that the hydrological regime, with factors such as flow velocity or substrate granulometry, is primarily responsible for the occurrence of a species (Chatenet et al., 2006; Bornette and Puijalon, 2011). A correct interpretation of the species composition based on physical, chemical or ecological deficits is therefore only realistic, if these factors are known for the focal species and taken into account when deriving water quality assessments. We therefore consider knowledge of the habitat preferences of individual species to be crucial. Not only in terms of its potential as indicator, but also in terms of species conservation.

Myriophyllum alterniflorum DC. (Haloragaceae) is described as characteristic of the order of Littorelletalia, but is also centred in the Ranunculion-Callitrichetum (Mucina et al., 2016). Its habitat is characterised by shallow, soft, oligotrophic waters (Haury et al., 2006), where it inhabits both, standing and flowing waters (IUCN, 2022). In the former, it seems to prefer rather intermediate conditions in terms of pH, calcium, nitrate, conductivity and carbonate hardness. It may therefore even indicate eutrophication when compared to communities dominated by *Litorella uniflora* (Klosowski and Szankowski, 2004). In the latter, the species prefers oligotrophic, oxygen-rich, shallow and lotic sites (Zueva et al., 2022).

It is noteworthy that some Floras describe *M. alterniflorum* primarily as a standing water species (Voss, 1985; Oberndorfer, 2001). Nevertheless, the species has been found to tolerate high flow velocities in upland rivers (Keruzoré and Willby, 2014). However, the literature is full of studies conducted in standing water only (Klosowski and Szankowski, 2004; Caffrey et al., 2006) and others that do not distinguish between lentic and lotic habitats (Harris et al., 1992). In Bavaria, the species occurs almost exclusively in running waters (Woschée et al., 2019) and there are only few studies that explicitly address species preferences in this habitat type (Wiegleb, 1984; Poschlod et al., 2010).

Globally and in Europe, *M. alterniflorum* is classified as a species of least concern according to the Red List of Threatened Species (IUCN, 2022). However, in some parts of its Palaearctic range, e.g. in parts of Canada and some North American states or several European countries (e.g. Czech Republic, Switzerland, Italy), *M. alterniflorum* has a conservation status of critically endangered, endangered or vulnerable (IUCN, 2022). In Germany, the species is currently critically endangered (Red List category 2, Haupt et al. (2018)). Acidification and eutrophication have been identified as key factors (Brandrud and Johansen, 1994).

With our study of the habitat conditions of *M. alterniflorum* in five different brooks of the Upper Palatinate Forest, we aim to contribute to this field of knowledge by answering the following question:

Which environmental variables and chemical factors are crucial for the occurrence of *M. alterniflorum* in running waters?

a) Which factors determine its distribution in the river Pfreimd?

b) Can these findings be transferred to other brooks in the Upper Palatinate Forest?

c) How does flow velocity interact with nitrogen pollution in flow regulated streams?

3.2 Material and methods

3.2.1 Study area

The studied brooks, Pfreimd, Ascha, Bohemian and Bavarian Schwarzach and Tirschenreuther Waldnaab, are located in north-eastern Bavaria, close to the German-Czech border (Figure 10). The area covers the German part of the Bohemian Massif, the border mountain range to the Czech Republic, and is the main distribution centre of *M. alterniflorum* in Bavaria (Woschée et al., 2019).

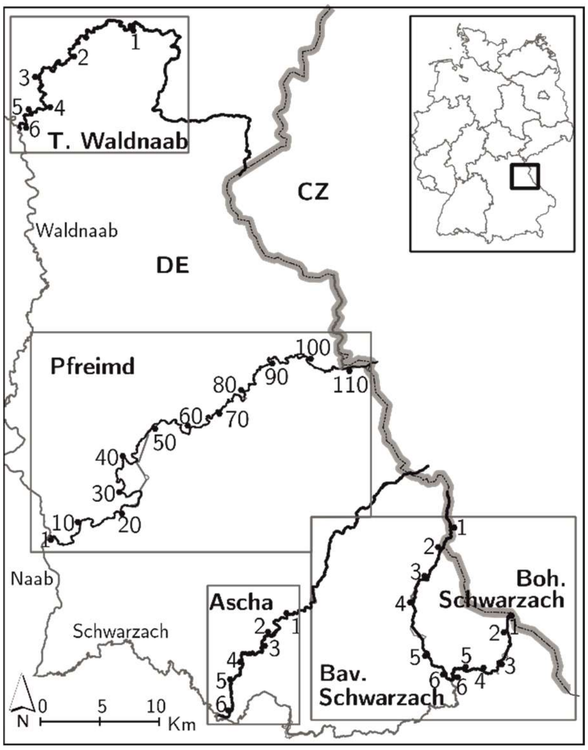

Figure 10: Distribution of sampling sites in the upper palatine forest close to the border between Germany (DE) and Czech Republic (CZ). Sampling sites are located in the brooks Pfreimd, Ascha, Bohemian and Bavarian Schwarzach and Tirschenreuther Waldnaab. Basic geodata reference: Bavarian Surveying

As these highlands consist of siliceous bedrock, the brooks studied are characterised by soft water. They are further described as cool, fast or moderately fast flowing with coarse to fine sediments (UBA, 2016).

The Pfreimd is the largest of the five rivers. With a length of 76.5 km, it flows more or less meandering in a south-westerly direction from its source near the Czech town of Lesná to its confluence with the river Naab. The water is naturally shallow and fast-flowing and varies in width from 5 to 10 (40) meters (WWA Weiden, 2018). On the German side, there are four larger and nine smaller weirs, which alter the flow velocity and create lakes up to 200 m wide.

The three brooks that join into the Schwarzach (Ascha, Bavarian and Bohemian Schwarzach) are similar in length and size. While the Ascha flows more or less freely through forests, agricultural areas and small villages on its 41.8 km way down to its mouth, the Bavarian and Bohemian Schwarzach have undergone several regulation measures on their shorter length of 25 km (Bayr. LfU, 2016). Both brooks are dammed to reservoirs and the catchment area of the Bavarian Schwarzach in particular has been largely deforested, with large areas of grassland often mown right up to the banks.

With a length of only 10.9 km, the Tirschenreuther Waldnaab is the smallest of the five brooks. Its meandering character has been modified in its upper and middle reaches, where it also flows through open land and settlements (Bayr. LfU, 2016). However, the last 6 km upstream to its mouth are free flowing through the forest or extensively used grassland. The chemical characteristics of all five brooks are given in Table 2.

Table 2: Chemical characterisation of all analysed brooks. Pfreimd – mean of measures from 110 samples; reference system – mean of measures from 6 samples per site. SD = standard deviation. * spearman rank correlation between *M. alterniflorum* coverage and the chemical variable, p-value and correlation coefficient rho. ** generalized linear model of *M. alterniflorum* occurrence (response) and the chemical variables as predictors. Estimates (est.) give direction of the relationship.

	value	pH	conductivity [µS cm^{-1}]	NH_4^+ [mg l^{-1}]	NO_3^- [mg l^{-1}]	PO_4^{3-} [mg l^{-1}]	Ca^{2+} [mg l^{-1}]
Pfreimd	mean	8.03	238.76	0.05	1.95	0.33	17.26
	SD	0.39	16.51	0.07	2.49	0.74	2.62
Ascha	mean	7.22	174.67	0.05	2.37	0.47	17.26
	SD	0.20	12.88	0.08	0.21	0.46	2.62
Bavarian	mean	7.28	137.33	0.03	1.92	0.36	9.17
Schwarzach	SD	0.12	14.45	0.04	0.33	0.17	2.38
Bohemian	mean	6.70	115.83	0.06	1.12	0.28	6.95
Schwarzach	SD	0.30	20.62	0.05	0.52	0.21	1.12
Tirschenr.	mean	7.46	274.00	0.02	1.98	0.58	17.88
Waldnaab	SD	0.32	10.30	0.01	0.44	0.18	1.66
M. alterniflorum	p	**0.00**	0.09	0.98	**0.02**	0.18	0.58
cover*	rho	0.36	0.18	0.00	0.26	0.15	0.06
M. alterniflorum	p	**0.00**	0.14	0.72	**0.03**	0.91	0.31
occurrence**	est.	2.57	- 0.02	-1.58	0.21	-0.05	0.12

3.2.2 Study design and sampled populations

During the summer months of July and August of 2012, 110 sections of 20 m length each were mapped every 500 m from the Czech border to the confluence of the Pfreimd and Naab rivers. In each section, vegetation composition and cover were determined on a five-level scale according to Poschlod et al. (2010). Species were determined down to species level, except for *Ranunculus* species, according to van de Weyer and Schmidt (2007). Six environmental variables, sediment class, depth, shading, flow velocity and turbidity, were assessed according to the scales given in Table 3.

Table 3: Environmental variables annotated at every section, classification and n number of sections matched with respective classes. Determination coefficient R^2 refers to the trend line in Figure 12, estimates (est.) give direction of relation and p-value significance as derived from logistic regression.

	A: sediment [1-5]	B: depth [cm]	C: shade [%]	D: flow [1-5]	E: turbidity [1-4]
1	gravel, stones (n = 15)	<25 (n = 11)	<10 (n = 30)	stagnant (n = 17)	clear (n = 18)
2	sand, gravel, stones (n = 37)	25-50 (n = 38)	10 – 25 (n = 22)	little (n = 53)	weakly (n = 30)
3	sand (n = 9)	50-75 (n = 17)	25 – 50 (n = 15)	middle, turbulences (n = 33)	moderate (n = 39)
4	mud, gravel, stones (n = 16)	75-100 (n = 18)	50 – 75 (n = 20)	high, turbulent (n = 6)	strong (n = 19)
5	mud, sand (n = 18)	100-125 (n = 7)	75 – 100 (n = 22)	-	-
6	mud (n = 14)	>125 (n = 18)	-	-	-
R^2	0.85	0.94	0.88	0.85	0.97
est.	-1.28	-1.48	-0.19	2.15	-2.18
p	0.00	0.00	0.31	0.00	0.00

Conductivity and pH were measured directly in the field using a DWD Multi 3320 multimeter with TetraCon® 325 and SenTix® 41 placed in the water in a central part of the section. For further chemical analysis in the laboratory, water samples of 500 ml each were taken from the central part of the section close to the river bed, filtered through a 0.2 µm nitrocellulose syringe membrane and frozen. A maximum of two months after sampling, the samples were analysed for ammonium, calcium, nitrate and phosphate using a Dionex® DX-120 ion chromatograph. We used the results from the Pfreimd as *reference system* to test whether they could be transferred to other sites. Therefore, the rivers Ascha, Bavarian- and Bohemian Schwarzach and Tirschenreuther Waldnaab, hereafter referred to as the *transfer system*, were mapped analogously in August 2020 at six representative sites each. Chemical analyses were carried out with pHotoFlex® Turb for phosphate, nitrate and ammonium according to the manufacturer's programme protocols 315 (ortho-phosphate), 314 (nitrate) and 312 (ammonium vario LR), and with ThermoElemental-ASX-510 atomic absorption spectrometer (AAS) for calcium.

3.2.3 Statistical analyses

The occurrence and abundance of *M. alterniflorum* in the Pfreimd were analysed in R 4.1.2 (R Core Team, 2021) along the environmental gradients obtained. We tested the normal distribution of our environmental variables with Shapiro-Wilk test. As this was not given and could not be obtained by transformation, we performed non-parametric tests. To determine which category of each environmental variable co-occurred with the highest mean cover of *M. alterniflorum*, we performed pairwise comparisons using the Wilcoxon rank-sum test with Bonferroni continuity correction from the R package 'stats' (R Core Team, 2013). The frequency of occurrence of *M. alterniflorum* between a) the categories of environmental variables, and b) the chemical measurements were calculated using logistic regression in R package 'mgcv' 1.8-34 (Wood, 2011).

In order to visualise differences in vegetation composition between the observed sections, we performed non-metric multidimensional scaling (NMDS) in the R package 'vegan' 2.5-7 (Oksanen, 2022). The maximum number of random starts was set to 100 and a three-dimensional NMDS was performed using the function 'metaMDS' with Bray-Curtis dissimilarity and species abundance per site as the primary matrix. Ordination performed with a Kruskal's Stress of 0.07, which is considered as a good representation of the observed distances between the individual samples in space (Clarke, 1993). To reveal more information about differences between the sites, we correlated environmental gradient and species vectors with the ordination axis using the function 'envfit' implemented in the R package 'vegan' R package. We limited the display of environmental vectors with a cut-off for R^2 at 0.3.

The same method was used to compare conditions in the *transfer system* with those at the *reference* from the Pfreimd.

The influence of water retention through the six weirs in the Pfreimd, in combination with pollution by elevated nitrate concentrations on the occurrence of *M. alterniflorum* was analysed using data on the estimated backlog length of the weirs, provided by the authority for water resources (WWA) Weiden. We implemented these data in ArcGIS (ESRI, 2020) and calculated the distance of our sampling sections from the weirs and the corresponding backlog. We fitted a linear regression model with *M. alterniflorum* coverage as the response and distance from backlog, as well as an interaction term of nitrate concentration and flow velocity as predictor variables. To visualise the results, we generated an effect-plot using the 'allEffects'-function of the R package 'effects' (Fox and Weisberg, 2019). This plotted the predicted effect of flow velocity on *M. alterniflorum* cover at different levels of nitrate concentration.

3.3 Results

The measured chemical variables differed between the analysed brooks (Table 2). pH ranged from 8.03 in the Pfreimd to 6.7 in the Bohemian Schwarzach and was positively correlated with the occurrence and cover of *M. alterniflorum.* Conductivity was highest in the Tirschenreuther Waldnaab (274 µS cm^{-1}), followed by the Pfreimd (239 µS cm^{-1}), Ascha (175 µS cm^{-1}), Bavarian (137 µS cm^{-1}) and Bohemian (116 µS cm^{-1}) Schwarzach. It was significantly positively correlated with *M. alterniflorum* cover, but not with its occurrence. Ammonium concentrations were always low (<0.1 mg l^{-1}) and did not correlate with the presence of *M. alterniflorum.* The highest nitrate concentrations were measured in the Ascha (2.4 mg l^{-1}) and the lowest in the Bohemian Schwarzach (1.1 mg l^{-1}). Other values were around 2 mg l^{-1}. Logistic regression showed a positive correlation between nitrate concentration and the occurrence of *M. alterniflorum*, while coverage was not affected. Phosphate and calcium were not correlated with *M. alterniflorum.* Phosphate concentration was also low (0.3 - 0.6 mg l^{-1}), calcium was more variable ranging from 7.0 mg l^{-1} (Bohemian Schwarzach) to 17.9 mg l^{-1} (Tirschenreuther Waldnaab).

3.3.1 Analyses of the river Pfreimd

NMDS ordination distinguished the vegetation composition of sections in the Pfreimd where *M. alterniflorum* occurred from those where it was absent (Figure 11). The vectors of environmental and species gradients showed that the species *Lemna minor* and

M. alterniflorum occurred at sites with opposite conditions. While L*. minor* sites were also correlated with turbidity, finer sediments and greater depths, *M. alterniflorum* sites tended to be correlated with higher nitrate concentrations and higher flow velocities. *Ranunculus* species also tended to be associated with *M. alterniflorum,* whereas the *Callitriche* species *C. hamulata* and *C. cophocarpa* were intermediate. The grey asterisks in the diagram represent the sites that were locaded within the backlog of weirs. These were also positioned opposite to *M. alterniflorum* sites and *L. minor* was more likely to occur here.

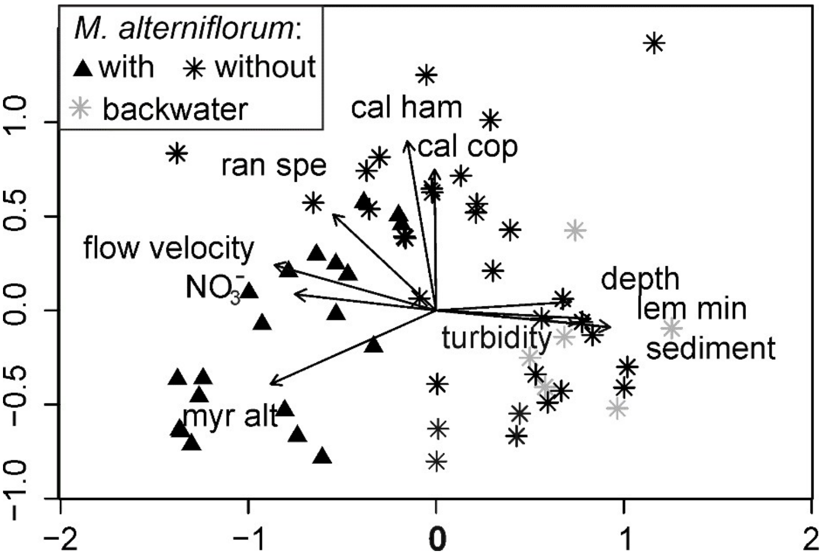

Figure 11: NMDS diagram of species composition in sampled sections in Pfreimd river. Sites were correlated with environmental and species vectors with a cut-off of 0.3. cal ham = *Calllitriche hamulata*, cal cop = *C. cophocarpa*, lem min = *Lemna minor*, myr alt = *Myriophyllum alterniflorum*, ran spe = *Ranunculus* spp. Scores of vectors are given in the appendix in Table A.2. It is distinguished between sites with and without occurrences of *M. alterniflorum.* Grey asterisks refer to sites located within the backlog of weirs.

3.3.2 Influence of single environmental variables in the Pfreimd and in the transfer system

Among the six classes of sediment types (Figure 12a), we found that *M. alterniflorum* reached the highest mean cover when the sediment consisted of sand, gravel and stones (class 2), followed by class 1, which consisted of gravel and stones. This is true for both, the Pfreimd and the *transfer system.* In the latter, however, *M. alterniflorum* reached a higher mean cover than in the Pfreimd. Class 3 sites were only occupied in the Pfreimd with a mean cover of 2.5 %. Finer sediments (classes 4 - 6) were not colonised. No significant differences were found between the mean cover of colonised classes 1 - 3, but the trend line over occurrence rate and sediment class showed a significant decrease towards finer sediment types.

Along the depth gradient (Figure 12b), *M. alterniflorum* decreased in both, mean cover and occurrence rates. The shallowest sites of less than 50 cm in depth (classes 1 and 2) had the highest mean cover and differed significantly from sites with > 75 cm depth (classes 4 - 6) where the species was very rare. In the *transfer system*, sites with a depth of 25 - 50 cm were more densely populated than in the Pfreimd. This negative correlation was highly significant.

The variable shading (Figure 12c) affected the mean cover of *M. alterniflorum,* but the pattern differed between the *transfer system* and the Pfreimd in the first two categories. Whereas in the Pfreimd, *M. alterniflorum* was most abundant and had the highest cover when 25 - 50 % of the area was shaded, in the *transfer system* cover was highest in sites with less than 10 % of shading and decreased continuously as shading increased. Shading of more than 75 % completely excluded the species. The differences in mean cover between class three and class five were significant. The frequency of occurrence rate showed a polynomial curve with a maximum in class 3, medium high shading (26 - 50 %), which was the most frequently populated.

Flow velocity (Figure 12d) showed a clear positive correlation with the occurrence and abundance of *M. alterniflorum.* While the species was completely absent from sites with stagnant flow (class 1), it had the highest cover in moderately high flow with turbulences (class 3) and the second highest in fast-flowing and turbulent waters (class 4). Mean cover differed significantly between class 3 and the first two classes where the flow was slow or stagnant. The correlation between occurrence rate and flow velocity was positive, linear and highly significant.

Turbidity (Figure 12e) was negatively correlated with the occurrence and abundance of *M. alterniflorum.* The species was predominantly found at sites without turbidity, where it also reached the highest abundance. In the *transfer system*, a high cover at medium turbidity was an outlier but did not affect the general pattern. Mean abundances in sites with clear water differed significantly from those with any degree of turbidity. The strong decrease in occurrence rate from clear to turbid water was highly significant.

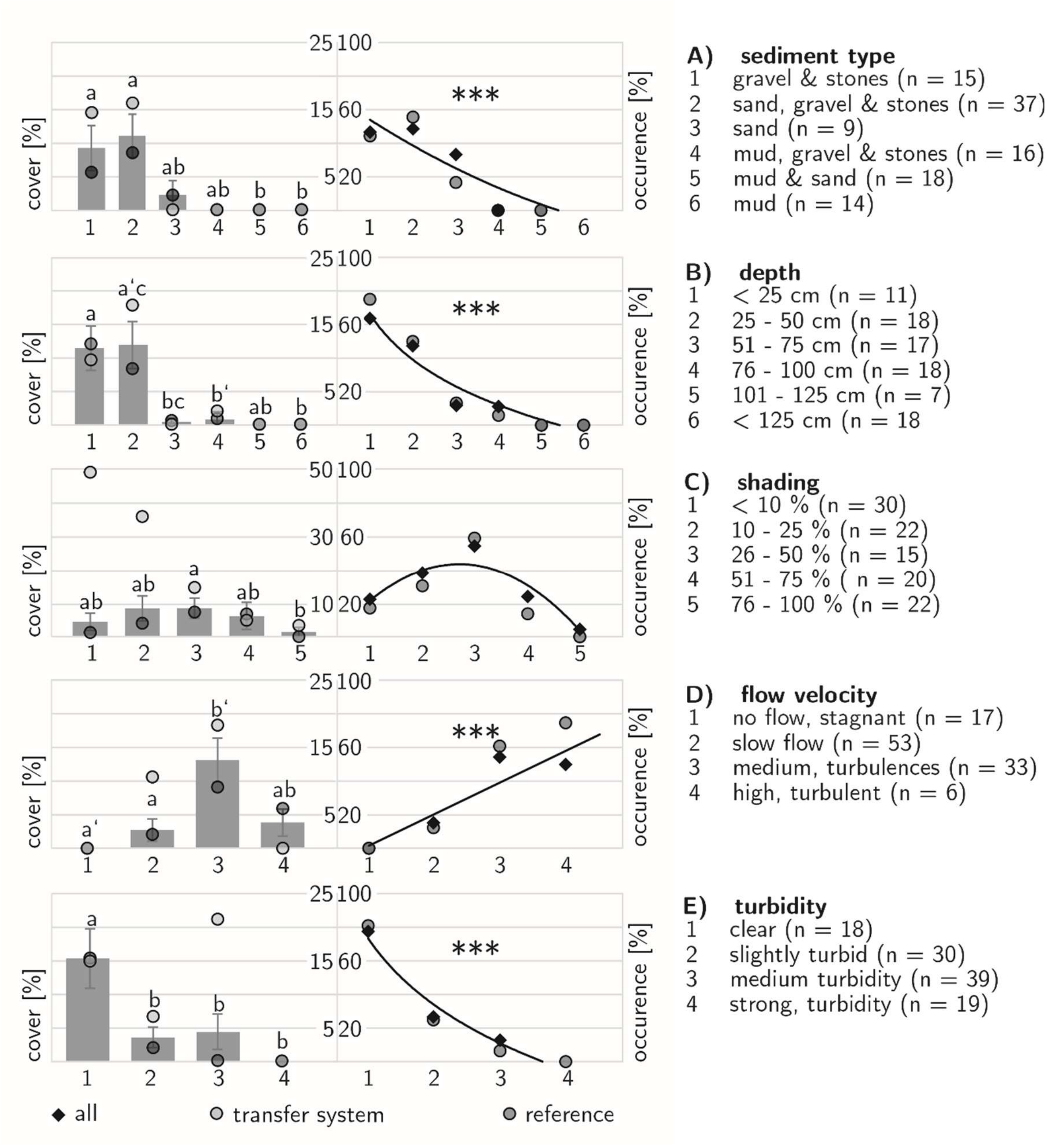

Figure 12: Abundance of *M. alterniflorum* along environmental gradients. Left: mean values of *M. alterniflorum* cover per class of each variable. All observations are represented by the bars and compared with pairwise Wilcoxon Rank Sum Test. Different letters mark significant differences ($p \leq 0.05$), values of $0.05 \leq p \leq 0.1$ marked with ‘. Right: Percentage of each class of the respective variable with *M. alterniflorum* occurrence. Significance level of logistic regression is $p \leq 0.05$: *. R^2, logistic regression estimate and p-value refer to all observed sites and are given in Table 3.

When compared on the basis of their environmental conditions, the differences between the observed brooks are greater than within each system. This is clear from the NMDS plot (Figure 13), where all the sites belonging to the same brook are encircled and show more or less distinctness from other brooks. Thus, sites from the Tirschenreuther Waldnaab are correlated with higher flow velocities, while those from the Bavarian and Bohemian Schwarzach are rather correlated with higher concentrations of ammonium concentrations and overlap with each other as well as with sites from the Ascha, which are more widely distributed, indicating a higher heterogeneity in between. This heterogeneity is more pronounced in the Pfreimd, which, however, has ten times more observations and contains sites correlated with higher conductivity, nitrate and calcium concentrations and others where turbidity and depth are more strongly represented.

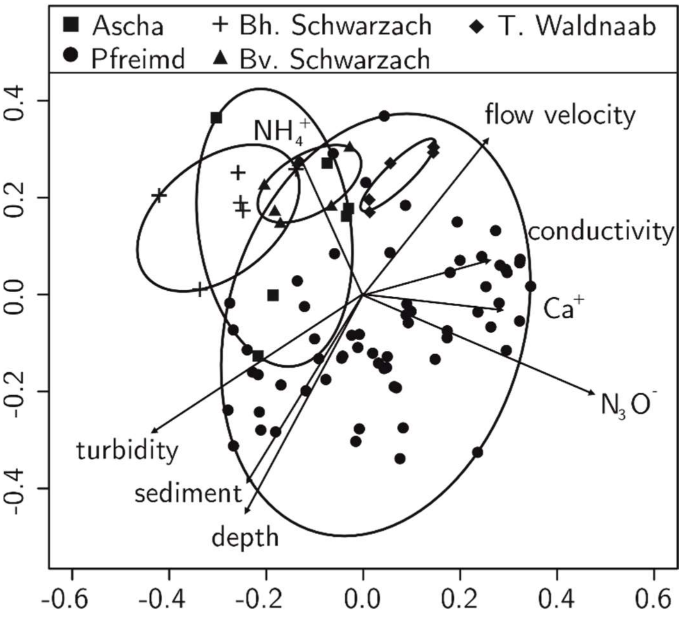

Figure 13: NMDS diagram of the sampled sections in all five brooks: Pfreimd, Bavarian (Bv.) and Bohemian (Bh.) Schwarzach, Ascha, Tirschenreuther (T.) Waldnaab, among environmental conditions. Different brooks are additionally enveloped with ellipses. Environmental vectors are correlated with a cut-off of 0.3, scores are given in appendix Table A. 3.

3.3.3 Influence of flow regulation on flow velocity and nitrate concentration

M. alterniflorum cover increased significantly with distance from the back of the weirs in the Pfreimd (Figure 14). The interaction term visualised in the right part of the figure shows that the positive relationship between flow velocity and *M. alterniflorum* cover was strongest when nitrate concentrations were high. At nitrate concentrations below 3 mg l^{-1}, flow velocity had no impact. As NO_3^- increased above 3 mg·l^{-1}, the correlation became weak, and at concentrations above 6 mg l^{-1} clearly positive.

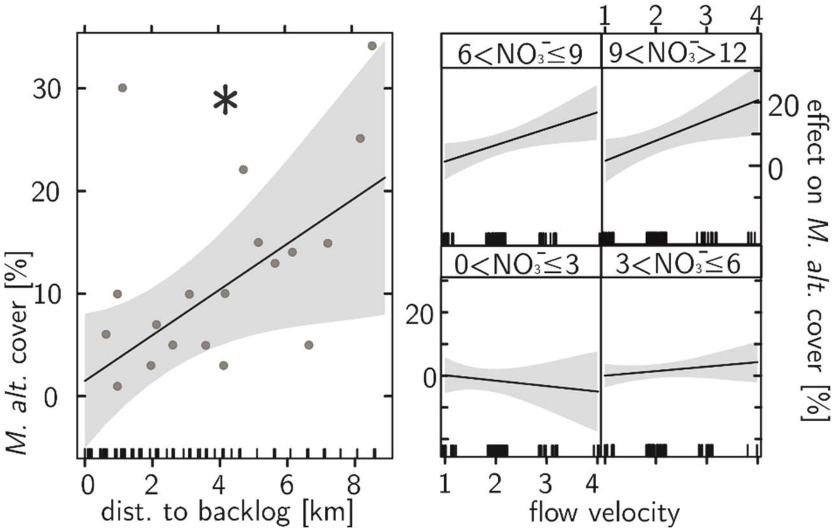

Figure 14: Cover of *M. alterniflorum* along distance from backlog of weirs in the Pfreimd and effect of flow velocity (class 1 - 4) at different levels of nitrate concentration as interaction. P-values: intercept = 0.82, dist. Backlog = 0.03, nitrate [mg l-1] = 0.46, flow = 0.28, nitrate*flow = 0.09.

3.4 Discussion

3.4.1 Habitat conditions

Among the chemical factors measured, we found a positive effect of pH and nitrate concentration on the occurrence of *M. alterniflorum.* This species is described as a typical soft water macrophyte (Riis et al., 2000) and as such, it is adapted to lower carbon availability and lower pH value. In lakes *M. alterniflorum* was shown to be limited by CO_2 below a concentration of 90 µmol l^{-1}. Increased CO_2 and nutrient availability promoted its growth leading to an increased competitiveness (Spierenburg et al., 2009). Similarly, elevated pH has been shown to promote *M. alterniflorum* in Lobelia lakes, where it replaces isoetid species (Ronowski et al., 2020). Although we did not measure CO_2 concentration or carbon availability, the positive correlation of *M. alterniflorum* abundance with pH value may indirectly indicate a better carbon supply and thus promote the species. However, it is unlikely that this will continue as conditions become more alkaline. Species adapted to high pH are often able to use bicarbonate in addition to carbon dioxide, which is not the case for *M. alterniflorum*, but for its sister species *M. spicatum* (Riis et al., 2000), which is replacing *M. alterniflorum* in the Naab River, the main river of the Pfreimd (Poschlod et al., 2010).

The environmental conditions favouring the occurrence of *M. alterniflorum* were i) high flow velocity, ii) very low to no turbidity, iii) sediment composition of sand, stones and rocks, iv) depth between 25 and 75 cm and v) shading between 25 and 50 %. Such factors are known to shape the vegetation community of a stream (Robach et al., 1996) and are therefore useful for describing macrophyte habitats.

The highly positive correlation of *M. alterniflorum* occurrence and abundance with flow velocity and its exclusion from sites with stagnant water sites is remarkable, given that the species is commonly found in lakes. Some floras describe slow-flowing waters as riverine habitats (e.g. Haines (2011), Lauber et al. (2018)). However, it has been reported from the Fennoscandian River Paz, that *M. alterniflorum* was the only macrophyte occupying sites with velocities of up to 1 m s^{-1} (Zueva et al., 2022). The ability to withstand the high mechanical stress of a turbulent and fast flow regime may be a key to the success of the species in riverine stands. It allows growth under low competition and allows the species to benefit from the enhanced nutrient and gas fluxes induced by water movement, which increase photosynthesis and plant growth (Bornette and Puijalon, 2011).

Sediment composition has been reported to influence on *M. alterniflorum.* Aiken (1981) showed that this species responds morphologically to different sediments. In rivers of the Limousin region, France, fine sediments accumulated in slow flowing sections and were indirectly related to plant chlorosis through increased turbidity and depth (Chatenet et al., 2006). Increased sediment input due to increased erosion in the environment could amplify this effect (Denic and Geist, 2015).

The factors of turbidity, depth and shading are among others related to light attenuation in aquatic ecosystems, which limits the occurrence of macrophytes (Middelboe and Markager, 1997). The optimum depth of 25 - 75 cm observed here could therefore be shifted to deeper zones when turbidity is reduced. Such an observation has been made in a German lake, where *M. alterniflorum* occurred up to 6 m depth, but was restricted to 3 m depth when turbidity increased (Vöge, 1993). Shading may interact with depth as a function of light availability and temperature. The optimum temperature for macrophyte growth is generally high (20 - 35 °C; Bornette and Puijalon, 2011). However, *M. alterniflorum* occurs in relatively cool waters below 20 °C, where it is thought to have a competitive advantage (IUCN, 2022). Extensive shading, as occurs naturally along the highland brooks of the Upper Palatinate Forest would keep the water cool even at shallower sites, which would explain the optimum observed here at intermediate levels of shading between 25 and 50 %. If the species were to migrate to deeper zones, where temperature and light are naturally lower, shading would be counterproductive. Such interactions make it necessary to broaden the range of species habitat requirements. This is also suggested by the regional pattern we observed when comparing the *transfer system* with the Pfreimd.

3.4.2 Comparison between the reference- and the transfer system

The comparison between the sampled brooks showed that there is a regional pattern, especially with regard to the conditions in which high cover of *M. alterniflorum* was found. Thus, slightly deeper zones of up to 50 cm, and greater exposure to sunlight, 75 - 100 %, showed a higher mean cover of the species in the brooks of the *transfer system* as compared to the pattern in the Pfreimd.

It was shown that the environmental conditions of the compared water bodies have a regional aspect which is related to their geographical location (see Figure 1). This reflects how multiple processes at different spatio-temporal scales shape the biodiversity of a particular area, including human activities in the local catchment (Allan et al., 1997).

Nevertheless, we see that the regional effect does not complement the general one. However, we infer that the formulation of extinction criteria would be more robust to describe a transregional distribution pattern. Based on our observations, these would be i) sediment with saprobic fraction, ii) stagnant flow, iii) strong turbidity, iv) shading > 75 % and v) depth > 100 cm.

3.4.3 Influence of flow velocity and nitrate in a regulated river

While *M. alterniflorum* commonly occurs in standing water habitats, we found that stagnation of flow velocity in running water was an extinction criterion for the species. Most likely, turbidity, depth and accumulation of pollutants play a role in these cases (Schmutz and Sendzimir, 2018). Flow regime is one of the most important drivers for biodiversity in running water systems (Bunn and Arthington, 2002). According to Wiegleb (1984), *M. alterniflorum* can only tolerate elevated nitrate levels if flow velocity is high enough, as otherwise it will be overgrown by algae. It is also likely, that competition from other macrophytes, such as *Elodea canadensis,* plays a role in stagnant streams with higher nutrient loads, as the growth and diversity of macrophyte species is negatively correlated with flow velocity (Madsen et al., 2001). In the Pfreimd, we observed a positive correlation between flow velocity and the occurrence of *M. alterniflorum* only with increasing nitrate concentration. Chatenet et al. (2006) analysed populations of *M. alterniflorum* for their reactivity to nutrient enrichment in rivers of the Limousin region in France, and described an adaptation of the species to nutrient enrichment. Our hydrochemical analysis showed, that *M. alterniflorum* is positively affected by increased nitrate levels when flow velocity is also high. This suggests, that it may not be a toxic effect of the elevated nitrate concentrations, as it can occur at high levels of around 10 mg l^{-1} (Li et al., 2020), but a competitive disadvantage with other species that causes *M. alterniflorum* to disappear in the course of eutrophication.

3.4.4 Conclusions

As stated before, the conservation status of *M. alterniflorum* is very heterogeneous. It is classified as a species of *least concern* (IUCN, 2022) globally and to a European extent. However, there are several regions where *M. alterniflorum* is classified as *endangered* or c*ritically endangered* (e.g. parts of Canada and some North American states). Examples of critical status in Europe are Switzerland (*endangered*), Czech Republic (*critically endangered*), Italy (*vulnerable*) or Germany (*highly endangered*) (IUCN, 2022).

For the German federal states of Bavaria, Lower Saxony and Schleswig-Holstein, the situation of *M. alterniflorum* has improved slightly in the post-millennial years. Long-term monitoring in the Pfreimd showed a continuous decline of the species from 1972 to 2004 (Poschlod et al. 2010). However, when we repeated the monitoring in 2020 (data unpublished), we also found a slight recovery as compared to 2004, which could be attributed to an improvement in hydro-chemical conditions. Nevertheless, the status is still far from that of 1972, when the monitoring started.

In addition to hydrochemical conditions, flow regime was found to be an important criterion for habitat suitability. Hydromorphological derogation has been identified as a major cause of biodiversity loss (Rolls et al., 2012). The effect of flow regulation on the occurrence of *M. alterniflorum* was increased with increasing nitrate concentrations. With sight towards conservation, this means that unregulated reaches where natural water flow can recover to some extent are needed in combination with reduced nutrient and sediment inputs. Buffer structures for terrestrial influxes are urgently needed, not only as part of riparian strips, but also at other potential sediment and pollutant sources, such as croplands, fish ponds or secondary drainage networks, which are often associated with modern transport systems (Geist and Auerswald, 2019). With these assertions, we go in line with authors, who arrogate the same for clear water species, such as the freshwater pearl mussel *Margaritifera margaritifera* (Denic and Geist, 2015; Grzybowski and Glińska-Lewczuk, 2019). The measures taken to implement the WFD have shown some effects in maintaining or even improving the ecological status of surface waters. However, in order to counteract the extinction of species linked to very good water quality, approaches with a broader impact are needed, including land use and management in the whole catchment.

3.4.5 Outlook and attempt to further research

What remains to be answered, is the question of differences in habitat requirements between standing and flowing water sites. In addition to possible morphological adaptations that allow resistance to high flow regimes, adaptations in the reproductive strategy would also be an important point. We never found any evidence of the species flowering in flowing water, whereas it is generally known to produce its alternate flowers from June to July (Harris et al., 1992).

In this context, it would be interesting to analyse the degree of genetic differentiation between standing and flowing water populations. Harris et al. (1992) analysed the genetic variation of different populations and found high levels of genetic diversity among and within populations of both, river and lake populations compared to other macrophyte species. According to the authors, this may be due to the high capacity of *M. alterniflorum* to produce viable seeds after wind pollination. The high genetic diversity found in these populations may be the basis for their adaptability to environmental changes, as observed by several authors (Aiken, 1981; Chatenet et al., 2006; Delmail et al., 2011). At the same time, *M. alterniflorum* has been described as highly sensitive to environmental change (Wiegleb, 1984). A switch to exclusive vegetative reproduction in running waters could reduce the level of genetic diversity, leading to a reduced ability to adapt to changing environmental conditions (Ellegren and Galtier, 2016), potentially making the species more vulnerable in its riverine stands.

4 The habitat of *Margaritifera margaritifera* in application of environmental metabarcoding

4.1 Introduction

The freshwater pearl mussel (*Margaritifera margaritifera*) is currently one of the world's most endangered freshwater organisms (Geist, 2005). The species historically thrived in oligotrophic streams with siliceous bedrock, but has experienced a rapid decline in recent decades. Besides the direct effects of pearl fisheries and predation, habitat alteration and loss of habitat are a major contributing factor (Bauer, 1988). Adverse impacts can come from within the catchment, where intensive agriculture or forestry, sewage treatment or other pollution causes eutrophication, acidification, siltation or toxification of water and interstitial space. In addition, direct impacts on the stream ecosystems such as damming, river regulation or dredging, affect aquatic habitats by altering river morphology, thus causing loss of natural river banks and impeding fish migration. Finally, the effects of climate change and associated species migration put pressure on natural pearl mussel habitats (Araujo and Ramos, 2000).

Comprehensive conservation efforts have been crucial in preventing the extinction of *M. margaritifera*. These efforts include in situ and ex situ management of populations (Moorkens, 2000), management of the catchment areas (Geist, 2010), and extensive research on the habitat and biology of the freshwater pearl mussel (i.e. Roscoe and Redelings (1964), Bauer (1987), Hastie et al. (2003), Täubert and Geist (2017)). Despite these efforts, the species' status remains critical across Europe and worldwide, with most populations being overaged and exhibiting limited recruitment (Geist, 2005).

A major component of habitat quality is the supply with suitable food resources (Hruska, 1999). *M. margaritifera* filter-feeds from small planktonic particles that float in the water (Baker & Levinton, 2003; Gramstad, 2014). There are indications that detritus particles are preferred by the mussel, although algae food is more efficient in increasing growth rates (Eybe et al., 2013). In mussel hatcheries, certain food mixtures are successfully used to feed juveniles. These are often composed of algae mixtures to supply fatty acids, detritus, which is collected in surrounded wetlands and sometimes *Chironomid* larvae as protein source (Geist et al., 2023). What remains unclear is which species may donate to the preferred diet of

M. margaritifera in its natural habitat and if the species thought to be relevant actually occur in the natural pearl mussel sites.

The inflow of terrestrial detritus into streams can be enhanced by floodplain meadows connected either directly to the stream or by drainage ditches. At such ditches, detritus is being collected for use in ex-situ mussel breeding which suggests that wet or periodically flooded meadows can contribute to the quality of pearl mussel habitats (Hruska, 1999). This type of land-water-connection has become rare as alluvial meadows have been progressively drained and converted to agricultural or construction land (Leibundgut, 2003). In addition, agricultural practices in general have changed over time and can affect physico-chemical parameters, particle composition and food webs of aquatic habitats. This has a direct impact on aquatic species assemblages and on taxonomic and functional diversity (Paz et al., 2023). Therefore, habitat assessments and conservation approaches must consider the constitution of the catchment area. Besides the physicochemical processes in the river and on the catchment scale, community feedback processes, which account for species-species interactions, are essential for habitat functionality. These considerations rest on two theoretical concepts: community theory, which focuses on direct species-species interactions and the metacommunity concept, which describes interactions within a connected set of local communities (Leibold et al., 2004). To account for all these levels – the constitution of land- and waterscapes, spatial patterns, and community assemblage – decisive efforts in data collection are needed.

Modern genetic approaches like environmental barcoding and metabarcoding are promising tools for overcoming these bottlenecks in order to advance community ecology. DNA metabarcoding allows for the simultaneous identification of entire assemblages through high-throughput sequencing. Initially developed in microbiology in the 1980s for marine sediment (Barnes and Turner, 2016), the method has become increasingly applicable due to advancements in next-generation sequencing (NGS) and information technology, coupled with decreasing costs. Despite its potential, the role of environmental barcoding and metabarcoding in applied nature conservation and ecology remains under investigation. These techniques have been proposed for detection of rare species, biodiversity assessments, species determination, and analysis of habitat connectivity (Harper et al., 2019). Comparisons with classical field methods have shown varying degrees of overlap, suggesting that both approaches may be complementary (Keck et al., 2022) and that sampling effort can significantly affect metabarcoding outcomes (Grey et al., 2018).

The applicability of environmental metabarcoding and its power to address ecological issues are examined in this study, focusing on the habitat of *M. margaritifera.* By incorporating data on land-use of catchment areas, physicochemical parameters from waterscapes, and community data via environmental metabarcoding, we aim to identify the factors that most influence and characterize these habitats. The following questions guide this research:

1. Is the community data derived from the applied environmental metabarcoding method suitable for characterising the habitat of *M. margaritifera*?
2. Which taxa are associated with functioning *M. margaritifera* populations and may these indicate the quality of *M. margaritifera* habitats?
3. Which of the associated taxa may contribute to the diet of *M. margaritifera* in it's natural habitats?
4. Which levels mostly shape the community structure in aquatic habitats?

4.2 Material and methods

4.2.1 Research area and site selection

In order to obtain supra-regional information on the freshwater pearl mussel's habitat, 10 areas have been selected along an approximately 300 km section of the EGB. The area extends from the border triangle between the German states of Bavaria and Saxony and the Czech region Bohemia in the north-west of the study area to the Czech-Austrian border in the south-east (Figure 15, Table 4). All locations belong to the geomorphological province of the Bohemian Massif, which covers most of the area of the Czech Republic and extends into southern Poland and the north-eastern parts of Bavaria. The Danube forms the Massif's southern boundary between its northernmost point near Regensburg and its local southern depression between Linz and Vienna. The area consists of crystalline bedrock of gneiss and granite, which was formed during the Variscan orogeny and subsequently weathered to form brown soils (Migon, 2008). The rivers in this area are characterised by soft water with neutral to slightly acidic pH.

Table 4: Climatic constitution of sampling regions and number (n) of sampled sites per region. Temperatures refer to annual averages for the years 2018 – 2022 and precipitation (precip.) to averages of yearly totals for the same period of time. Longitudinal (longit.) and latitudinal (latit.) coordinates as well as altitude (altit.) refer to the centroid of the sampling region.

nr.	name	ID	n sites	temp. [°C]	precip. [mm]	longit.	latit.	altit. [mASL]
1	Huschermühle	HU	14	8.4	661	12.1320	50.2576	598
2	Grenzbach	GR	3	8.7	791	12.0721	49.8765	576
3	Waldnaab	TW	3	8.6	624	12.2010	49.8238	470
4	Pfreimd	PF	1	9.0	653	12.2661	49.5336	506
5	Biberbach	BI	2	8.78	703	12.6251	49.4139	499
6	Perlenbach	RE	3	10.26	667	12.3209	49.0416	480
7	Wolfsteiner Ohe	WO	2	9.38	1074	13.4726	48.7664	396
8	Ranna	RA	2	8.86	1007	13.7248	48.6466	705
9	Blanice	VO	3	6.10	768	13.9663	48.9303	782
10	Maltsch	MA	3	7.9	761	14.5652	48.6337	717

As this project focused on the border triangle, most of the sites are located there. Three different classes of sample sites have been defined:

a) active: sites where *M. margaritifera* occurs in stable populations
b) extinct: sites where *M. margaritifera* used to occur but has gone extinct
c) detritus: sites that could function as detritus resources for the nutrition of *M. margaritifera*

For category a), we selected only sites that were monitored continuously in the context of conservation programs. For category b), we excluded sites, where it was known that one-off events, such as desiccation, mechanical destruction or toxic influx had caused population extinction. For category c), we selected wetlands associated with pearl mussel habitats, most of which were also used for the collection of detritus for feeding in pearl mussel hatcheries.

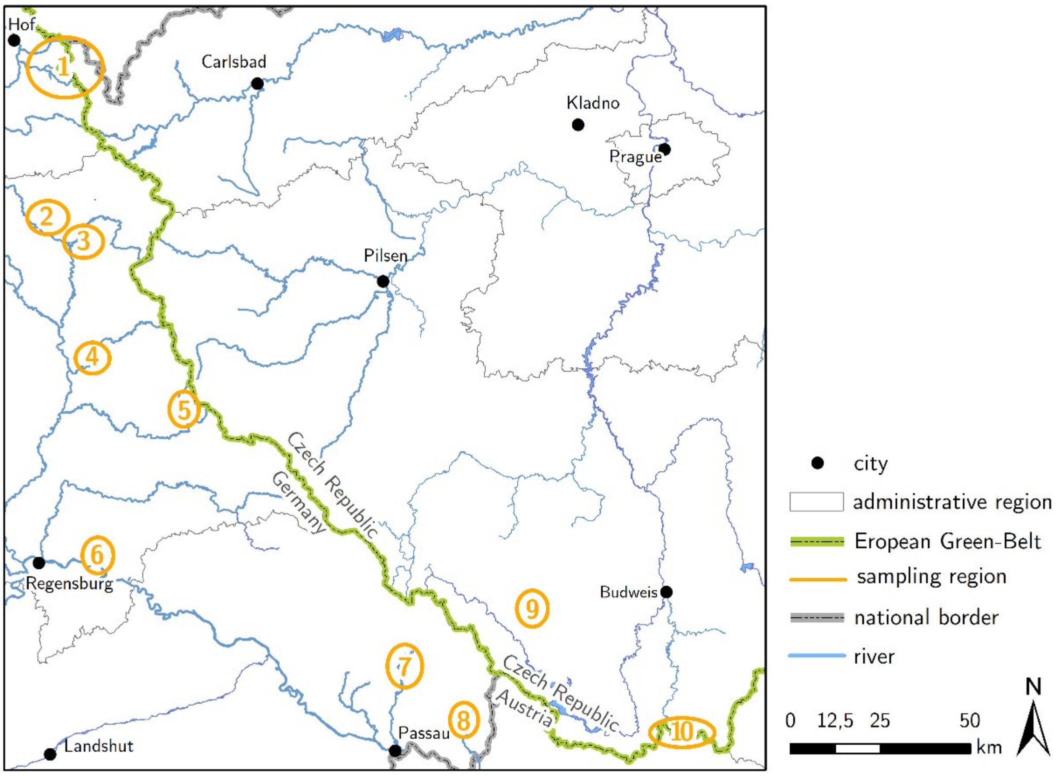

Figure 15: Study area and sampling sites along the European Green-Belt.

Based on these criteria, 36 sites were selected from 10 different areas: 14 sites of category a), 13 sites of category b) and 10 sites of category c). The numbers of sites per region, the climatic conditions and the location of the regions are given in Table 4.

4.2.2 Land-use in the watershed

In order to calculate the influence of land use on the biotic and abiotic conditions in the sample sites, the type of land use was reconstructed upstream from the sample sites within the catchment of the sampled water. For active and extinct sites, the reach buffer according to Allan (2004) was used as the spatial scale (Figure 16). For this, the segment length of the sampling waters and contributing streams was limited to 1 km upstream from the sample site and buffered by 200 m to the left and right.

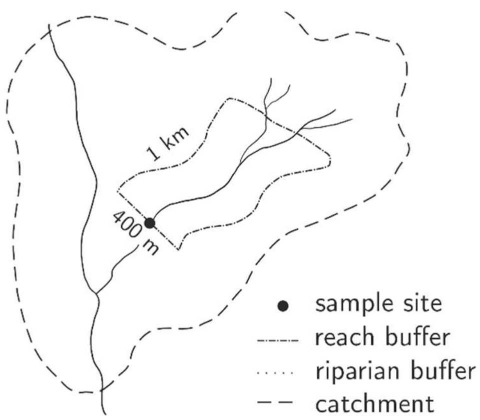

Figure 16: Reach buffer as spatial extent, in which land-use was regarded as influential to stream conditions at the sample site. Adapted from Allan (2004).

For the extent of the river catchment, the 4th order catchment was used, as obtained from ISCS - VODA for the Czech sites[1] and from the LfU Bayern for the Bavarian sites[2].

A radial buffer of 1 km was chosen for detritus type sites, as the contribution to these wetlands is not directional. For the reconstruction of the land use types, existing layers were used and checked for plausibility with digital orthoimagery at a resolution of 1:20 000. For Bavarian sites, the layer 'ATKIS tatsächliche Nutzung' was used. For Czech sites, data from the Natura 2000 maps of the years 2013 and 2021 provided by the Nature and Landscape Protection Agency of the Czech Republic (AOPK, © AOPK ČR 2022/© CzechGlobe 2022) were used.

4.2.3 Sampling design

Each of the 36 sites reflected a river segment of 20 m which was visited twice in the summer of 2021, once in June and once in August. At each site, pH, conductivity and O_2 concentrations were measured three times, upstream, midstream and downstream, using a DWD Multi 3320 multimeter with TetraCon® 325 and SenTix® 41 probes which was placed at mid-diameter just above the river bed. At each measuring point, a water sample

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[1] https://voda.gov.cz/?page=rozvodnice-4-radu-mapa

[2] https://www.lfu.bayern.de/gdi/wms/wasser/grundlagen_fliessgewaesser

was taken for turbidity measurement using a WTW pHotoFlex® Turb photometer. For eDNA analysis, 2 subsamples of 250 mL each were collected every meter along the reach from the downstream end using a long-handled sampling device to allow sampling without entering the water. All 40 subsamples were collected in sterile plastic bottles and pooled in a container lined with a fresh garbage bag for each site. A 20 % chloral bleach solution (Danklorix®) was used to sterilise the plastic bottles, which were incubated for 5 minutes and then rinsed with deionised water. The pooled sample, totalling 10 L, was filtered through sterile 0.8 µm nitrocellulose syringe filters until the filter was blocked or a volume of 1.2 L was reached. Remaining water was then pressed out of the filter before a 0.5 mL buffer solution consisting of 490 µL TES buffer (N-[Tris(hydroxymethyl)methyl]-2-aminoethanosulforic acid, Avantor®) and 10 µL proteinase K was added with a second syringe. The filters were then sealed with capsules and stored refrigerated in labelled bags until further processing.

Ten milliliters of the filtered water were collected in a labelled Falcon tube and kept cool until laboratory analysis for Ammonia, Nitrate, Phosphorous and Calcium. The pHotoFlex® Turb was used for chemical analyses of orthophosphate, nitrate, and ammonium according to the manufacturer's programme protocols 315, 314, and 312 respectively. In addition, calcium analysis was carried out using the ThermoElemental-ASX-510 AAS (for device settings see appendix Table A 7).

Furthermore, habitat parameters such as shading intensity in percent, minimum and maximum water depth and width in centimeters, as well as flow velocity on a four-step scale were assigned to each sample site. Other parameters were macrophyte species cover and identity, sediment composition, and riverbank material.

In order to check the influence of the sampling period, a survey of 10 sites of all types, randomly distributed over the study area, was repeated in August of 2022 using the same sampling scheme as described above.

4.2.4 Sample processing and analysis of eDNA

The analyses of the filter samples was carried out by Sinsoma GmbH ©. A white room with separate pre- and post-PCR laboratories was used. After incubation of the samples for 12 h at 56 °C for lysis, DNA extraction was performed with a Bioprint-96-extraction robot using the BioSprint-96-DNA-Blood&Tissue Kit (Qaigen, Hilden, Germany) and the protocol of the supplier.

Four different group-specific primers were selected for amplification and sequencing in order to specifically detect DNA from macrozoobenthos (MZB), terrestrial and aquatic plants and algae. Two pairs of primers were used for MZB: 1. The primer fragment COI-5P (313 base-pairs mINTCOI1F and HCO2198, Leray et al. (2013), targeted to the Folmer region cytochrome c oxidase I (COI) gene, further referred to as MZB-I. 2. A combination of fwhF2 (Vamos et al., 2017) and EPTDr2n (Leese et al., 2021), also targeted to the COI gene, further referred to as MZB-II. For the identification of algae, all samples were analysed using an assay with primers EK-565F and 18S-EUK-1134R-UNonMet, which target to the 18S region of the genome and were developed at the technical university (TU) Dresden by Felix Grunicke (unpublished). These will be referred to as 18S primers. For aquatic and terrestrial vascular plants, an assay optimised for the ITS2 (internal transcribed spacer 2) region by Sinsoma GmbH has been amplified. This will be referred to as the ITS2-primer.

The applied sequencing technique reads many sequences at once. It is therefore commonly referred to as *metabarcoding* or *next-generation sequencing*. The sequences found were clustered into *operational taxonomic units* (OTUs) according to their similarity. These OTU's were then assigned to species by comparison with open public sequence databases (here Genebank® NCBI and BOLD Systems). As different sequences may refer to the same organism, the bioinformatic alignment provides an average percentage match between the generated sequence and the databases. A minimum match of 99 % has been established to be sufficient for species identification. Taxa with lower matches were removed from the dataset. The number of sequences that can be assigned to an organism reflects the amount of this organism's DNA of this organism in the sample. This is influenced by species abundance, but also by species activity, life cycle and season (Barnes and Turner, 2016). For this uncertainty, this value has not been considered in further analysis.

In addition to the metabarcoding analyses, all sites of type 'active' or 'extinct' were specifically screened for *M. margaritifera* DNA using a species-specific primer pair. Detection of an amplified fragment is by capillary electrophoresis, which gives back *relative fluorescence units* (RFU) that indicate the presence of the target DNA and can be used as a measure of the amount of DNA present.

4.2.5 Data processing and statistics

Comparative analyses of physicochemical parameters comparing active and extinct sample sites were calculated with Kruskall-Wallis rank sum test with r (R core team, 2013). The significance level was set at 0.05.

For descriptive analyses, sampling regions, sampling periods, sampling site types and primer pairs were compared based on the uniqueness or overlap of detected species pools. For this, simple Venn plots were generated using the r packages ggvenn (Linlin, 2023) and UpSetR (Gehlenborg, 2019).

The qualitative community analysis was conducted with a scalable joint species distribution model (sjSDM). This approach is based on joint species distribution models (JSDMs), which are useful for elucidating spatial variation in community composition caused by the influences of environmental factors, biotic relationships, and potentially spatially organised residual correlations. With JSDMs, species-species relationships are calculated separately for each pair of species in a dataset, making it computationally expensive as the number of species increases. This becomes relevant for the very species-rich datasets we create when working with environmental metabarcoding. In sjSDM, this problem is circumvented by offloading the computation to graphical processing units (GPUs) using a Monte Carlo approach (Pichler and Hartig, 2021a). For this, metabarcoding taxa lists were transformed into a 1-0 taxon matrix containing all taxa occurring in more than five samples. As there was little overlap between seasonal recaptures (see appendix Figure A 10), and since the fitted model takes into account the influence of location (longitude, latitude and altitude of sampling site) as spatial variable, recaptures were treated as single samples in the following analyses. The final taxa list included 640 taxa in 81 samples. Environmental variables were reduced to 6, excluding intercorrelations, using the ggpairs function in the r package GGally (Schloerke et al., 2021). The remaining variables were a) percentage of arable land in the catchment upstream from the sampling site, b) nitrate concentration, c) water temperature, d) turbidity, e) pH and f) type of site. Of all categories of land use, arable land was selected as it is considered to have a high impact on aquatic habitats (IPBES, 2019). The r package sjSDM (Pichler and Hartig, 2021b) was used to calculate associations between species, environment and space. A post hoc ANOVA (analysis of variance) was used to assign sites and species to the levels by which they were most influenced. This resulted in ternary plots for sites and species where the corners of the triangle are represented by community, space and environment (see also Leibold et al. (2022)). The species association matrix provides correlations for each species pair in form of correlation coefficients r^2. This was combined with species-environment regression results which, for each species and each variable, provide estimates, standard errors and p-values characterising their relationship. In a further step, the r^2 value between each species and *M. margaritifera*, ranging from -1 to 0 (negative associations) and from 0 to 1 (positive associations), was tested in another linear regression model against the estimates (estimated slope of the regression line) for the environment variables. This tested which environmental variables had a positive or negative effect on the communities positively or negatively associated with *M. margaritifera.* In order to explicitly consider pearl mussel-related species, r^2 values were restricted to values ≥ 0.25 for positively and ≤ -0.25 for negatively associated species. Residual diagnoses were performed post hoc with the r package DHARMa (Hartig, 2022) to show that the distributional assumptions of the regression model were correct.

4.3 Results

4.3.1 The physicochemical constitution of active and extinct sites

Among the physicochemical parameters measured from water samples in the laboratory (Figure 17, a-c), nitrate concentration showed significantly higher values at sites, where the freshwater pearl mussel had become extinct. Ammonia concentration did not discriminate between extinct and active sites, and phosphate concentration showed isolated higher values in active sites, but concentrations varied widely in both types so that these differences were not significant. All other values (Figure 17, d-g) were measured directly on site. Here, extinct sites showed marginally significantly higher pH and significantly higher conductivity. Turbidity was slightly higher at extinct sites, but with greater variance and not significant. Oxygen concentration did not differ between the two types of sites, being always around 8 µg L^{-1}.

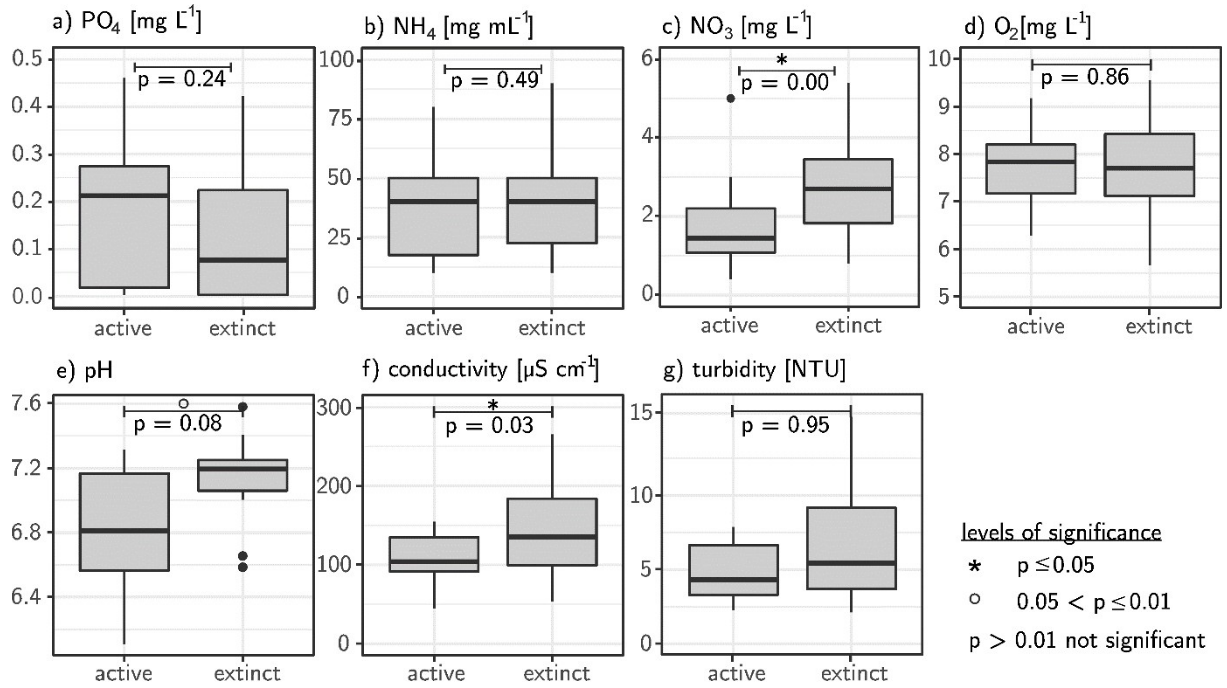

Figure 17: Chemical and physical parameters a) phosphate (PO_4), b) ammonia (NH_4), c) nitrate (NO_3), d) oxygen (O_2), e) pH, f) conductivity and g) turbidity, compared between active and extinct sites. P values were calculated with Kruskall-Wallis test. P-values and significance is shown between the boxes: significance = $p \leq 0.05$ (*), low significance = $0.05 < p \leq 0.1$ (O), no significance = $p > 0.1$.

4.3.2 Diagnostic detection of *M. margaritifera*

One sample from each of the 14 active as well as from the 13 extinct sites was tested for freshwater pearl mussel DNA. This resulted in seven positives and 20 negatives (Figure 18). All positives were from active sites. Pearl mussel DNA was not found at extinct sites, nor on seven of the 14 active sites tested. Positive results were obtained from the active sites at Blanice (VO, RFU = 2.67), Ranna (RA, RFU = 0.36), Grenzbach (GR, RFU = 0.3), Biberbach (BI, RFU = 0.48) and three sites from Huschermühle (HM1, RFU = 0.13, HM6, RFU = 0.23 and HM7, RFU = 0.08). For site description see section 4.2.1, P. 73, Table 4 and Figure 15.

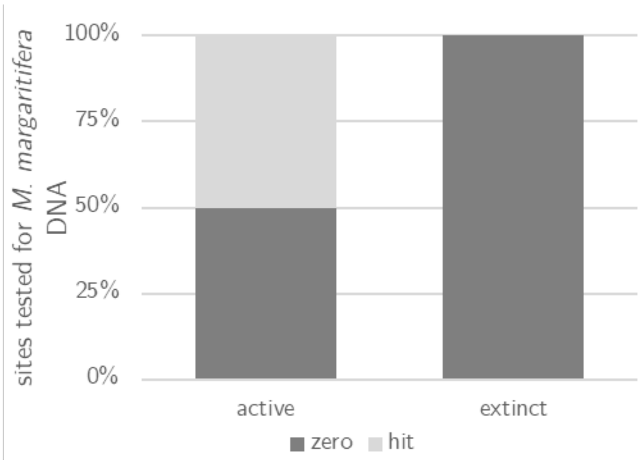

Figure 18: Share of sites with positive (hit) and negative (zero) diagnostic screening for DNA of *M. margaritifera*.

4.3.3 Species associations with *M. margaritifera*

Of the 640 taxa that were included in the sjSDM model, 83 were positively associated ($r^2 \geq 0.3$) with the occurrence of *M. margaritifera* and 17 were negatively associated ($r^2 \leq -0.3$). As shown in Figure 19, the positively associated taxa were mostly algae (41 taxa). They also showed the strongest correlations, as reflected by the highest r^2 values. 16 algal taxa were correlated with an $r^2 > 0.5$ (Table 5) belonging to the *Scenedesmaceae* (9), *Coccomyxaceae* (2) *Monodopsidaceae* (1) *Trebouxiaceae* (1), *Goniochloridaceae* (1), and *Oocycstaceae* (1) families. Still strongly correlated, with r^2 between 0.3 and 0.5 were four more taxa of *Scenedesmaceae,* two *Oocystaceae,* two *Chlorellaceae*, two *Stephanodiscaceae,* two *Selenastraceae* and one taxon each of *Hydrodictyaceae, Thalassiosiraceae, Goniochloridaceae, Melosiraceae, Aulacoseiraceae, Botryococcaceae, Chattonellaceae, Acrochaetiaceae,* *Neochloridaceae, Melosiraceae, Mychonastaceae, Naviculaceae, Monodopsidaceae.* Among vascular plants, there were 23 taxa from eleven families correlated with $r^2 \geq 0.3$, most of which were terrestrial tree species. These were *Alnus spp.*, *Juglans spp.*, *Tilia cordata and T. platyphyllos, Juglans spp., Populus nigra* and eight taxa from the genus *Prunus.* Also present were the herbs *Cicera alpina* and *Ranunculus parnassiifolius*, the amphyphytic taxa *Veronica beccabunga* and *Impatiens glandulifera* and the macrophytes *Ranunculus fluitans, R. tripartitus, Callitriche* spp. and *Myriophyllum alterniflorum.* Among the protozoa, seven taxa from seven families were correlated with an $r^2 \geq 0.3$. There were eleven fungal taxa from ten families with an $r^2 > 0.3$ and as the only metazoan the mayfly *Baetis rhodani.*

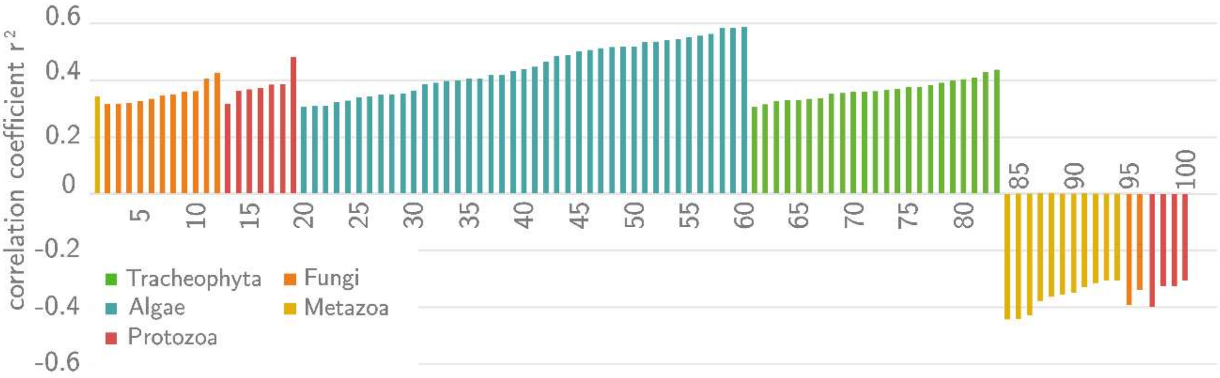

Figure 19: Numbers of species that were positively and negatively associated with the occurrence of *M. margaritifera* with an $r^2 \geq 0.3$ or ≤ -0.3, sorted by the groups of species targeted for with the selected primer combinations in the same order as species lists in Tables 8 and 9.

Strong negative associations with $r^2 \leq -0.3$ were found for 17 taxa (Table 6). Eleven of these belonged to the metazoan group, where the family of the *Chironomidae* was mainly represented by six taxa. The other families were *Leuctridae*, *Phoridae*, *Caeciliusidae* and *Hydridae* which were all represented by one taxon, and finally there was one taxon from the order of *Harpacticoida.* There were also two negatively associated fungi, one from the genus *Darkera,* a soil organism, and the species *Aphanomycetes euteiches*, an enteric pathogen. The remaining four taxa negatively associated with *M. margaritifera* belong to the protozoan group. These are the unicellular eucaryotes *Amphisiella pulchra*, *Rhogostoma cylindrica* and two unknown representatives of the genera *Cyclidium* and *Vorticella.*

Table 5: Taxa positively associated with *M. margaritifera* occurrence with $r^2 \geq 0.3$, arranged within their target group with ascending r^2 value analogous to the taxon order in Figure 19. The column 'taxon' refers to the highest taxonomic resolution to which a metabarcoding derived OTU could be assigned.

nr.	taxon	family	r^2
Metazoa			
1	*Baetis rhodani*	*Baetidae*	0.34
Fungi			
2	*Leohumicola verrucosa*	*Calloriaceae*	0.31
3	*Pseudogymnoascus pannorum*	*Pseudeurotiaceae*	0.31
4	*Globisporangium ultimum*	*Pythiaceae*	0.32
5	*Sydowia polyspora*	*Dothioraceae*	0.32
6	*Leohumicola levissima*	*Calloriaceae*	0.33
7	*Antarctomyces pellizariae*	*Thelebolaceae*	0.34
8	*Blastobotrys*	*Trichomonascaceae*	0.34
9	*Phytophthora gonapodyides*	*Peronosporaceae*	0.35
10	*Verticillium nonalfalfae*	*Plectosphaerellaceae*	0.36
11	*Edenia gomezpompae*	*Pleosporaceae*	0.40
12	*Synchaetomella acerina*	*Chaetomellaceae*	0.42
Protozoa			
13	*Colpodella*	*Colpodellidae*	0.31
14	*Adelina*	*Adeleidae*	0.36
15	*Hoplorhynchus*	*Actinocephalidae*	0.36
16	*Colpodellaceae*	*Colpodellaceae*	0.37
17	*Heteromita globosa*	*Heteromitidae*	0.38
18	*Cryptosporidium*	*Cryptosporidiidae*	0.38
19	*Paulinella micropora*	*Paulinellidae*	0.48
Algae			
20	*Hydrodictyaceae*	*Hydrodictyaceae*	0.31
21	*Cyclostephanos invisitatus*	*Stephanodiscaceae*	0.31
22	*Stephanocyclus*	*Thalassiosiraceae*	0.32
23	*Chloroidium viscosum*	*Oocystaceae*	0.32
24	*Tetraedriella subglobosa*	*Scenedesmaceae*	0.48
25	*Melosira varians*	*Melosiraceae*	0.34
26	*Tetradesmus deserticola*	*Scenedesmaceae*	0.34
27	*Fistulifera saprophila*	*Naviculaceae*	0.35
28	*Chlorellaceae*	*Chlorellaceae*	0.35
29	*Neodesmus pupukensis*	*Scenedesmaceae*	0.36
30	*Aulacoseira subarctica*	*Aulacoseiraceae*	0.38
31	*Chlorella desiccata*	*Chlorellaceae*	0.39
32	*Botryococcus braunii*	*Botryococcaceae*	0.39
33	*Chloroidium*	*Oocystaceae*	0.40
34	*Chattonella*	*Chattonellaceae*	0.40
35	*Audouinella hermannii*	*Acrochaetiaceae*	0.40
36	*Nannochloropsis limnetica*	*Scenedesmaceae*	0.41
37	*Neochloris terrestris*	*Neochloridaceae*	0.41
38	*Desmodesmus intermedius*	*Scenedesmaceae*	0.43
39	*Monoraphidium*	*Selenastraceae*	0.44
40	*Melosira ambiqua*	*Melosiraceae*	0.44
41	*Mychonastes*	*Mychonastaceae*	0.46
42	*Kirchneriella irregularis*	*Selenastraceae*	0.36
43	*Desmodesmus costato-granulatus*	*Scenedesmaceae*	0.48
44	*Ranunculus parnassiifolius*	*Ranunculaceae*	0.30
45	*Scenedesmus quadricauda*	*Scenedesmaceae*	0.50
46	*Trebouxia jamesii*	*Trebouxiaceae*	0.50

Continuation of Table 5: Taxa positively associated with *M. margaritifera* occurrence with $r^2 \geq 0.3$, arranged within their target group with ascending r^2 value analogous to the taxon order in Figure 19. The column 'taxon' refers to the highest taxonomic resolution to which a metabarcoding derived OTU could be assigned.

nr.	taxon	family	r^2
Algae			
47	*Chloroidium laureanum*	*Oocystaceae*	0.51
48	*Scenedesmaceae*	*Scenedesmaceae*	0.51
49	*Selenastraceae*	*Selenastraceae*	0.51
50	*Desmodesmus armatus*	*Scenedesmaceae*	0.52
51	*Tetradesmus*	*Scenedesmaceae*	0.53
52	*Trachydiscus minutus*	*Goniochloridaceae*	0.53
53	*Desmodesmus communis*	*Scenedesmaceae*	0.54
54	*Tetradesmus obliquus*	*Scenedesmaceae*	0.54
55	*Desmodesmus denticulatus*	*Scenedesmaceae*	0.55
56	*Choricystis parasitica*	*Coccomyxaceae*	0,55
57	*Desmodesmus*	*Scenedesmaceae*	0.56
58	*Nannochloropsis*	*Monodopsidaceae*	0.58
59	*Desmodesmus abundans*	*Scenedesmaceae*	0.58
60	*Choricystis limnetica*	*Coccomyxaceae*	0.58
Tracheophyta			
61	*Tilia cordata*	*Malvaceae*	0.30
62	*Ranunculus parnassiifolius*	*Ranunculaceae*	0.30
63	*Juglans spp.*	*Juglandaceae*	0.31
64	*Alnus spp.*	*Betulaceae*	0.32
65	*Ranunculus tripartitus*	*Ranunculaceae*	0.33
66	*Ranunculus fluitans*	*Ranunculaceae*	0.33
67	*Tilia platyphyllos*	*Malvaceae*	0.33
68	*Prunus yedoensis*	*Rosaceae*	0.33
69	*Ranunculus aquatilis*	*Ranunculaceae*	0.35
70	*Populus nigra*	*Salicaceae*	0.35
71	*Impatiens glandulifera*	*Balsaminaceae*	0.36
72	*Myriophyllum alterniflorum*	*Haloragaceae*	0.36
73	*Circaea alpina*	*Onagraceae*	0.36
74	*Callitriche spp.*	*Plantaginaceae*	0.37
75	*Prunus dulcis*	*Rosaceae*	0.37
76	*Prunus cornuta*	*Rosaceae*	0.37
77	*Prunus domestica*	*Rosaceae*	0.38
78	*Rosaceae*	*Rosaceae*	0.39
79	*Prunus virginiana*	*Rosaceae*	0.40
80	*Prunus spp.*	*Rosaceae*	0.40
81	*Prunus avium*	*Rosaceae*	0.41
82	*Veronica beccabunga*	*Plantaginaceae*	0.43
83	*Prunus padus*	*Rosaceae*	0.43

Table 6: Taxa negatively associated with *M. margaritifera* occurrence with $r^2 \leq -0.3$, ordered within their target group with ascending r^2 value analogous to the taxon order in Figure 19. The coloumn 'taxon' referres to the highest taxonomic resolution to which a metabarcoding derived OTU could be assigned.

nr.	taxon	family	r^2
Metazoa			
84	*Rheocricotopus effusus*	*Chironomidae*	-0,44
85	*Heterotanytarsus apicalis*	*Chironomidae*	-0,44
86	*Krenosmittia halvorseni*	*Chironomidae*	-0,43
87	*Leuctra nigra*	*Leuctridae*	-0,38
88	*Krenopelopia binotata*	*Chironomidae*	-0,36
89	*Phoridae*	*Phoridae*	-0,36
90	*Valenzuela*	*Caeciliusidae*	-0,35
91	*Hydra viridissima*	*Hydridae*	-0,33
92	*Micropsectra appendica*	*Chironomidae*	-0,32
93	*Corynoneura fittkaui*	*Chironomidae*	-0,31
94	*Harpacticoida*	*NA*	-0,31
Fungi			
95	*Darkera*	*Phacidiaceae*	-0,40
96	*Aphanomyces euteiches*	*Saprolegniaceae*	-0,34
Protozoa			
97	*Amphisiella pulchra*	*Amphisiellidae*	-0,40
98	*Rhogostoma cylindrica*	*Rhogostomidae*	-0,32
99	*Cyclidium*	*Cyclidiidae*	-0,32
100	*Vorticella*	*Vorticellidae*	-0,30

4.3.4 Impact of environmental factors

The affinity of all taxa to the selected environmental variables was calculated in the sjSDM model, which provided p-values, describing significance and estimates and also explaining the strength and direction for each species-environment relationship. To determine the affinities of pearl mussel-related species to their environment, the correlation coefficients r^2 of the taxa listed in Table 5 and 5 were tested in a regression model against the estimates of each taxon to each environmental variable. As negatively related species with an $r^2 \leq -0.3$ were not sufficient in number to run a linear regression model, the cutoff for r^2 was reduced to 0.25. The affinity, expressed by the estimate value, of species that are positively correlated with freshwater pearl mussel occurrence is highly significantly negative for the extent of arable land in the water shed (Figure 20a). Most of the species highly correlated with the occurrence of *M. margaritifera* have negative estimates for the variable 'arable field'. The affinity of pearl mussel associated species to nitrate concentration at the sampling sites tends to be marginally significant in a positive direction. Most of the species positively correlated with *M. margaritifera* have positive estimates for the variable 'nitrate' (Figure 20b).

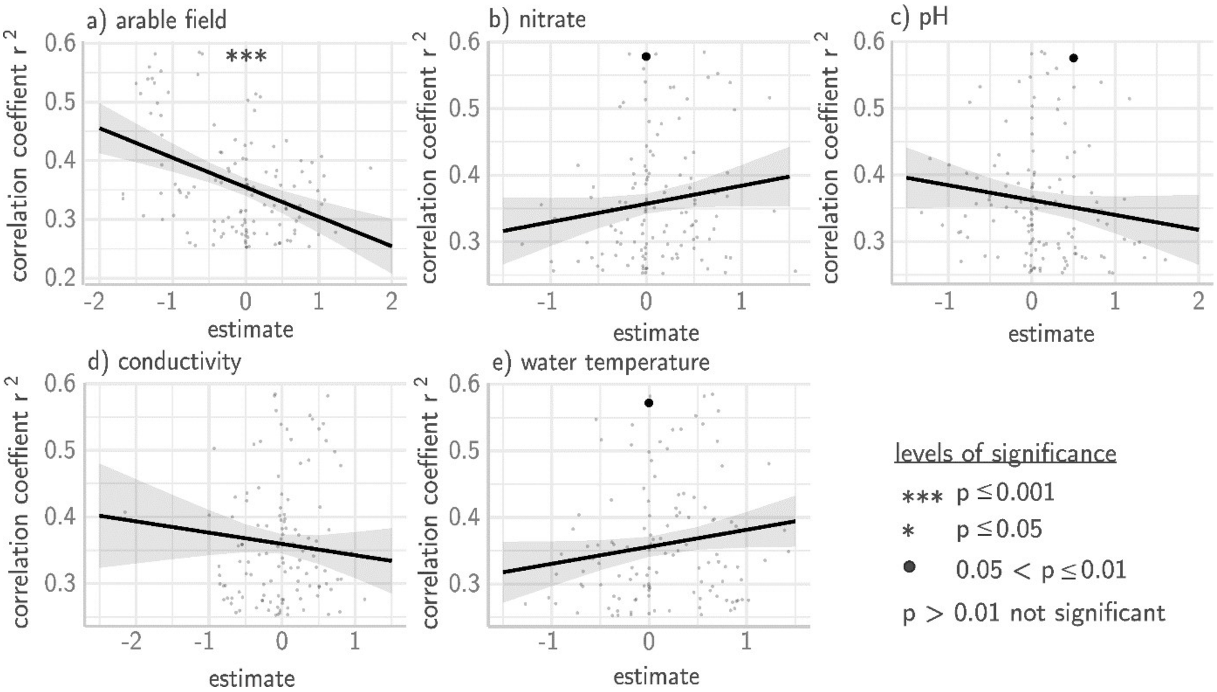

Figure 20: Linear regression outputs of positive species correlation with *M. margaritifera* (r^2) and their estimates for the selected environmental variables.

A marginally significant negative relationship was found for pH estimates and r^2, hinting that species less correlated with *M. margeritifera* tend to prefer higher pH values (Figure 20c). Positive and negative estimates for conductivity were more or less equally distributed between species with high and with low correlations with *M. margaritifera.* The relationship was not significant, but the regression line shows a slightly negative trend (Figure 20d). The relationship between water temperature estimates and correlation with *M. margaritifera* was marginally significantly positive, indicating that higher temperatures tend to be preferred by pearl mussel related species (Figure 20e).

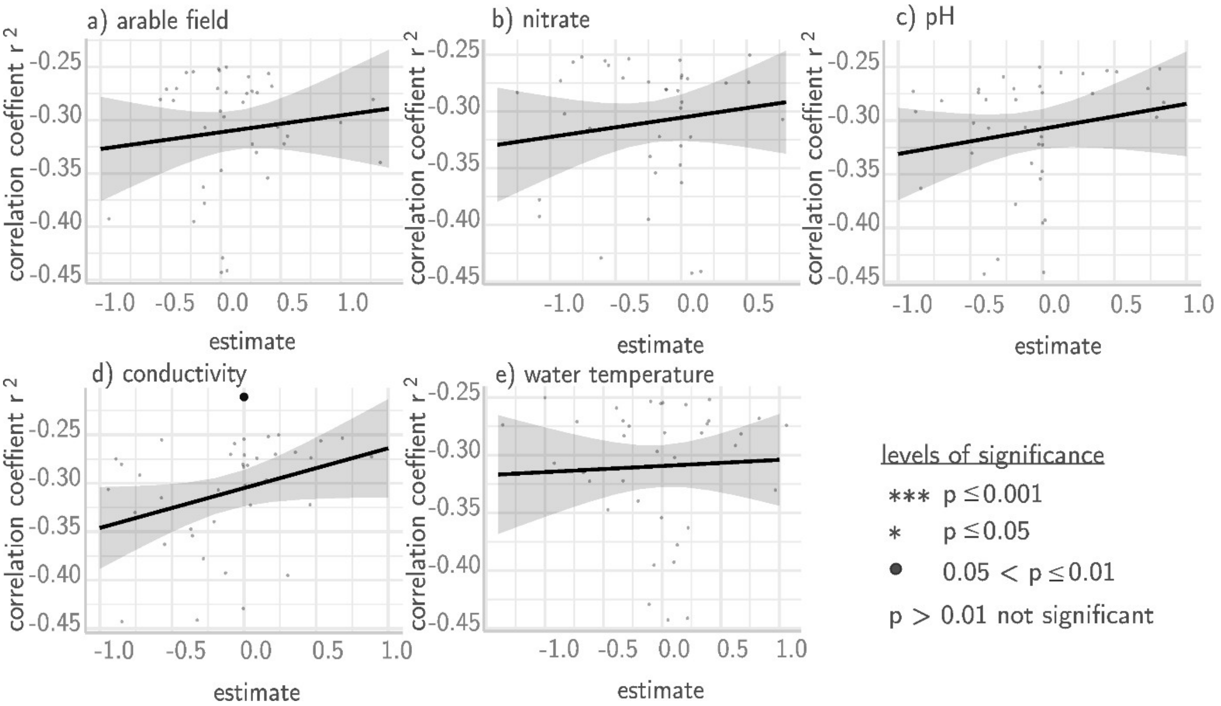

Figure 21: Linear regression outputs of negative species correlation with *M. margaritifera* (r^2) and their estimates for the selected environmental variables.

Species negatively correlated with *M. margaritifera* occurrence with $r^2 \leq -0.25$ showed no significant relationship with four of the five environmental variables tested (Figure 21). For all variables, the regression line tends to be slightly positive, only for the conductivity variable this relationship is marginally significant (Figure 21d).

4.3.5 Levels to shape community structure

The ternary plots of the internal community structure as revealed by the ANOVA model (Figure 22) show the share of variance explained by the community (C), the environment (E) or the geographical position (S). The left part of the plot shows the communities of taxa according to the sites where they were detected. This is reflected by the dots in the plot. There is a clear tendency towards the upper apex representing the community aspect. A few sites are also located in the middle of the triangle and along the left leg reflecting the environmental gradient. None of the sites was positioned clearly towards the spatial variable in the right corner. The distribution of individual species across the ternary plot differs to some extent from that of the sites. Looking at the right triangle in Figure 22, the majority of points is clustered in the central part. Along the left leg of the triangle, there is another, smaller concentration of dots which are influenced by a gradient between community and environment, excluding space, and another concentration along the right leg is influenced by a gradient from space to community, excluding the environment. There are a few taxa clearly positioned in the upper apex toward the community variable. Fewer are positioned towards the environmental variable in the left corner and only one dot with small correlation is positioned near the space variable in the right hand corner.

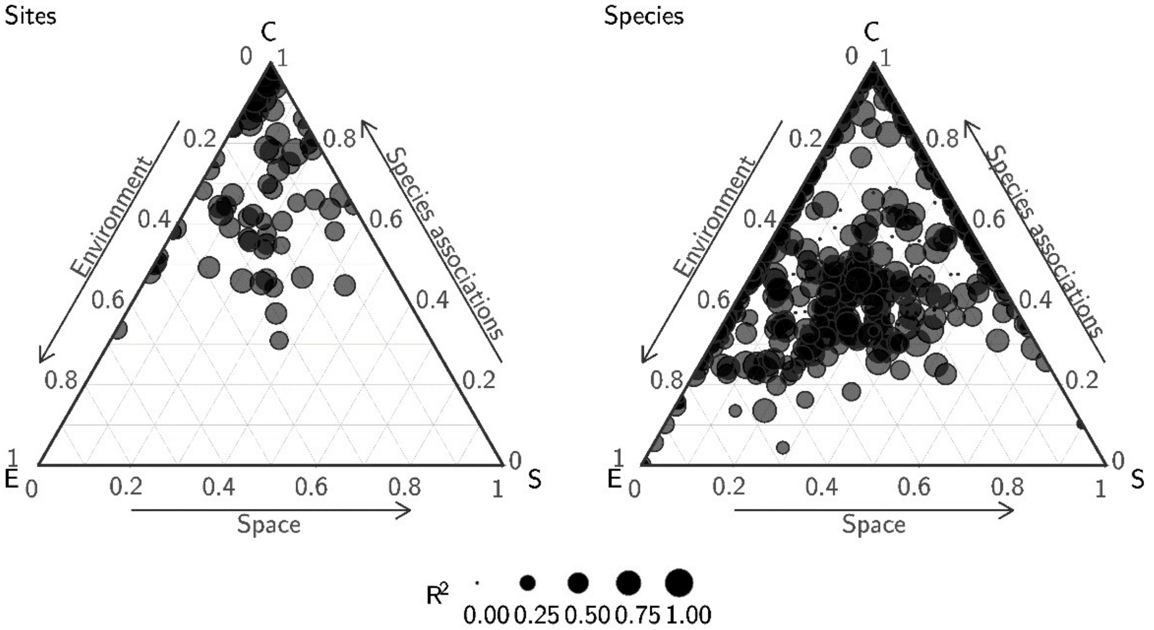

Figure 22: Ternary plots of the community internal structure, evaluated with anova model built on the outcome of the sjSDM model. The size of symbols corresponds to the models R^2 and the position refers to the proportion of explained variation ascribed to environmental factors (E - lower left), spatial influences (S - lower right), and species associations (C - apex).

4.4 Discussion

The freshwater pearl mussel (*Margaritifera margaritifera*) is an ecologically important and highly sensitive species, which is often considered to be an indicator of pristine freshwater habitats. In this study, sites with active *M. margaritifera* populations were compared with sites where the pearl mussel had gone extinct, and sites where detritus was collected for ex situ pearl mussel breeding, focusing on physicochemical parameters and species composition, using environmental metabarcoding.

This revealed differences in the physicochemical constitution of the different types of sampling sites, with nitrate and conductivity being significantly higher at sites where *M. margaritifera* had gone extinct as compared to sites with active pearl mussel populations. Diagnostic screening for *M. margaritifera* DNA from both of these types of sites revealed positive results at only 50 % of sites where the species was known to occur, indicating potential limitations in detection methods. The application of scalable joint species distribution modeling (sjSDM) identified a set of taxa either positively or negatively associated with *M. margaritifera*, providing insights into the community structure of these habitats. It also became evident that these communities are differently influenced by environmental factors such as land use in the watershed. Finally, these findings suggest that community interactions play a more significant role in shaping community structure than environmental or spatial variables.

These results underscore the complex interplay between environmental factors and biotic interactions in shaping the habitats of *M. margaritifera* and highlight the importance of considering community-level dynamics in conservation efforts.

4.4.1 The physicochemical constitution of sampling sites

Active and extinct sites were chosen in a way, that habitats were similar to each other in terms of river morphology and environment. Also, each of both sites were represented in each region with the exception of the site at the Pfreimd, where the expected population no longer existed and only an extinct site was sampled. The physicochemical comparison between the two types of sites showed significant differences for nitrate concentration and conductivity, and a marginally significant difference for pH, all of which were higher at the extinct sites. At active sites, nitrate concentrations were between 0.4 and 5 mg L^{-1} with a mean of 1.7 mg L^{-1}

and for conductivity a mean of 112 µS cm^{-1} with a range between 42 and 232 µS cm^{-1} was measured. These values are just at the upper edge of the benchmarks for freshwater pearl mussel habitats which allow nitrate values below 1.7 mg L^{-1} (DIN Standards Committee Water Practice, 2007). For conductivity, values below 100 µS cm^{-1} are suggested (Skinner et al., 2003). Anyway, in Bavaria, only between 10 to 15 % of pearl mussel streams measured values below the proposed benchmark for nitrate in 2004 and almost 80 % exceeded these by more than 50 % (Sachteleben et al., 2004). Also a more recent study by Stöckl et al. (2020) revealed, that none of the Bavarian pearl mussel streams reached 'good' habitat quality (category A, NO_3-N $<$ 1 mg L^{-1}) and only 24 % reached a 'moderate' habitat quality (category B, NO_3-N $<$ 1.5 mg L^{-1}). Also, Geist and Auerswald (2007) measured conductivity values below 150 µS cm^{-1} at 46 sites with functional pearl mussel populations, with a mean below 100 µS cm^{-1}, and Bily and Simon (2007) found long term existing populations at mean conductivity values above 90 µS cm^{-1}. It is thought, that inorganic subsoil compounds originating from human land use or traffic, leading to higher conductivity values are more harmful to *M. margaritifera* than organic ones due to the geological subsoil and there seems to be some degree of adaptability of the species towards increased conductivity and nitrate values (Bily and Simon, 2007). However, increasing nitrate concentrations reduce population size and reproduction (Sachteleben et al., 2004) as they go along with increased mortality of juveniles and adults due to eutrophication (Bauer, 1988) and a threshold of 90 µS cm^{-1} is regarded as limit for successful reproduction (Bily and Simon, 2007). This was also reflected by the population status in the study of Stöckl et al. (2020), where no population held a proportion of juvenile mussels ($<$ 10 years) of more than 20 % and in only 14 % of all populations juvenile mussels were found at all. The fact that both, nitrate with a mean of 2.7 mg L^{-1} and conductivity with a mean of 176 µS cm^{-1} were increased way above the discussed limits for *M. margaritifera* in extinct sites points to eutrophication as an important driver for species extinction in the study area.

Concerning pH values a limit for successful reproduction was described at pH 6 (Bily and Simon, 2007). Slightly acidic to neutral pH values between 6.2 and 7.3 are characteristic for the soft water habitats *M. margitifera* lives in. Although values below these limits are found in pearl mussel populated sites, strong or permanent acidification at values below 5 leads to shell degradation and therefore puts the species at risk (Degerman et al., 2013). In this study, pH values were slightly increased at extinct sites, ranging between 6.5 and 7.8 with a mean at 7.2. At active sites they were between 6.3 and 7.6 with a mean at 7. Thus, mean values for both types of sites are within the natural range and maximum values from both exceeded the range. Higher pH values are not known to depict a direct threat to *M. margaritifera*, though they can mediate a shift in the ammonium (NH_4^+) to ammonia (NH_3) ratio towards higher NH_3, which is toxic for aquatic organisms. Also, increased pH values can indicate increased calcium concentrations, which can also have direct toxic effects on *M. margaritifera* and can in the long-term increase mussel growth, which goes along with a shortened life span and a reduced reproductive success (Boon et al., 2019). Anyway, in this study only marginal differences in pH values were found and calcium and ammonia concentration did not differ between both types of sites, which suggests that pH is not critical for the habitat quality in the study area.

4.4.2 Diagnostic detection of *M. margaritifera*

The diagnostic detection of *M. margaritifera* at active and extinct sites was a step towards evaluating the reliability of the eDNA outcomes and towards proving the absence of the pearl mussel from extinct sites. There was no positive prove of pearl mussel DNA at extinct sites, which is in accordance with the previous data research and declaration by the regional pearl mussel experts who helped to select the sites. For active sites, there was also no positive prove for 50 % of the tested sites, despite the fact, that only sites were chosen, where presence of *M. margaritifera* was affirmed by experts and by own sight. Especially for small organisms DNA metabarcoding inventories of plankton, microphytobenthos and macroinvertebrates have shown notable differences to traditional methods, resulting rather in a complementary relationship of both (Keck et al., 2022). Environmental factors such as high flow velocities or floods, as well as seasonality can for instance decrease eDNA concentrations and produce false negatives (Curtis et al., 2021). Thus, some organisms per se release higher amounts of DNA at certain times and therefore would be overrepresented in a sample, regardless of the species abundance. The same could be true for times of low species activity so that an actually present organism could be overseen. This suggests that eDNA data should be regarded as presence only data and not, as commonly suggested, as prove for presence or absence of a species.

4.4.3 Species associations with *M. margaritifera*

The study of species associations and co-occurrences across different taxonomic groups provides insights into ecological interactions and environmental influences on biodiversity. Positive associations often arise from mutual interactions, shared environmental preferences, facilitation, or indirect benefits. Negative associations may result from competitive interactions, allelopathy, or different habitat preferences. Predation or parasitism can cause both, positive and negative associations. These interactions occur between species from different taxonomic groups and at different trophic levels, further complicating community dynamics. These relationships illustrate the interconnectedness of species and the importance of preserving diverse and functioning ecosystems.

With respect to food resources, algal species are of particular interest. Algae are thought to be the main source of essential fatty acids in freshwater food webs (Baker and Levinton, 2003). In particular, the provision of PUFAs is mediated by algal species which increase the nutritional value of stream detritus (Grunicke et al., 2022). Among the taxa associated with *M. margaritifera* occurrence above the r^2 threshold of |0.3|, algae associations were exclusively positively correlated, accounting for 59 % of all positively correlated taxa above this threshold. Most of these were benthic freshwater green algae or diatoms. Seventeen of the positively associated algal taxa have been shown to be digested by *M. margaritifera*, either in a feeding experiment (Baker and Levinton, 2003), as a common component of mussel diet in pearl mussel hatcheries (Eybe et al., 2013) or they have been found in the guts of free-living *M. margaritifera* individuals (Komulaynen, 2021). These were species of the genera *Cyclotella, Scenedesmus, Nannochloropsis* and *Aulacoseira* and the species *Melosira varians.* Further, a total of eight positively correlated taxa are diatoms which are regarded as essential components for pearl mussel nutrition due to their heigh ability in PUFA production (Grunicke et al., 2022). The strong positive correlations of these taxa with the occurrence of *M. margaritifera* indicates, that algal species play a role in mediating habitat suitability for *M. margaritifera.* This could be due to their nutritional value, as mentioned above. Even for taxa not previously related with the freshwater pearl mussel, some, such as the green algae *Desmodesmus denticularis* or *Tetradesmus obliquus,* are known and studied in contexts of production of biofuel or food for their potential to generate PUFAs (Soares et al., 2019; Oliveira et al., 2021) suggesting their ability to provide a nutritious food resource. Other functional benefits could be derived from antimicrobial and antioxidant exudates as they are attributed to *Neodesmus pupukensis* (Omomowo et al., 2020). Despite these beneficial effects that the detected algal species might have on the freshwater pearl mussel habitats, strong presence of algal species is generally often associated with poor water quality, as many algal species develop rapid growth under high nutrient conditions, resulting in algal blooms which can cause anoxic conditions in the water. However, we did not find any algal species that were strongly negatively correlated with *M. margaritifera*, indicating that none of the sampled sites was affected by these effects.

The second most strongly represented taxa group of positively associated species were vascular plants. These account for 33 % of all positively associated taxa and also have no negative associations. Vascular plants also play a role in the nutrition of the freshwater pearl mussel, as they may contribute to the detrital part of the pearl mussel diet. According to Bauer and Wächtler (2001), the main component of the diet of *M. margaritifera* is detritus with particles smaller than 40 µm, rather than live algal cells, a fact which distinguishes the species from the majority of large freshwater bivalves. According to Hruska (1999), such detritus mostly comes from extensively used grasslands in the catchment area and enters the pearl mussel habitat through small water veins in the crenal zone of a stream. Taxa that have been discussed as providing important nutritional components are mainly species from the family of *Poaceae* in general. Hruska (1999) mentions in particular the species *Alopecurus pratensis* and *Poa trivialis* as important components of the riparian vegetation. These taxa were found in both active and extinct sites by DNA metabarcoding in this study, but were not positively associated with *M. margaritifera* in the sjSDM model. Herein, strong positive correlations were found for deciduous trees, especially those of the genus *Prunus,* and macro- and mesophytes such as *Ranunculus* species *Myriophyllum alterniflorum* or *Veronica beccabunga.* The rhizosphere of riparian trees and macrophytes, together with algae, contribute to the autochtonous or stream detritus which has been analysed by Grunicke et al. (2022) as opposed to riparian detritus as a food resource for the freshwater pearl mussel. It was shown that the PUFA content of stream detritus exceeded that of riparian detritus, resulting in higher growth rates of juvenile pearl mussels fed on the former. The strong positive correlations of the above taxa may therefore be due to their function in providing nutritious food for the freshwater pearl mussel. Of the positively associated plant taxa, four are included in the Bavarian Red List (RL) of endangered vascular plants (Scheuerer and Ahlmer, 2003). These were *Ranunculus tripartitus* (RL 0)*, Ranunculus parnassiifolius* (RL 1), *Ranunculus fluitans* (RL 3), *Populus nigra* (RL 3) and *Myriophyllum alterniflorum* (RL 2). All of these species were also found on sites in the Czech Republic, where only *M. alterniflorum* (RL 2) and *P. nigra* (RL 1) are included in the Red List (Grulich, 2012). The association of *M. margaritifera* with these endangered species may be due to the fact that they share the same habitat and are therefore simultaneously affected by its degradation. It is also possible that there is an interdependent relationship between these species. In this case, the threat to one species indirectly affects the species that depend on it for food, shelter or other ecological functions (Colwell et al., 2012). However, the presence of *R. tripartitus* and *R. parnassiifolius* is suspect as these species are not typical for the focal ecosystem. These results highlight the importance to carefully consider the limitations of metabarcoding when interpreting the results.

The positive associations of *M. margaritifera* with the taxonomic groups of Metazoa, Protozoa and Fungi may be due to reasons other than food and prey relationships. Of the seven positively associated protozoan taxa, three are parasitic and parasitise the intestines of macroinvertebrates (*Adelina*), Odonates (*Hoplorhynchus*) or Vertebrates (*Cryptosporidium*). Three are free living and predatory (*Colpodellacae, Colpodella and Heteromita globosa*) and one is photosynthetic (*Paulinella micropora*). These taxa were not previously related to the freshwater pearl mussel. Parasites can act as ecosystem engineers and have direct impacts on biodiversity (Hatcher et al., 2012). In this way, they act on the same level as predators in shaping species communities. The relationship of *M. margaritifera* and such species could therefore be of this indirect nature. However, the partially parasitic character of six of the positively related fungi (*Pseudogymnoascus pannorum, Globisporangium ultimum, Sydowia polyspora, Phytophthora gonapodyides, Verticillium nonalfalfae* and *Synchaetomella acerina*), is again likely to affect the food web. *P. gonapodyides* for example is known from forest streams, where it decomposes leaves. It can be saprophytic or a mild pathogen causing root rot (Aram and Rizzo, 2019). In this way, it mediates the decomposition of plant material, which is one step in the provision of detritus. This may also be the role of saprophytic fungi in general. In contrast, the genus *Leohumicola* is a mycorrhizal partner often occurring in communities with presence of *Ericaceae* (Hambleton et al., 2005). A positive association of these species with *M. margaritifera* could thus indicate indirectly a positive association with species of *Ericaceae* in the surrounding of *M. margaritiferas* habitat. Anyway, little is known about potential relationships between freshwater bivalves and fungi and a more profound discussion in this field would need further investigation. From the group of metazoans, only the mayfly *Baetis rhodani* reflected a positive association. The larva is bound to flowing waters and feeds on algae and detritus. Its ecological niche therefore overlaps with that of the freshwater pearl mussel. As the mayfly nymph is an important prey for salmonid species (Dahl and Greenberg, 1999), the presence of this species indicates a nutritional basis for the host fish of the pearl mussel glochidia and may therefore function in the life cycle of *M. margaritifera*.

Negative association patterns between different taxonomic groups have mostly been analysed in terms of interspecific competition leading to spatial segregation. Furthermore, habitat requirements or predation can shape community structure leading to segregation or aggregation of species (Cordero and Jackson, 2019). Among negatively associated taxa, the metazoan group was the most abundant. Eleven taxa, of which six belonged to the family of *Chironomidae*, showed strong negative correlations. This seems at odds the fact, that chironomid larvae are often added to food mixtures in pearl mussel hatcheries. The negatively associated chironomid species are rheophilic (*Micropsectra* sp., *Rheocricotopus* sp.), crenophilic (*Corynoneura fittkaui*, *Krenopelopia* sp., *Rheocricotopus* sp.) or cold-stenophilic (*Heterotanytarsus apicalis*) and are characteristic to forest springs (Orendt, 2000). The Bavarian Red List of endangered *Chironomidae* (Orendt and Reiff, 2003) lists two of these as endangered (RL 3, *C. fittkaui*) and strongly endangered (RL2, *Krenopelopia binotata*). These species are not associated with the occurrence of *M. margaritifera* in "active" sampling sites, but are, associated with "detritus" sampling sites, indicating a good conservation status of these forest springs and highlighting their potential as detritus source according to Hruska (1999).

The negative association of protist taxa with *M. margaritifera* is difficult to interpret, as little is known about their role in biocenosis and in food webs. There was one species known from brackish waters (*Amphisiella pulchra*, Chen et al. (2013)), one species inhabiting the rhizosphere of agricultural crops (*Rhogostoma cylindrica*, Dumack et al. (2017)) and two broadly distributed genera known from terrestrial, marine and freshwater habitats as well as from clean or waste waters (*Cyclidium, Vorticella* GBIF.org (2024)). It is therefore likely, that these taxa belong to habitats whose constitution is different from those of the freshwater pearl mussel, for reasons which were not included in the model, but can explain the negative association with the species.

Furthermore, two fungal taxa, both of which are phytopathogens, were negatively associated with the freshwater pearl mussel. First the genus *Darkera,* which is known to cause needle blight in conifers, resulting in premature shedding of needles (Whitney et al., 1975). As these needles decompose, they can release humic acids, which may contribute to soil acidification. This acidification could adversely affect nearby freshwater ecosystems, which may explain the observed negative association with the freshwater pearl mussel. The second is the species *Aphanomycetes euteiches*, which causes root diseases in legume roots and is facilitated by high nitrogen concentrations, which it generates itself by causing the decomposition of rhizobial nodules (Wu et al., 2018). It could therefore also negatively affect the habitat of *M. margaritifera.*

As previously mentioned, the levels of interaction presented here have not been shown or analysed before, and any attempted interpretations remain to be proven. Nevertheless, these results highlight the complexity of community structures. Environmental metabarcoding combined with high performance data science, such as the applied sjSDM approach has the power to reveal such community structures holistically. The data obtained can further be used to predict species occurrence and community response to environmental alteration, which will be increasingly important in the face of climate change.

4.4.4 Impact of environmental factors

The regression model of the correlation strength of species associated with *M. margaritifera* and their estimated responses to environmental factors reveals several relationships that influence the freshwater pearl mussel community structure. In particular, a significant negative correlation with the presence of arable fields in the catchment area, marginally significant positive correlations with nitrate concentration and water temperature, and slight negative correlations with pH and conductivity were observed for taxa positively associated with *M. margaritifera*, but not so for those with negative associations.

The negative association between arable fields and the biocenosis of the freshwater pearl mussel underscores the detrimental effects of agricultural activities on aquatic ecosystems. The percentual cover of arable land in the catchment of a river has been shown to induce shifts in macroinvertebrate communities towards generalist species with high tolerance to a variety of environmental conditions (Karaouzas et al., 2007). Arable fields are often sources of nutrient runoff, pesticides, and sediments, which can degrade water quality and habitat conditions essential for mussel survival (Araujo and Ramos, 2000). Agricultural runoff typically increases nutrient levels, particularly nitrates and phosphates, which can lead to eutrophication. Eutrophication depletes oxygen levels and promotes algal blooms, directly affecting mussel physiology (Bauer, 1988). However, these typical symptoms of eutrophication were not detected in the sampling sites. Furthermore, there was a marginally significant positive correlation between nitrate concentration and freshwater pearl mussel community. As stated in section 4.4.1 nitrate levels were higher in extinct sites than in active ones but they never exceeded the limits for freshwater pearl mussel streams stated in the literature. Moderate increases in nitrate also enhance the nutrient level which may promote the species in oligotrophic waters (Piggott et al., 2012). It was shown for example, that the macrophyte *M. alterniflorum,* which was also positively associated with *M. margaritifera,* profits from an increased nitrate level when flow velocity is high enough to prevent algal growth (see chapter 3, Sattler and Poschlod, 2023). Therefore it may be stated, that the community of *M. margaritifera* may profit from increased nitrate concentrations, if these remain beneath a certain threshold and if they occur in combination with fast flowing waters. For negatively associated taxa, however, the signal is weaker indicating an indifferent impact of the measured nitrate concentrations on these communities. Furthermore, nitrate concentrations are generally correlated with conductivity. The correlation curve of conductivity with the association strength of species to *M. margaritifera* was adversely not significant and points in a negative direction. Therefore, substances other than nitrate may have contributed to the measured conductivity levels and impacted the community negatively. As stated above, agricultural runoff may also contain pesticides, insecticides and fungicides, which were identified as most dominant stressors for river biota (Schürings et al., 2024) as they directly reduce the biodiversity of stream ecosystems (Beketov et al., 2013). However, the negatively associated taxa showed a marginally significant positive response to increased conductivity, reflecting the fact that taxa with strong negative associations with *M. margaritifera* tend to have negative and those with weaker negative associations tend to have positive estimates for conductivity. Schürings et al. (2024) showed that the land use type "agriculture" has different effects on different taxa groups, depending on the crop type and the associated agricultural practice. However, this cannot be explained by the resolution of the data used in the context of this study and would therefore require further research.

The variables pH and water temperature reflected marginally significant positive responses of taxa with positive associations to *M. margaritifera.* These showed preferences of lower pH values reflecting the slightly acidic to neutral pH conditions typical for pearl mussel streams. The slight positive correlation of these taxa with water temperature suggests, that parts of the community of *M. margaritifera* might benefit from moderate increases in water temperature. Warmer temperatures can enhance overall metabolic rates, growth, and reproduction. For *M. margaritifera* itself, higher temperatures are connected with increased growth rates and shortened life span (Bauer, 1987). Extreme or sustained high temperatures however can also reduce dissolved oxygen levels, and as a result the abundance of the Brown Trout as host fish for *M. margaritifera* (Geist, 2005). Anyway, overall temperature fluctuations were between 9.5°C and 21.2°C and therefore lay within the range acceptable for pearl mussel streams, which is between 0°C and 25°C (DIN Standards Committee Water Practice, 2007).

4.4.5 Levels to shape community structure

The ANOVA model of the internal metacommunity structure indicates that biotic interactions play a more significant role in shaping community structure compared to environmental or spatial factors. This contradicts the assumption of common analytical frameworks in metacommunity ecology that spatial and environmental factors dominate community assembly (see Leibold et al., 2022). Moreover, in a meta-study by Inoue et al. (2017) on interactions of mussel populations and fish community, SDM explained mussel occurrences exclusively by abiotic factors. The responses of the community to environmental factors, as described in section 4.4.4, may thus be overlayed, or must at least be supplemented by the impacts of biotic interactions within the community (see sections 4.3.3, 4.4.3). The importance of biotic interactions over environmental and spatial factors has been emphasized in several theoretical frameworks and empirical studies. Niche theory, for example, posits that species co-exist by occupying different niches and using different resources as a result of biotic interactions (Hutchinson, 1957). According to community assembly rules, species interactions and environmental filters jointly determine community composition. The relative importance of these factors can vary across systems and scales whereas in certain ecosystems, biotic interactions can be more critical than abiotic factors (Chase, 2003). These interactions occur within and across local communities and at different spatial scales, and involve interactions within a network of local communities, known as a metacommunity, which further influence colonisation and extinction (Leibold et al., 2004). The use of aquatic samples, as applied in this study, is likely to cover both, the local and the meta community level, as in river ecosystems, transport distances of DNA fragments vary species specifically and reach up to 10 kilometres (Deiner and Altermatt, 2014). The levels on which interactions in this case have occurred may therefore not be broken down any further.

Single species, however, did not completely follow the same distribution pattern as sites in the ternary plots shown in Figure 22 (page 89). Whereas some species were equally influenced by species associations and were located in the upper part of the triangle, some were clearly environmentally driven and the majority of species was located in the central part of the triangle indicating similar impacts of the three levels of influence. Biotic interactions are often more pronounced at the community level due to the integration of multiple species interactions, leading to emergent patterns that dominate community structure. In contrast, environmental and spatial factors may have more direct and measurable effects on individual species, resulting in a more balanced influence on species distributions (Leibold et al., 2022).

The ANOVA results reveal a complex interplay between biotic interactions, environmental conditions, and spatial factors in shaping (meta-)community structure. While biotic interactions predominantly shape the community as a whole, individual species distributions are similarly affected by biotic, environmental, and spatial factors. This discrepancy underscores the need for a multifaceted approach in community ecology that considers different scales of biological organisation and the context-dependent nature of ecological processes.

4.5 Conclusions

eDNA metabarcoding has demonstrated significant power in detecting communities within aquatic ecosystems, providing a comprehensive snapshot of biodiversity. This technique can be confidently interpreted for presence data, though it should be approached with caution for absence data and is limited in providing information about species abundance. The sjSDM approach is particularly well-suited for analysing the large datasets generated by metabarcoding, revealing complex community structures and species interdependencies. However, the bulk sampling method inherent in eDNA metabarcoding poses challenges for fine-scale spatial differentiation.

The findings of this study indicate that species interactions play a crucial role in shaping community structure, emphasizing the importance of considering these interactions in conservation efforts. For instance, the observed interactions of *M. margaritifera* with algae and vascular plants can be explained by their function as a food source. However, the associations with other taxonomic groups, such as fungi, metazoa, and protozoa, remain speculative and require further investigation in order to understand their functional roles within the community.

M. margaritifera exists within an interacting cross-taxonomic community that shares similar habitat preferences, and it is associated with other endangered species. This association underscores the freshwater pearl mussel's role as both an indicator and an umbrella species and it should be given further consideration in conservation efforts. To gain a deeper understanding of the complex ecological dynamics that operate within pristine aquatic ecosystems, future research should focus more on the entire community. The use of eDNA metabarcoding, in conjunction with sophisticated bioinformatics techniques such as the applied sjSDM approach, represents a promising approach for unravelling the underlying relationships between habitat and community interactions.

5 Historical meadow irrigation and its relation with the habitat of the freshwater pearl mussel *M. margaritifera*

Historical meadow irrigation used to be widely distributed in the cultural landscapes but is nowadays almost forgotten (Leibundgut, 2003). The peak period of meadow cultivation and flourishing pearl fishing occurred concurrently during the 18th century (Hessling, 1859; Poschlod, 2017). Also, there is indication, that flooded meadows play an important role in contributing terrestrial detritus as important food resource to habitats of the freshwater pearl mussel (Hruska, 1999). Therefore, we conducted a spatial analysis to test, whether areas, where conceptual meadow irrigation was practiced overlap with the distribution of the freshwater pearl mussel. This study was published in the journal Limnologica in 2023 (Sattler et al., 2023) and is presented in the first part of this chapter (5.1, P. 104). All text, figures and tables were taken directly from the manuscript, references were incorporated in the bibliography of this thesis. In the second part (5.2, P. 110), a closer look to functional interactions between historical meadow irrigation and the habitat of *M. margaritifera* is taken. Herein, effects on the meadow as agricultural good and on adjacent brooks as potential habitats for *M. margaritifera* were measured on different scales. Both parts are presented as separate subchapters.

5.1 Meadows and mussles – the relation between historical irrigation systems and the habitat of the freshwater pearl mussel *Margaritifera margaritifera* (L.)

The section literally quotes an article authored by Julia Sattler, Sabine Fischer and Peter Poschlod, published in January 2023 in the journal *Limnologica* 98, doi: 10.1016/j.limno.2022.125994.

Abstract

Conceptual meadow irrigation is a historically prominent land use practice, that contributed decisively to the landscape. It enhances land-water-connectivity and might directly interact with habitats of *Margaritifera margaritifera* (L.). The aim of the presented study was to investigate, if there is a spatial correlation in the distribution of conceptual meadow irrigation and *M. margerigtifera*. We therefore digitalized the distribution of both in the German Federal Republic in GIS. This revealed a 68% overlap of map quarters with records of the Freshwater Pearlmussel with those, where conceptual meadow irrigation was practiced. With our findings we want to draw the readers attention to the potential of this land use practice for ecosystem functioning.

5.1.1 Introduction

The freshwater pearl mussel *Margaritifera margaritifera* (L.) is today one of the most endangered limnic organisms (Geist, 2010). Its high demands on water quality and habitat equilibrium roused many researchers interest which led to a profound understanding of its biology and ecology (Jungbluth et al., 1985). However, due to these high quality needs, it hardly survives the radical changes exerted by mankind throughout the Anthropocene (Täubert and Geist, 2017). Its strong decline was observed over the last 100 years, and since the end of the 20th century, the species is listed as endangered in IUCN 1996 Red Data Book (Araujo and Ramos, 2000) and is protected by the Annexes II and V of the Flora Fauna Habitats Directive (FFH, EU-Directive 92/43/EEC). Whilst the direct impacts through pollution and river regulation are ubiquitous in the discourse of pearl mussel protection (Araujo and Ramos, 2000), a major component of habitat quality is the supply with suitable food resources. *M. margaritifera* filter-feeds from small floating particles such as derived from algae, terrestrial and aquatic detritus (Bauer and Wächtler, 2001).

The influx of terrestrial detritus to brooks is enhanced in alluvial meadows or by draining ditches that pervade them. From such ditches, detritus for the ex-situ breeding of mussels is being collected (Hruska, 1999). Thus, wet or periodically flooded grasslands may contribute to the quality of freshwater pearl mussel (FPM) habitats (Brauns et al., 2021).

The connectivity between terrestrial and aquatic habitats has become scarce in modern times, e.g. through the loss of floodplains by regulation measurements. Historically though, this association even contributed decisively to the cultural landscape. Before mineral fertilizers started to be applied, the conceptual flooding of meadows with water from adjacent brooks was an adequate way to fertilize grasslands (Leibundgut, 2003; Poschlod, 2017) and to elongate the vegetation period or to control vermin and pest (Hassler, 1995). In this process, brooks were impounded periodically by small weirs to let the water pervade the adjacent, inclined meadow. Thereby, nutrient and sediment loads were contributed to the soil (Hassler, 1995). Moreover, the partially filtered water seeped down to the ground water, elevating its level (Leibundgut and Binggeli, 1984). The proportion of water that was not held back by the meadow usually re-entered the river system via a channel at the bottom end of the meadow. In this system, the meadow functions as sediment and nutrient trap. On the one hand, this advances grassland species and accelerates the production, on the other hand it provides clear and oxygen rich water to downstream waters (Cook et al., 2017). As such, conceptual meadow irrigation (IM) could perform an important ecosystem service also to *M. margaritifera*, which is highly dependent on clear, oxygen-rich waters and at the same time, threatened by siltation and colmation of the river bed (Denic and Geist, 2015). Furthermore, it is known, that the detritus composition of waterbodies can be influenced by the kind of land use practice in the basin (Merlo-Galeazzi and Zambrano, 2014).

The here discussed agricultural practice resembles floodplain processes (Cook et al., 2017) which contribute detritus as essential food resource to FPM habitats (Brauns et al., 2021).

The golden ages of grassland cultivation were the 18th and 19th century. In these times, complex watering systems with an own legislation and profession developed (Poschlod, 2017). At the same time, the pearl fishing industry was at its maximum indicating a broad distribution of FPM in the brooks (Hessling, 1859). Hence, IM and FPM overlap temporally in their occurrence.

Given the aforementioned findings, we hypothesize, that if there also is a spatial overlap between both variables, *M. margaritifera* may have profited from the beneficial effects of conceptual meadow irrigation. To find out, if IM and FPM co-occurred in former times, we conducted a distribution analysis on historical and present occurrences of both at the extend of Germany.

5.1.2 Material and methods

As a basis for this analysis, we used a map of conceptual meadow irrigation from 1937 (Böhm, 1990). We transferred the datapoints to the grid of topographical map quarters of Germany with ArcGIS 10.8 (ESRI, 2020). For gathering the distribution of FPM in past and present, we made an extensive literature study which we supplemented with distribution data from the authorities of the federal states of Germany. As basal layer for the generated map, we implemented areas, where brooks of the LAWA waters type 5 'rough material rich, softwater brooks on silicious bedrock' (Pottgiesser, 2018) exist. This type of brooks indicates a geographical predisposition for both variables. We then extracted all map quarters that had the records of interest and calculated the share of overlap in MS Excel.

5.1.3 Results

As can be seen in Figure 23, the areas where the freshwater pearl mussel occurs in higher densities are those, where also IM was practiced. We found, that in 68 % of all map quarters in Germany that have records of FPM, IM was also practiced and in 86 % of those occur brooks of LAWA type 5 (Figure 24). From those quarters where FPM was not recorded, there is only a share of 28 % with IM, in 20 % brooks of LAWA type 5 occur and 63 % have neither of both.

With these findings, we demonstrate that, besides the temporal, there also is a spatial overlap between conceptual meadow irrigation as historically important type of land use and the occurrence of the strongly endangered freshwater pearl mussel.

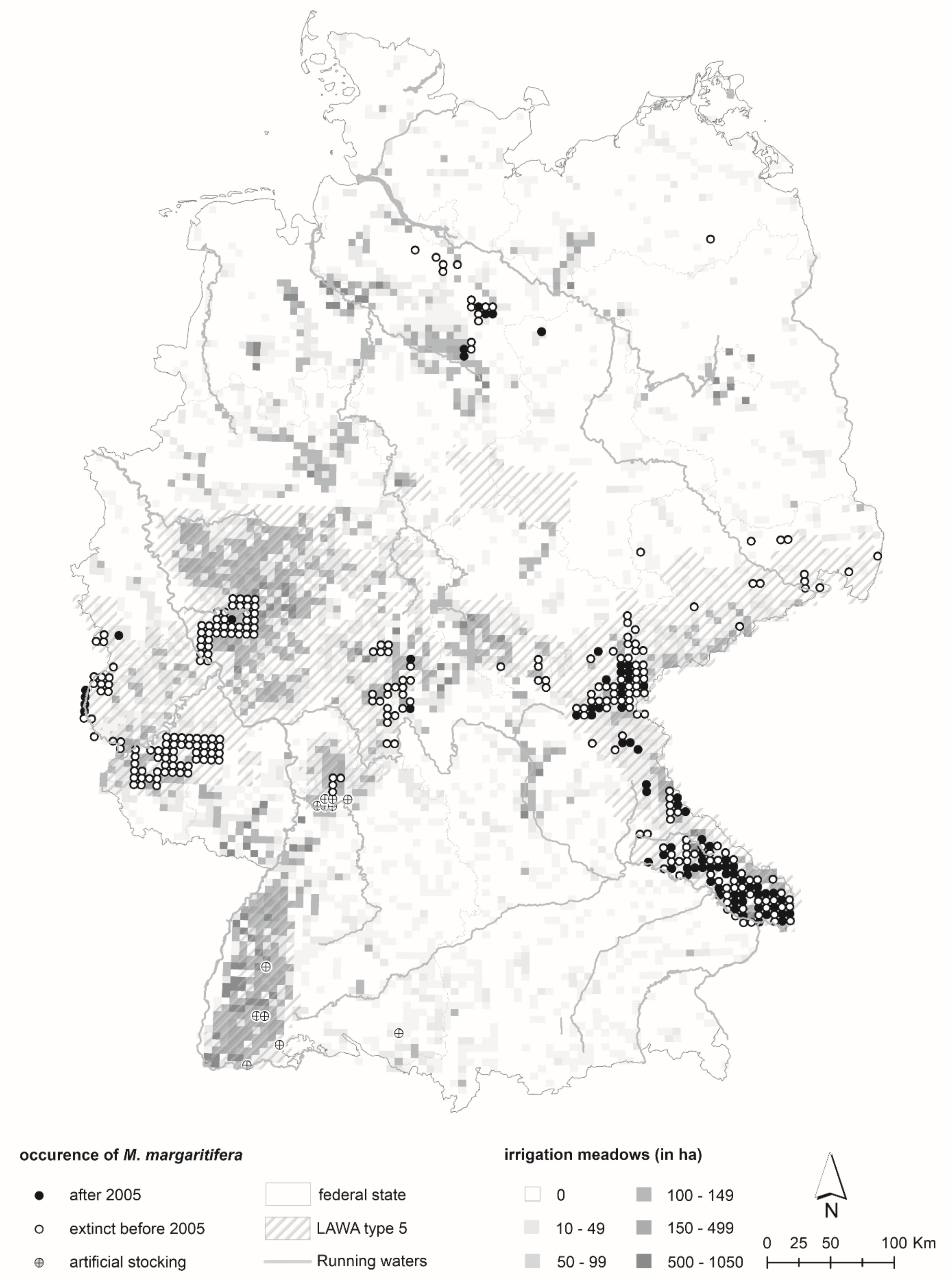

Figure 23: Distribution of *M. margaritifera* from literature and databases with records between the years 1600 and 2018, of conceptual meadow irrigation with records published by C. Troll (1937) and of brooks of LAWA type 5 (rough material rich, softwaters, on silicious bedrock). Resolution is given per quarters of the 1:25.000 grid of topographic maps in Germany (12.5 ×12.5 km2). References for the distribution of *M. margaritifera* are provided in the appendix.

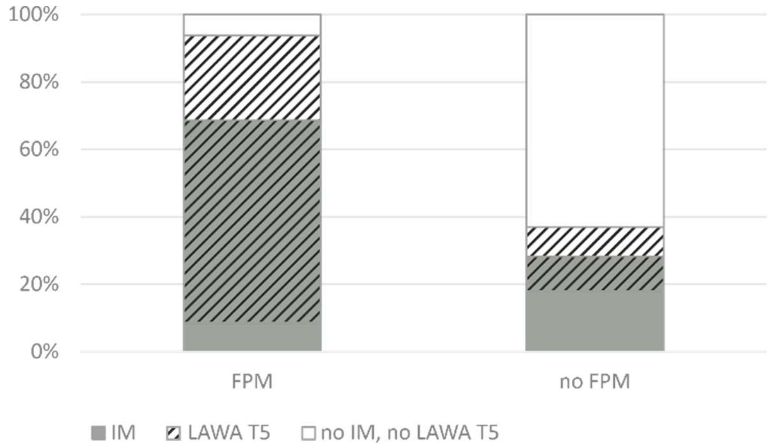

Figure 24: Quarters of the 1:25.000 grid of topographic maps in Germany (12.5 ×12.5 km) with and without occurrences of the freshwater pearl mussel (FPM) and their proportionate share of co-occurrence with practiced meadow irrigation (IM) and brooks of LAWA type 5 (rough material rich, softwaters, on silicious bedrock).

5.1.4 Discussion

Clearly, the presented results reflect a mere correlation, and a functional nexus remains to be proven. However, we see a great and often underestimated potential of historical land use types as concepts for nature conservation. In central Europe, land has been shaped by humans for more than 7,000 years, whereby a natural landscape has been continuously converted into a cultural one (Poschlod, 2017). For the longest period of time, the quest for higher productivity caused a diversification of landscapes. As it allowed a co-evolution of the species, which adapted to the newly created habitat niches, it even increased biodiversity (Poschlod, 2014). Only throughout the very recent years, the technical progress, e.g. the availability of artificial fertilizers, modern pump and sprinkler devices or heavy agricultural machinery, made redundant those very specialized ways of land use. This inverted the often synergistic effects of land use and biodiversity and went along with a vast extinction of species (Newbold et al., 2019). Conceptual meadow irrigation may be seen exemplary for the described development. Devised to increase productivity in grassland management, it created a high structural diversity which gave habitat to many species (Everard, 2005). It provides a large variety of ecosystem services, e.g. by contributing a high potential for water retention, functioning as trap for silt, phosphorous and nitrate content, improving the soil and increasing the production (Hassler, 1995). Anyway, throughout the 20th century, the irrigated areas in Germany declined from more than 250,000 to a few thousand hectare (Leibundgut, 2003). For reasons of nature conservation and maintenance of cultural heritage, conceptual meadow irrigation has been re-established in some areas (Leibundgut and Kohn, 2014a). With the here presented postulation, we want to underline the recognized potential of this historical type of land use and raise awareness to its functional contributions to habitats of the freshwater pearl mussel.

5.1.5 Conclusion

We have outlined that there is a temporal, as well as a spatial overlap in the distribution of freshwater pearl mussels and irrigation meadows. Further, the functional traits provided by IM match the habitat demands of FPM in many aspects. The proceedings in climate change and species extinction urge for progressive and holistic approaches that combine land use and nature conservation. The potential of this unconventional land use practice for water retention and clearance, improvement of soils and valorisation of FPM habitats should therefore highly be considered for further investigation and practical implementation.

5.2 Historic meadow irrigation - effects on grassland communities, soil conditions and adjacent brooks as potential habitats for *M. margaritifera*

5.2.1 Introduction

Historically, the economic use of land has been much more determined by location and abiotic conditions. Grasslands, with their constant leaf production and shallower root horizons, have a higher demand for surface area near water than arable land. At the same time, they are less sensitive to, and may even benefit from, high groundwater levels and periodic flooding. Therefore, fluvial valleys or moist locations in general have always been preserved for grassland cultures as the only possible type of land use (Hejcman et al., 2013). As the "mother of the field", grasslands were essential for survival, as they fed the cattle which provided food for the population and fertilizer for the fields. Therefore, grasslands were carefully managed using sophisticated techniques (Poschlod, 2017).

The concept of flood pulse irrigation led to the creation of water or irrigation meadows in river valleys from the Alps to the lowlands. Grasslands were systematically flooded with water from adjacent brooks, providing them with water and nutrients. In the days before the use of mineral fertilizers, cattle manure was preserved for use on arable land. Conceptual irrigation was therefore the only way to offset the otherwise unidirectional nutrient flow from grasslands mediated by the constant removal of biomass (Konold et al., 2004). Furthermore, in arid regions, such as the Mediterranean, it has allowed the agricultural use of grasslands, while in cold regions with longer frost seasons it has extended the growing season, allowing earlier harvesting or grazing (Renes et al., 2020). Other benefits may include the reduction of vermin and pests (Ewald and Klaus, 2009).

Concepts of irrigation were various, including water logging with different types of weirs or active pumping. Irrigation systems could be individual meadows, where simple weirs were used to log the water and flood the meadow before flowing back into the same brook. Other systems make use of the natural slope of a meadow, where water is introduced via a canal at the top, penetrates the meadow as it runs downhill and is intercepted in another canal at the food, which returns it to the main canal. Complex mosaics of meadows and canals designed to optimize flow rates and fertilizing functions were also developed, mostly in the 18th century. They are all based on the principle that water must be kept moving in order to avoid ponding in the meadow and with it the creation of anoxic conditions (Renes et al., 2020).

Until the 20th century, the use of conceptual meadow irrigation was widespread in Europe, with a variety of techniques adapted to local conditions (Leibundgut and Kohn, 2014a). However, systematic river regulation from the 19th century onwards released these stocks for agricultural use. With the conversion of meadows into arable land and the availability of mineral fertilizers, the meadow lost its key role in the agricultural cycle. As a result, water meadows fell into disuse and eventually disappeared from the landscape (Renes et al., 2020).

In recent decades, there has been a growing interest in water meadows from different directions. From geologists interested in the technique itself or its effects on the physicochemical constitution of the soil (Cook et al., 2017), from cultural science and heritage stakeholders aiming to preserve water meadows as cultural good (UNESCO, 2019), or from ecologists analyzing the functional relationships of conceptual irrigation with grassland communities and biodiversity (Riedener et al., 2013; Schirmel et al., 2014). All this has contributed to a broader knowledge of this agricultural practice and its impacts on landscapes and habitats. Today, water meadows are suspected of providing a wide range of ecosystem services. Sediment and nutrient loads in water bodies often put aquatic habitats at risk (Denic and Geist, 2015). In conceptual meadow irrigation, the irrigation water penetrates the meadow in such a way, that fine particles and nutrients are sedimented and deposited on the soil surface. This improves soil fertility and reduces erosion potential due to the constant and uniform nature of this treatment. The water that returns to the water system is clarified and enriched with oxygen (Cook et al., 2017). It also raises the water table, making these sites more resistant to aridification (Leibundgut and Kohn, 2014a).

As shown in a map about the distribution of conceptual meadow irrigation published by Böhm (1990), there is a concentration of this agricultural practice in Low Mountain Ranges, on areas of bedrock or in the so called rain shadows of them. As such, there is a spatial overlap of areas with conceptual meadow irrigation and the distribution of the freshwater pearl mussel *M. margaritifera* (Sattler et al., 2023). This correlation was analyzed in the previous chapter and depicts the first baseline for a potential functional interaction with the habitat of *M. margaritifera.*

M. margaritifera is one of the most endangered freshwater organisms in the world (Geist, 2010). It is adapted to oligotrophic, fast running, soft-waters on silicious bedrock with clear and oxygen rich interstitial, composed of rough material (Bauer, 1988). Adults sit in the sediment and filter feed from small particles that float in their surrounding waters. To successfully reproduce, the pearl mussel depends on the presence of the brown trout *Salmo trutta* or the Atlantic salmon *Salmo salar*, which may host the mussel larvae for one winter until it reaches the stage of a juvenile mussel (see also subchapter 2.1). Juvenile mussels spend five to seven years buried in the sediment, where they strongly depend on an oxygen rich, well perfused interstitial (Bauer, 1987).

River regulations, which alter the physical constitution of running waters and their beds prevent migration of fish and induce eutrophication, which promotes algal growth leading to oxygen poor conditions, siltation and colmation in sediments, depict great risks for *M. margaritifera.* So do mechanical destruction e.g. pearl fishing or tourism and pollution with toxic wastes from industry or agriculture. All these have caused around 90 % of *M. margaritifera* populations to go extinct throughout the 20th century (Geist, 2010). Another key factor is the paucity of suitable nutrients such as riparian or terrestrial detritus (Brauns et al., 2021) which origins from wetlands or meadows and is contributed to aquatic habitats via some kind of land-water connectivity (Hruska, 1999).

Given these facts, we hypothesize that conceptual meadow irrigation

i) enhances soil fertility and thereby impacts grassland communities by promoting growth of nutrient and moisture indicating species

ii) improves habitat conditions for *M. margaritifera* by

   a) increasing oxygen concentrations in water and interstitial of the discharging water
   b) reducing fine sediment loads and thereby siltation in the discharging water and
   c) promotes mussel growth by contributing terrestrial detritus to the discharging water.

5.2.2 Material and methods

To investigate the hypotheses, an abandoned irrigation meadow in the Bavarian *Fichtelgebirge* near the town of *Wunsiedel* was restored (Figure 25). The meadow was divided into a treatment site, where flood irrigation was applied twice a year in 2021 and 2022, and a control site without irrigation (section 5.2.2.2). Vegetation was mapped three times in May, before irrigation in 2021 and after one year of irrigation in 2022 (section 5.2.2.3). Soil samples were taken from each vegetation plot and analysed for nutrient composition (section 0). Filter samples were taken from the effluent at the treatment and control sites to analyse its chemical condition and eDNA components (section 5.2.2.8). Bioindication with juvenile mussels was carried out in situ, and detritus was collected and analysed for nutrient content and also used to carry out bioindication under controlled conditions ex situ (sections 5.2.2.5 and 5.2.2.6). Sediment traps and turbidity loggers were installed along with irrigation periods and water chemistry parameters were measured (section 5.2.2.7).

5.2.2.1 Study region

The Fichtelgebirge is a low mountain range in Upper Franconia, in the north-eastern Bavaria, Germany. At 578 m above sea level, the average annual temperature in the region between 1991 and 2020 was 7.4 °C and the average annual precipitation was 786.2 mm. The *Wender Bach* is a small brook that originates in a forest about 2.5 km south of the meadow and contributes to the *Röslach* after about 6.5 km. At the southern end of the meadow, a canal feeds a small reservoir, from which a second canal runs parallel to the brook at the food of the sloping meadow. The structures of old irrigation systems are well visible in the brook.

The meadow is mown twice, at the end of May and in the end of July. As the meadow is under a special nature conservation contract (Vertragsnaturschutzprogramm, VNP), it has not been fertilized for at least 15 years. The average distance between the brook and the ditch at the treatment site is 40 m and the total area of the meadow is 6.5 km^2. The slope of the meadow is 7 % at the treatment site and 4 % at the control site (https://abag.lfl.bayern.de/). Erosivity is calculated with an R-factor1 of 123, which reflects the intensity and quantity of all erosion-inducing rainfall events at a site, and a length-slope (LS) factor (Panagos et al., 2015) of 0.74. Despite very high rainfall in June and July of 2021, there was no waterlogging at the site. Water infiltrated regularly, which is a prerequisite for the application of conceptual irrigation. The summer of 2022 was extremely hot and dry (DWD, 2022) and this area was also affected by drought, but brook and ditch contained enough water to allow irrigation.

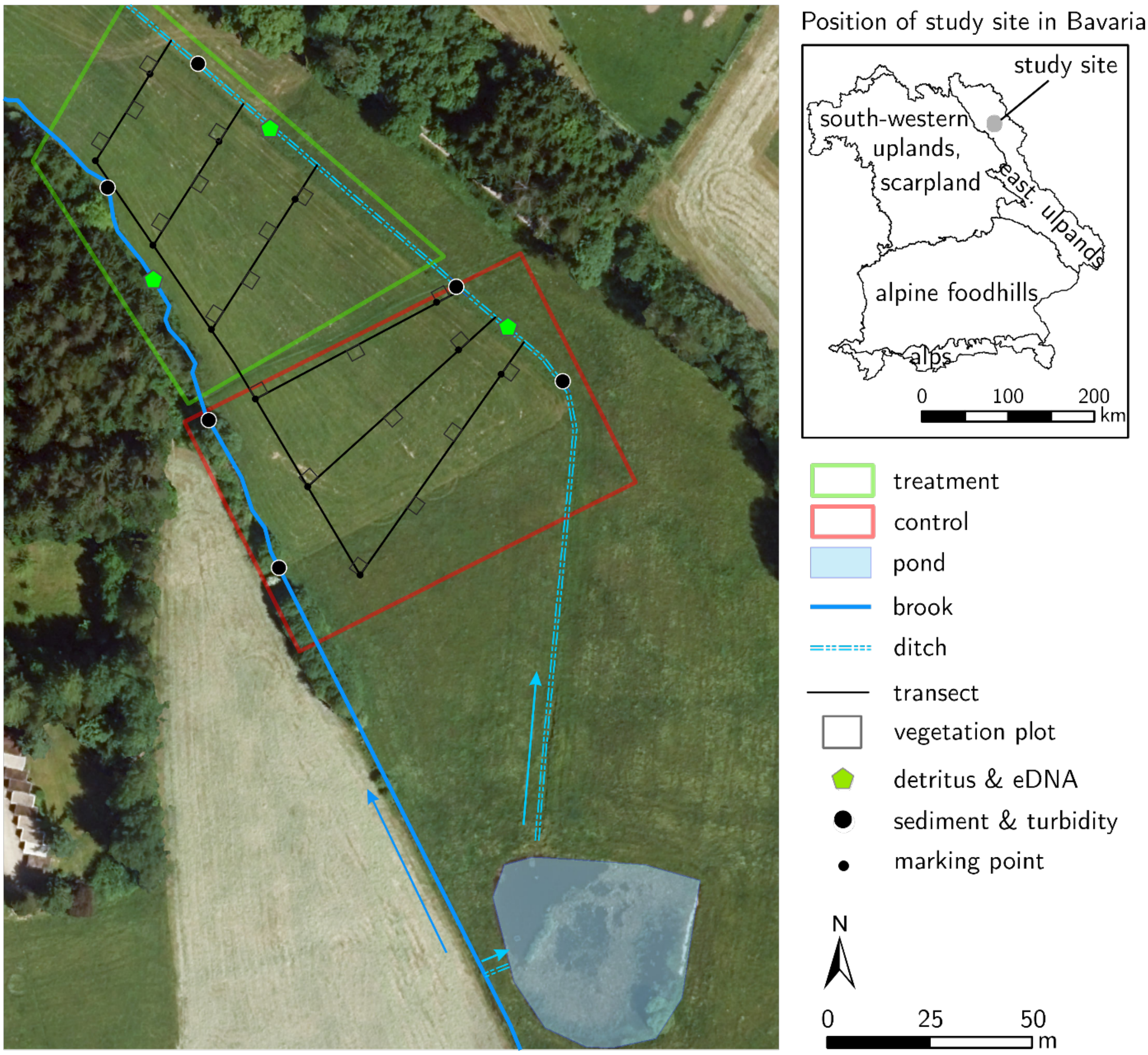

Figure 25: Detailed plan of the re-established irrigation meadow, position of treatment and control site, position of sample sites and location in the eastern uplands of Bavaria. Background map: digital ortho photo (DOP), Bayerische Vermessungsverwaltung.

5.2.2.2 Irrigation

Irrigation was carried out twice a year in 2021 and 2022, immediately after cutting in May and July, for 10 days each. The *Wender Bach* was dammed with sand bags, starting at the upstream end of the treatment site. Following the historical irrigation practice at the site, the sacks were moved 5 m downstream each morning throughout the period to irrigate the meadow section by section. This irrigated a segment approximately 10 m wide, so that each section of the treatment side was irrigated for 48 hours per irrigation period. In 2021, the irrigation periods were 6 to 17 June and 3 to 12 August, and in 2022 they were 10 to 19 June and 30 July to 8 August.

5.2.2.3 Installation and vegetation mapping of semipermanent plots

The meadow was divided into a treatment site located downstream of the irrigation water and a control site located upstream of the treatment site. From the baseline, that was drawn at the top end of each meadow parallel to the irrigation water, three transects were projected across the slope of the meadow at each site. The start and end point of each transect were marked with metals and the GPS position was recorded. Along the transects, 10 plots of 2x2 m^2 each were distributed at defined distances on both sides, always starting with the first plot to the left. The distances given refer to the starting point of the longitudinal axis of the plot. The end point of each transect also marks the start point of the last plot of the transect (Figure 25, Figure A 11).

Each plot was mapped before the first irrigation period in May 2021 and after one year of irrigation in May 2022. The vegetation layers herb, grass, moss, litter and bare ground were estimated in percent coverage, herb and grass additionally in cm height. Species were recorded on an extended Braun-Blanquet scale (Table 7).

Table 7: Advanced Braun-Blanquet scale for vegetation relevées and translation to %-range and mean for calculation.

Scale	%-range	%-mean
r	< 1 %, single individual	0,5
+	< 1 %, several individuals	0,5
1	1-5 %	3
2a	6-15 %	10,5
2b	16-25 %	20,5
3	26-50 %	38
4	51-75 %	63
5	76-100 %	88

5.2.2.4 Soil samples

Soil samples were taken in 2022 before the first irrigation (24 May) and 6 to 8 weeks after each irrigation period (25 July and 26 September). Following the standard DIN ISO 10381 (Ad-hoc Arbeitsgruppe Boden, 1996), five sub-samples were taken from each 2x2 m^2

vegetation plot from a depth of 15 cm using a Prückhauer corer, mixed and stored in labelled plastic bags in cold boxes. On the same day, the bags were placed in the drying cabinet and dried at 40 °C to constant weight. This was usually achieved within three to four days. The dried samples were then stored at 4 °C until further analysis.

Phosphorous and potassium concentrations, pH and conductivity were analysed according to the VDLUFA (2016) protocol. Samples were sieved on a test sieve with a mesh size of 2 mm according to DIN ISO 11464 (DIN Standards Committee Water Practice, 2007). For the determination of pH in water (H_2O, active pH), 10 mg of soil was weighed into a PVC bottle. 25 ml of pure water was added and stirred every 20 minutes with a glass rod. After one hour pH was measured with a WTW Multi 3320 multimeter with a SenTix© 41 probe. After adding another 25 ml of pure water, the conductivity was measured with a DWD Multi 3320 multimeter with SenTix® 41 probe. The determination of pH in calcium chloride ($CaCl_2$, potential pH), was carried out in the same way, but instead of water, a 10 mM $CaCl_2$ solution was added to the soil. Phosphate and potassium were analysed according to VDLUFA (2016) with a calcium-acetate-lactate (CAL) extraction. The CAL solution consisted of 154 g calcium lactate ($C_6H_{10}CaO_6$ x 5 H_2O), 79 g calcium acetate $(CH_3COO)_2$ and 179 ml pure acetic acid (100 %) in 2 l pure water. The solution was diluted 1:5 before 100 ml was added to 5 g of soil. After shaking for 90 minutes at 20 rpm, the samples were filtered through soil filters (type 113P, #CA10.1) into 100 ml PVC bottles. For photometric determination of phosphate, 4 ml of CAL extract was placed in a glass test tube to which 5.2 ml Millipore water and 0.4 ml ammonium molybdate solution (5g $(NH_4)_6Mo_7O_{24}$ in 100 ml Millipore water) were added. After mixing, 0.4 ml reduction solution (1,25 g ascorbic acid, 350 mg stannous chloride-dihydrate ($SnCl_2$ * $2H_2O$) and 40 ml hydrochloric acid (HCl) in 100 ml Millipore water) was added and mixed again. After incubation for 10 minutes, the absorbance was measured photometrically at 580 nm against the blank. For the determination of potassium by AAS (ThermoElemental-ASX-510), 1 ml of the CAL extract was transferred to glass test tubes, mixed after the addition of 9 ml Millipore water and measured by AAS against a potassium standard (Merck, ß$_K$ = 100 mg l^{-1}).

The carbonate nitrogen rate was determined by elemental analysis using a vario micro cube (Elementar Analysensysteme GmbH). 10 mg of the samples were weighed in tin foil and, after addition of 3 mg of tungsten trioxide (WO_3), combusted at 1150 °C for 90 minutes with the addition of oxygen. Foreign gases were separated in a reduction tube filled with copper and silver wool. The target gases were then separated by chromatography and measured using a thermal conductivity detector. The intensity of the detector signal can be converted to elemental concentration.

5.2.2.5 Detritus collection and analysis

Detritus was collected from the brook, control and treatment sites before and after irrigation. For this, the standard method of Simon et al. (2017) was used. Half of the detritus samples were used for the ex situ feeding experiment and the other half was dried and used for chemical analyses, to determine the organic matter content in terms of loss on ignition (LOI, DIN EN 12879, 12880) and the concentration of potassium (K), manganate (Mn), sodium (Na), calcium (Ca), magnesium (Mg) and phosphate (P) with atomic spectroscopy.

Samples were weighed in ceramic crucibles, dried in a muffle furnace at 550 °C for four hours, weighed again, then calcined at 980 °C for a further four hours and weighed again. The differences between the dry weight and the ash weight was used to calculate the LOI according to the following formula:

$$LOI = \frac{m2 - m3}{(m3 - m0)} * 100$$

where m2 is the dry weight, m3 is the ash weight and m0 is the crucible weight. Of the remaining ash, 100 mg was weighed and taken up in 20 ml of HNO_3 [2 mol l^{-1}], filtered and measured by atomic spectroscopy for K, Mn, Na, Ca, Mg and P.

5.2.2.6 Bioindication

The in-situ and ex-situ bioindication followed the method standard of Simon et al. (2017). For the in-situ experiment, one hole plate per sampling site was loaded with ten one-year-old juvenile mussels and placed in the water at the brook, treatment and control site at the start of the irrigation treatment. Prior to the experiment, the size of each mussel was measured under a binocular microscope. At the end of the ten-day experimental period, the hole plates were removed and mussel survival and size were measured again to calculate survival and growth rates. For the ex situ experiment, the same set of hole plates was placed in separate tanks at the *Huschermühle* hatchery. For ten days, each set was fed 100 ml of detritus collected from the respective field site in 900 ml of water. Again, survival and growth rates were determined after ten days.

5.2.2.7 Sediment traps, turbidity loggers and water chemistry

At a total of six sites, three in the brook and three in the ditch, distributed upstream (control), central and downstream (treatment), sediment traps and turbidity loggers were placed before the irrigation period and left in place until irrigation was completed (Figure 25). Before and after the irrigation periods in June and August 2022, abiotic water conditions and substrate quality were recorded at three sites free water and in the interstitial respectively. Redox potential (Eh, in mV), which is a good proxy for long-term oxygen supply (Geist and Auerswald, 2007), oxygen concentration (O2 in mg l^{-1}), temperature (T, in °C), conductivity (Lf, in µS cm^{-1}) and pH were measured directly in free running water (FW) and in the interstitial from a water sample collected with a syringe from 5 cm depth according to Pander et al. (2015). Turbidity (TURB, in NTU) was measured in three replicates and sediment penetration resistance (Pen, in kg cm^{-2}) was taken from 20 randomly distributed points. During the irrigation period, turbidity was recorded every 15 min at the upstream (us) and downstream (ds) ends of the brook (WB) and the ditch (G) using automatic turbidity loggers (OBS-3A, Campbell Scientific, Logan, USA).

Sediment traps were installed at each sampling site according to Hoess and Geist (2021), with the volume of the trap following the width of the water (5 litres in WEB, 2 litres in G). Sediment traps were dug into the bottom of the waters so that the top of the trap was level with the bottom of the water. The traps were activated for two sampling intervals, from 7 to 22 June and from 26 July to 8 August 2022, during which sediment was collected. Deposition samples were processed in the laboratory by wet sieving into size fractions $>$ 20 mm, 6.3 - 20 mm, 0.2 - 6.3 mm and 0.85 - 2.0 mm. Each fraction was dried at 102 °C and weighed to determine its proportion of the total sample.

5.2.2.8 eDNA-analyses

At each site where bioindication was conducted and detritus collection was carried out, filter samples were also collected for eDNA analyses using the method described in chapter 4. This was done at the end of every irrigation period. Samples were analyzed with ITS2, 18S and Leray primers to determine plant and algal components.

5.2.2.9 Data analyses

Data were analysed using univariate and multivariate statistical methods with R (R core team, 2013). For vegetation composition analysis, the R-package 'vegan' (Oksanen, 2022) was used to perform NMDS (see also chapter 3) with Bray-Curtis dissimilarity and a threshold for random starts of 100. This produced two convergent solutions after 30 trials and a stress of 0.15, which can be considered a good fit (Clarke, 1993). The cut-off for correlation of environment and species vectors was set at $R^2 \geq 0.35$ and p-value at ≤ 0.05. Original distances of data points correlate with ordination distances in Spearman rang correlation with $R^2 = 0.8$.

Mean weighted Ellenberg indicator values (EIV, (Ellenberg et al., 2001) were calculated for each plot and observation year. The Shapiro-Wilk test was applied to check for normal distribution and Levene's test from package 'car' (Fox and Weisberg, 2019) was applied to check for homoscedasticity. As both were given, linear regression was performed using the 'lm' function from 'stats' package (R core team, 2022) to test whether treatment and control sites differed significantly in terms of EIV for moisture (F) and nitrogen (N).

NMDS, as described above, was also carried out on soil samples. Two convergent solutions were found after 20 trials in three dimensions with a stress value of 0.06, which can be considered a very good fit (Clarke, 1993). Vectors of chemical variables were correlated with a cut-off of $R^2 \geq 0.35$ and a significance level of $p \leq 0.05$. Original distances of data points correlated with ordination distances in Spearman rang correlation with $R^2 = 0.98$.

From bioindication experiments, growth rates were statistically compared using the pairwise Wilcoxon rank-sum test with Bonferroni adjustment. For survival rates, logistic regression using the 'glm' function was used. Both methods are implemented in the R-package 'stats' (R core team, 2022).

5.2.3 Results

5.2.3.1 Vegetation composition

Vegetation mapping revealed a total of 38 species, 37 with a mean of 19.5 per plot in the treatment and 34 with a mean of 17.4 per plot in the control site. The ordination diagram (Figure 26) shows the differences in vegetation composition between the years 2021 and 2022. The asterisks, representing relevés from 2021 are more to the left the diagram and correlate with the vector for 'bare ground' and the EIV for light (L). The circles marking 2022 data points are more concentrated on the right part of the diagram and correlate with grass height and cover of *Trifolium pratense, Poa pratensis* and *Taraxacum* spec. The comparison between the treatment (black) and the control (grey) plots shows that the 2021 plots (asterisks) are closer together and there is no clear distribution pattern of the data points. The 2022 sites (circles) show a wider distribution and the grey points (control) are more in the lower part and correlate with *Alchemilla* spec. and *Anthoxanthum odoratum*, while the black circles are more in the upper part and correlate with *Agrostis stolonifera, Anemone nemorosa* and EIV for moisture (F).

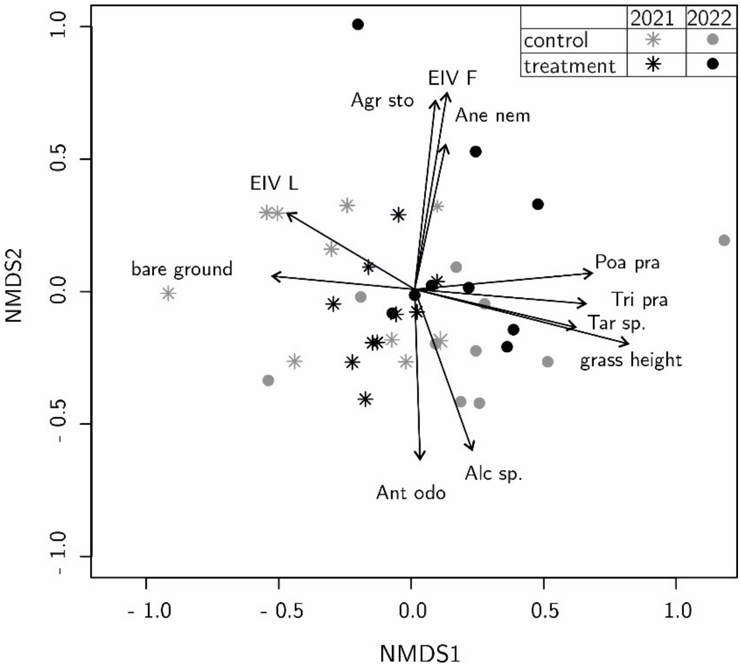

Figure 26: NMDS diagram of the vegetation composition of the irrigated meadow. A distinction is made between the treatment and control plots and between the 2021 and 2022 sampling years. Ordination was performed with a stress of 0.15, observed dissimilarity and ordination distances correlate with a Spearman rank correlation of 79.8 %. The cut-off for the correlation of species and environmental vectors was 0.35s and the significance threshold was $p \leq 0.05$. EIV_L = Ellenberg indicator value for light, EIV_F = for moisture, grass height is given in cm and bare ground in % cover.

The mean EIV for nitrogen has decreased slightly on the control site between the years 2021 and 2022 (Figure 27a). On the treatment site, it was lower than on the control site in 2021, although there was a slight increase compared to 2022, so that the median was higher than on the control site. These differences were not significant. The EIV for humidity (Figure 27b) was also higher on the control site in 2021. It increased at both sites until 2022, this increase was stronger and significant at the treatment site. All other differences were not significant.

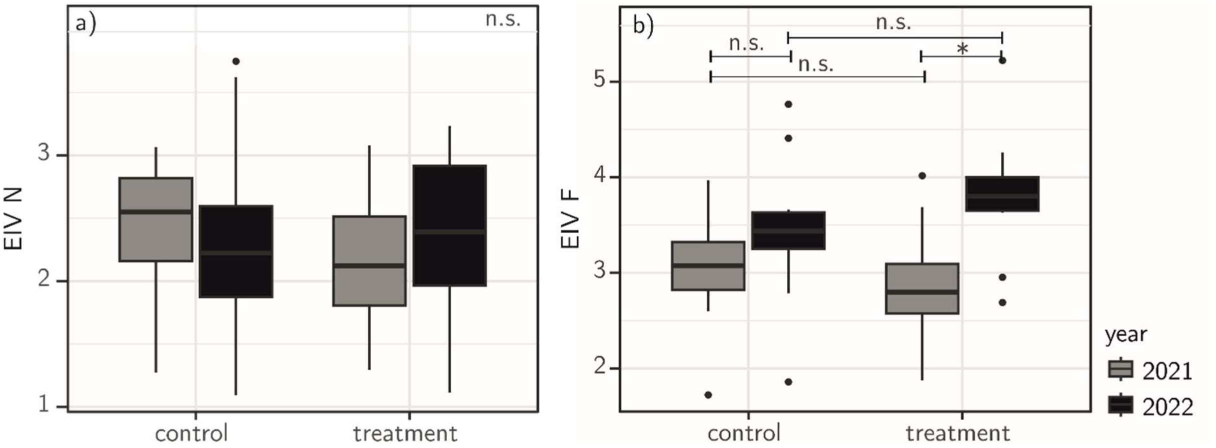

Figure 27: Ellenberg indicator value (EIV) for nitrogen (N, a) and moisture (F, b) on the treatment and control site within the observation period in 2021 and 2022. P-values were calculated using linear regression between sites in the same year and within sites over the observation years. The significance level was set at $p \leq 0.05$. Regression coefficients are given in Appendix Table A 10.

5.2.3.2 Soil samples

Soil chemistry changed during the observation period between May and September 2022 in both, the treatment and the control sites (Figure 28). Samples taken before the first irrigation in May 2022 (asterisks) are further to the left of the diagram and are interspersed between the treatment (black) and control (grey) sites. The conductivity vector is correlated with these samples. The dots and triangles representing samples taken after the first and the second irrigation periods are more on the right-hand side of the graph, which is negatively correlated with conductivity. Grey dots (control, after the first irrigation) and grey triangles (control, after the second irrigation) are spread more widely over the diagram than the black dots representing the treatment site and are oriented more towards the lower part of the diagram. Black dots and triangles are oriented more towards the central or upper part of the diagram.

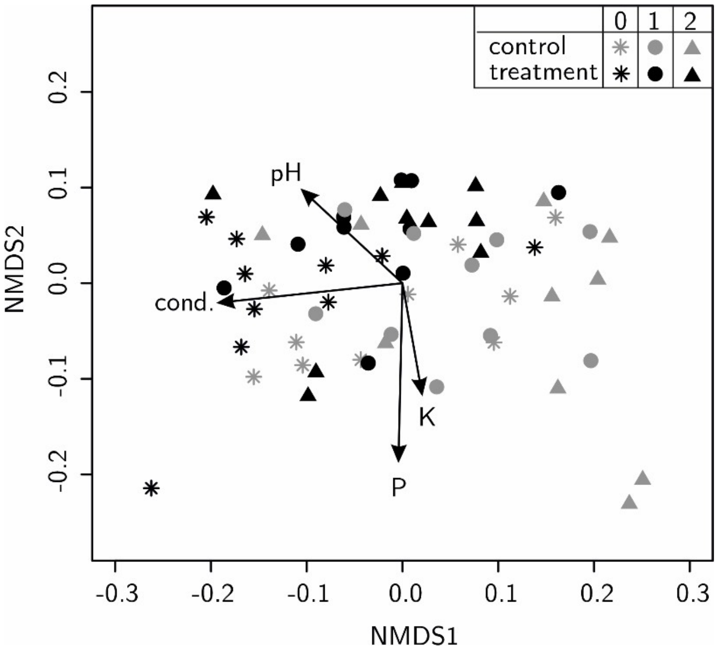

Figure 28: NMDS graph of soil samples from the treatment and control sites before first irrigation (0), after first (1) and after second irrigation (2) in 2022. Vectors for potassium (K), phosphorus (P), pH and conductivity (cond.) were correlated with a cut-off of 0.35 for R^2. Stress was at 0.05.

Regarding the individual measured parameters (Figure 29), the general pattern of parameter change in response to irrigation is similar at the control and treatment sites. However, the variance of the measured values at the treatment site is not as large as at the control site.

The potential pH (Figure 29a) was higher at the treatment site. The median remained almost constant at the control site, although the variance increased towards lower values. At the treatment site, there was a drop between the first and the second sample, then it remained constant. Phosphorus (Figure 29b) was higher at the control site. It decreased at both sites, but more clearly at the treatment site due to the lower variance. Nevertheless, single high values at the treatment site represent outliers. Potassium concentration (Figure 29c) was initially slightly higher at the treatment site. It then increased at the control site and decreased slightly at the treatment site. Conductivity (Figure 29d) was higher at treatment site and decreased at both sites during the observation period. The carbon-nitrogen ration (Figure 29e) was initially slightly higher at treatment site. The median decreased continuously, but to a small extent, at the control site. At the treatment site there was a decrease between the first and second sample and then an increase in the third sample.

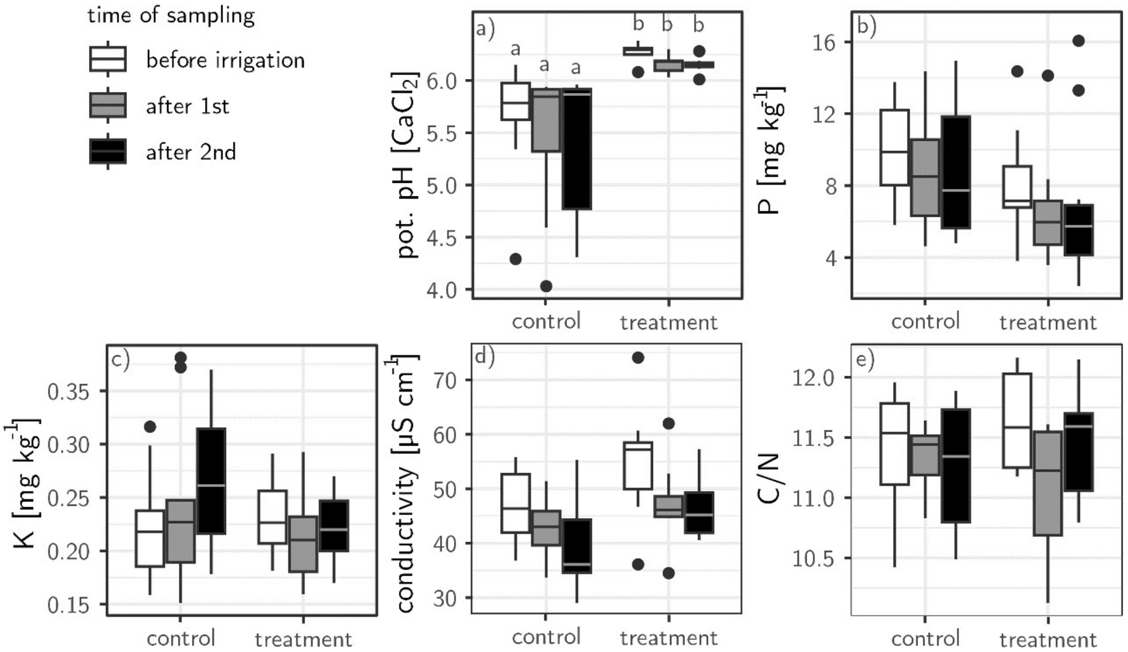

Figure 29: Soil chemical parameters measured before, after first and after second irrigation in 2022. Potential pH measured in calciumchloride, phosphorus (P), potassium (K), conductivity and carbon-nitrogen-ration (C/N). Different letters stand for significant differences ($p \leq 0.05$), no letters for no significance ($p > 0.05$). For p-values, means and standard deviation, see Table A 11.

5.2.3.3 Abiotic factors and sediments

The oxygen content in the free water was similar at the treatment and control sites. In the brook, more than 9 mg L^{-1} oxygen was measured, which was higher than in the ditch (treatment and control, Figure 30a). In the interstitial the oxygen concentration was generally lower than in free water (Figure 30b). Again, the highest values were measured in the brook (8 mg L^{-1}), followed by the treatment site (6 mg L^{-1}). The control site had the lowest values (7 mg L^{-1}). This is also reflected by the delta O_2 (Figure 30c), which shows that the differences between free water and interstitial are lowest in the brook and at the treatment site (1 - 1.5) and highest at the control site (4 - 4.5). Redox potential was highest at the treatment site in free water and in interstitial (Figure 30d, e). In free water, the measured values show greater variance at the control site, which includes values similar to those of the treatment site and those more than 100 mV lower. Also in the brook, one site showed a similar variance to those from the control site, but with higher medians. The other two sites from the brook were slightly lower than in the treatment site, but with similar variance (Figure 30d). In the interstitial the values measured at the control site are more than 100mV lower than at the treatment site. Again there were some outliers with very high and very low values were measured (Figure 30e). In the brook, the interstitial redox potential readings were intermediate between the treatment and the control site. Delta redox (Figure 30f) was lowest at the treatment site and higher and more variable at the control site and in the brook.

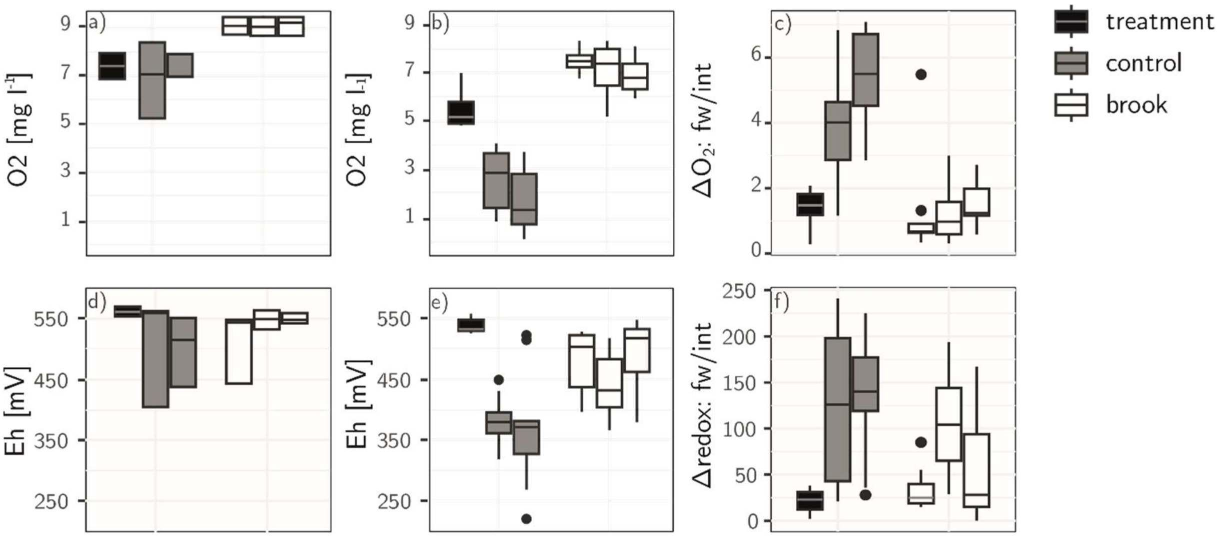

Figure 30: Oxygen concentration in mg l^{-1} and redox potential Eh in mV in free water and interstitial and relation of both at three sites in brook and ditch where one was affected by irrigation (treatment) and two were not acting as control.

The water chemical parameters show on the one hand a seasonal variance for all values except phosphate (PO_4), which was below the threshold of 0.06 mg L^{-1} for all sites after the first measurement (Figure 31b). For nitrate (NO_3), calcium (Ca), pH and conductivity, the values measured in June are always higher than those measured in August. For nitrate (Figure 31a), the values in the ditch (treatment and control) are always higher than in the brook. After the first irrigation, the concentrations in the treatment site were only half of those in the control site. This difference decreased during the following three irrigation periods, so that the last samples have almost the same values. Phosphate concentrations after the first irrigation in June 2021 were similar at the treatment site and the brook, and slightly higher at the control site. For all other samples, as mentioned above, the values were below the detection limit (Figure 31). Calcium concentrations were also lowest in the brook. After the first irrigation period they were about 4 mg L^{-1} higher in the treatment site than in the control, this difference was largely reduced over the following three irrigation periods (Figure

31c). pH values show a different pattern in the treatment site compared to the brook and the control, which show a similar oscillation over the seasons, with higher values in June and lower values in August. For the treatment site, this pattern is also visible, but after the drop in 2021, values remained at lower levels throughout the summer of 2022 (Figure 31d). The temperature showed a different variation for each sampling site (Figure 31e). In the brook and at the control site, it decreased between the first (June 2021) and the second (August 2021) irrigation period, while at the treatment site it remained at a constant lower level (14.4 °C). In the following two treatments, June 2022 and August 2022, the temperature at the stream and control site increased steadily, at the treatment site it remained low for the June treatment and then increased to 19.8 °C for the August treatment, where it exceeded the brook temperatures but remained two °C lower than the control site. Conductivity in the brook was low and with small fluctuations. At the treatment and control sites there were greater differences between the high June values and the lower August values. It was similar but slightly higher at the control site than at the treatment site (Figure 31f).

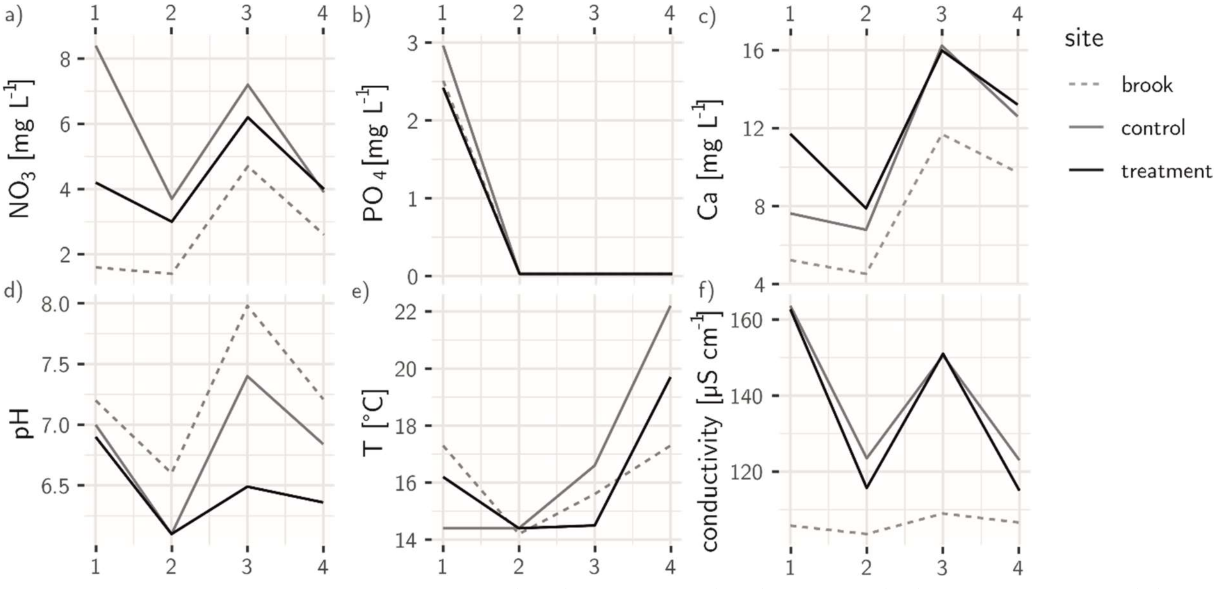

Figure 31: Water chemical parameters nitrate (NO_3), phosphate (PO_4), calcium (Ca), pH, temperature (T) and conductivity measured at three sites after each irrigation period 1: June 2021, 2: August 2021, 3: June 2022 and 4: August 2022.

The highest proportion of fine sediment was trapped at the control site (Figure 32) for both irrigation periods considered. However, in June (Figure 32a), the highest proportion of fine sediment was trapped at a site in the brook. The structure trapped at the treatment site was always between the brook and the control, in June it was more similar to the control and in August (Figure 32b), it was closer to the brook. The treatment and control sites converged at a mean corn diameter of about 5 mm, but the brook also contained sediment with 10 to 15 mm in diameter.

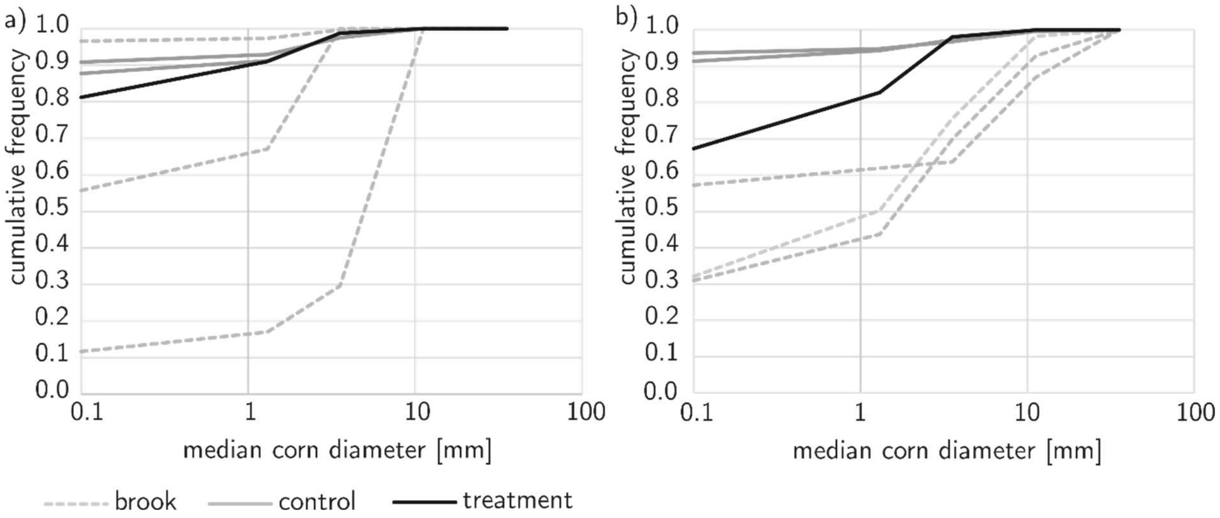

Figure 32: Structure of sediment, deposited during a) the 3rd (June 2022) and b) the 4th (August 2022) irrigation period in sediment traps in the brook, treatment and control sites.

Penetration resistance varied considerably between all sampling sites (Figure 33). By far the highest values were measured in the brook, where they ranged from 0.5 to 1.8 kg cm^{-2}. Values in the ditch were much lower and differences between control and treatment are not as obvious. Nevertheless, the values from the treatment site were significantly elevated compared to the control site. Means, standard deviations and p-values are given in Table A 16.

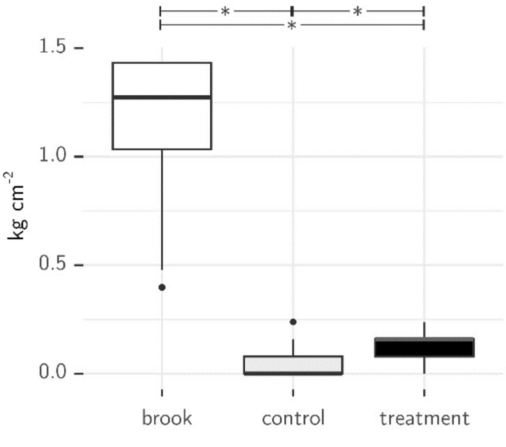

Figure 33: Penetration resistance in kg cm^{-2} measured after each irrigation period at each sampling site 20 times in an area of 1 m^2.. For p-values, means and standard deviation, see Table A 16.

5.2.3.4 Bioindication

The results of the in-situ and ex-situ bioindication experiments differ from each other and in between the test periods. In the ex-situ experiment (Figure 34), where detritus collected from the three sampling sites was fed to juvenile mussels in the hatchery, it is clear that the detritus collected from the brook had the lowest growth rates for each test period. Mussels fed with detritus from the control site, showed on average the same growth rates as those fed with detritus from the treatment site in June 2021 and in August 2022. For the other two test periods, August 2021 and June 2022, detritus collected from the treatment site induced the highest growth rates. Overall, detritus from June 2021 induced the highest growth rates, followed by August 2022. Growth rates from the experimental periods in between were similar at around 10 %. Survival was 100 % for all sites in 2021, lower in June than in August in 2022, always with the highest losses for the control site, and the highest rate from the treatment site with a constant 70 % survival. Differences were not significant in the pairwise Wilcoxon test (For p-values, means and standard deviation of growth rates see appendix Table A 13 and for regression coefficients of survival rates see Table A 14).

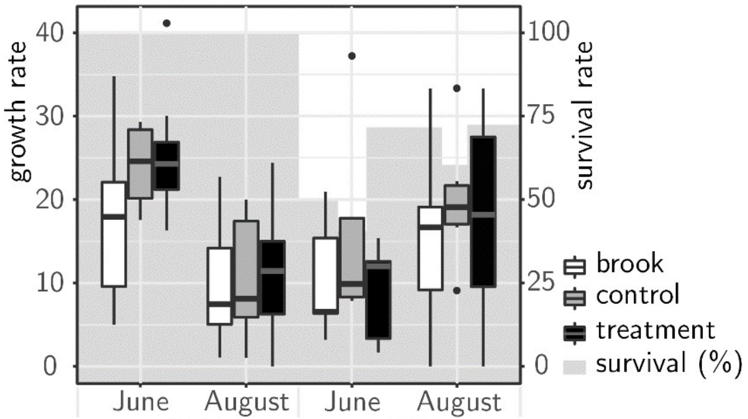

Figure 34: Growth- and survival rates of the ex-situ feeding experiment with detritus from treatment site, control site and brook, collected after the irrigation periods in June and August of 2021 and 2022.

In the in-situ experiment, growth and survival rates were much lower than with ex-situ feeding (Figure 35). The best results were obtained with mussels exposed to the treatment site. The difference is greatest after the first test period in June 2021 and still clear after August 2021. In June 2022 the treatment site and the brook showed almost the same results, while the control site had the lowest mean growth rate. Survival varied between sites and times. In the brook it increased from 50 % (June 2021) to 53 % (August 2021 and June

2022), in the control site it decreased from 65 % (June 2021) to 50 % in the other two periods and in the treatment site, it increased from 50 % (June 2021) to 60 % (August 2021) and to 65 % in June 2022. Differences were not significant in pairwise Wilcoxon test.

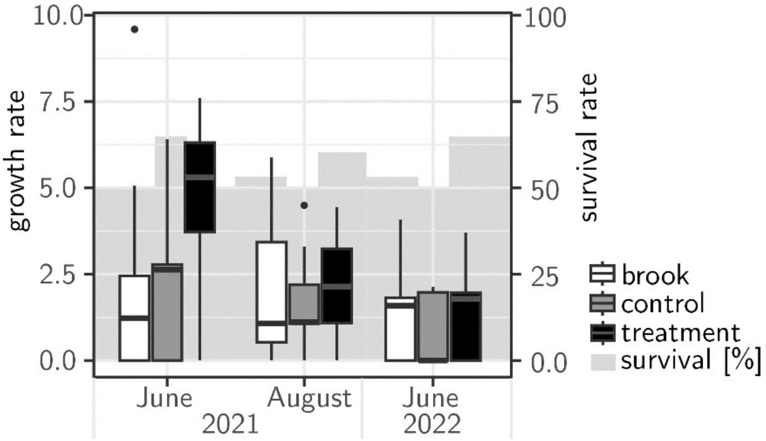

Figure 35: Growth and survival rates of the in-situ bioindication experiment with mussels on hole plates placed at the treatment site, the control site and in the brook, during the irrigation periods of June and August 2021 and June 2022.

Comparing the survival rate by experiment (Figure 36), it is clear that the detritus collected from the brook and the treatment site resulted in the same mean survival rate, although there were lower losses among mussels fed with detritus from the treatment site. The control site had the highest deficit. When placed directly on site in hole plates, the highest survival rate was observed for mussels in the treatment site with an average of 60 %. Again, the control site was the most variable but with the lowest mean of 50 %. Mussels in the brook showed the least variability and an intermediate position of mean survival rate at 55 %. The differences were not significant in logistic regression (Table A 14 for regression coefficients).

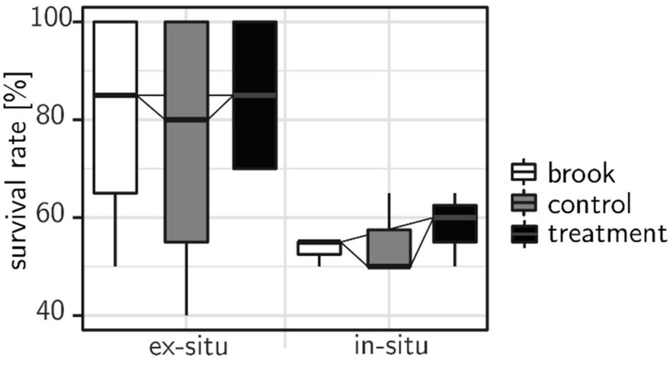

Figure 36: Total survival rates of ex-situ and in-situ bioindication at meadow irrigation experiment. Results from all observation periods were compared between sample sites.

5.2.3.5 Detritus analyses

Chemical analyses of the collected detritus revealed some differences between the sample sites. Calcium (Ca) concentration was strongly increased at the treatment site compared to the baseline measurement before irrigation and to the brook and control site (Figure 37, a). At the brook, samples after irrigation were similar to the baseline and at the control site a decrease was observed. Measures of LOI showed constant concentrations at all sites before irrigation (Figure 37, b). After irrigation, there was a decrease in the brook and the control site, while the organic load was similar or slightly increased in the detritus collected from the treatment site. Other analysed components were magnesium (Mg), phosphorus (P), potassium (K) and sodium (Na). These showed no or only marginal differences between the sampling sites and are shown in the Appendix Figure A 12. The observed differences were not significant in the pairwise Wilcoxon test (Table A 15).

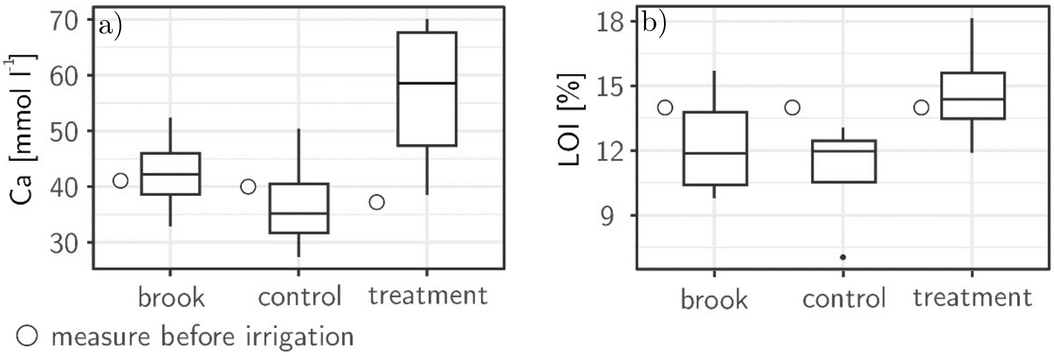

Figure 37: Chemical analyses of detritus samples for calcium (Ca, left) and organic matter in terms of loss of ignition (LOI, right).

5.2.3.6 eDNA analyses

Samples for eDNA analyses recorded a total of only nine taxa in the brook, and thus only the treatment and control sites are compared in the following section. Figure 38 illustrates the numbers of taxa recorded, grouped by functional categories. These include *Algae* (comprising taxa from the kingdoms *Chromista* and *Viridiplantae*), *Fungi*, *Metazoa* (with multicellular animals), *Protozoa* (heterotrophic organisms from the kingdom *Chromista*), and *Tracheophyta* (all vascular plants). A total of 280 species was found on the treatment site and 197 on the control site. While *Tracheophyta* and *Metazoa* are the most abundant at the treatment site, a larger proportion of *Algae* and *Fungi* contribute to the species pool at the control site. In Figure 38b, the species representations are further subdivided into the groups that are thought to contribute significantly to the diet of *M. margaritifera*. These are *Algae* and *Fungi*, which are assumed to provide essential fatty acids, species from the *Chrinomidae* family, which are thought to provide proteins, and grasses of the *Poaceae* family, which are the main component of pearl mussel detritus. *Algae* and *Fungi* were found to be more abundant at the control site while representatives of *Chironomidae* and *Poaceae* were more abundant at the treatment site. It should be noted that these data only represent the presence of the organisms and not their abundance.

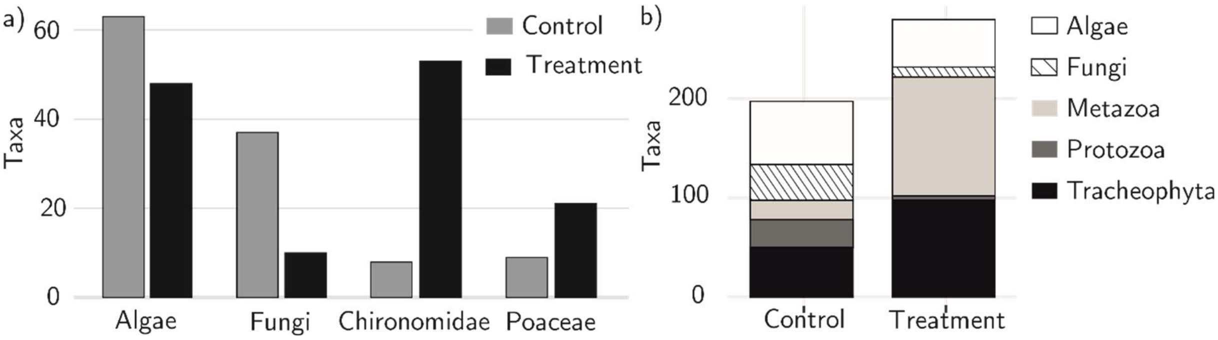

Figure 38: Taxa detected with environmental metabarcoding at treatment and control sites of the irrigation experiment. A) species numbers from taxonomic groups with nutritious relevance for *M. margaritifera,* b) Number of species per taxonomic group.

5.2.4 Discussion

5.2.4.1 Impacts of irrigation on grassland communities

Vegetation analyses revealed several differences between treatment and control sites in response to irrigation. Despite the limited observation period of two years, the treatment site exhibited a significant increase in moisture indicating species. This is in line with the finding of Gerlach et al. (2023) regarding long-term flood-irrigated meadows. A slight increase was also observed at the control site, which may be attributed to high precipitation levels experienced during the summer of 2021 (DWD, 2021). The nitrogen-indicating species also exhibited variations between the sites, although not statistically significant. An increase was observed at the treatment site and a decrease at the control site between 2021 and 2022. Flood irrigation has been demonstrated to stimulate plant nitrogen uptake (Leonardson et al., 1994) and to enhance carbon input, soil organic carbon and nitrogen levels within the meadow (Kenngott et al., 2021). The analysis of soil samples revealed no significant differences in carbon-nitrogen ratios between treatment and control sites throughout the irrigation periods. Gerlach et al. (2023) found in their study that mineral nitrogen availability was even reduced in soils from irrigated sites. Given that the meadow in this experiment has not been fertilised for a minimum of 15 years, it is plausible that the vegetation has been N-limited to some extent. This may have resulted in the additional nitrogen supplied by irrigation being taken up directly by the vegetation, leading to an increase in N-indicating species in the treatment site whereas they decreased at the control site. Other vegetation changes reported from long-term irrigated sites, such as the promotion of legumes and rosette plants (Müller et al., 2016) or that of graminoids (Gerlach et al., 2023), were not found here. It is possible that these effects become visible after a longer period of treatment. It is also assumed, that in low mountain ranges on bedrock, where streams are generally low in chalk and are more often nutrient poor, the fertilising effect of the treatment was not the main objective (Hassler, 1995), but rather the extension of the vegetation period or the improvement of soil texture were beneficial (Cook et al., 2017). The only significant difference in soil parameters was pH, which was higher at the treatment site and very constant across all measurements. Values ranged from 6 to 6.3 at the treatment site, which is similar those we measured at the ditch that runs along the foot of the site, but lower than those measured in the above running brook. Given that the values from the control site were between 4 and 6, the soil can generally be considered to be rather acidic. In such environments, flooding is likely to elevate soil pH, whereas it could decrease it if soil pH is high (Ponnamperuma, 1984). Raising the pH to around 6 is considered beneficial for the quality of hay meadows (Kirkham et al., 2014). Thus, continued irrigation could improve the economic value of the site in the long term.

5.2.4.2 Relationship between flood irrigation and parameters of *M. margaritifera* habitat quality of adjacent brooks

Oxygen concentration and redox potential were measured in free water and interstitial space in the second year of irrigation. According to Moorkens (2000), the accepted standard for dissolved oxygen in free water of *M. margaritifera* streams is at 9 mg O_2 L^{-1}. This level was only recorded in the brook, whereas the treatment and control sites exhibited similar values of around 7 mg L^{-1}. This corresponds to a 17 % reduction in oxygen concentration between the irrigation carrier and ditch, which is consistent with the finding from Cook et al. (2017). However, the values observed at the treatment and control sites were similar, indicating that irrigation did not result in a change in free water oxygen concentration. In the interstitial space, oxygen concentrations were elevated by approximately 2.5 mg L^{-1} in the treatment site as compared to the control, resulting in a delta-O_2 of 1.5, which is similar to that observed in the brook (1.0) and indicates a good sediment perfusion. The difference between the treatment and the control site is, however, more pronounced when regarding the redox potential. Here, the highest and most constant measurements for interstitial redox were taken at the treatment site with ~540 mV, which was almost as high as that of free water with ~560 mV. These values are even higher than those measured by Geist and Auerswald (2007) in streams with functioning and reproducing pearl mussel populations, who found 530 mV in free water and 510 mV in the interstitial. The discrepancy between the free water and interstitial measurements at the treatment site was 3.5%, which aligns with the CEN standard for redox potential in pearl mussel streams (DIN Standards Committee Water Practice, 2007). This suggests a markedly enhanced perfusion of the sediment in comparison to the elevated and variable values observed at the control site and in the brook.

Nitrate concentrations were consistently above the benchmark for pearl mussel streams of 0.5 mg L^{-1} given by Bauer (1988) and accepted for central Europe (Sachteleben et al., 2004). The values were higher in June than in August. The treatment site, with values between 3 and 6 mg L^{-1}, was at an intermediate position between the brook (1.5 – 4.6 mg L^{-1}) and the control (3.9 – 8.2 mg L^{-1}). In Bavaria, only between 10 and 15 % of pearl mussel streams have nitrate concentrations below the proposed benchmark, with almost 80 % exceeding these values by more than 50 % (Stöckl et al., 2020). This indicates a significant agricultural impact on water quality. However, there is no evidence, that elevated nitrate levels exsert a direct toxic effect on mussels. It has rather been proposed that the accumulation of organic matter in the interstitial space, resulting from eutrophication, leads to anaerobic conditions and, subsequently, to increased juvenile mortality (Sachteleben et al., 2004). Despite nitrate levels exceeding the suggested benchmarks, oxic conditions were observed in interstitial space, indicating that nitrate may not be a limiting factor the presence of *M. margaritifera* at this site. Also, phosphorus concentrations were high at all sites after the first irrigation period. However, after the second irrigation, they dropped below the detection threshold of 0.06 mg L^{-1}, which is in line with the recommendations for pearl mussel streams (Moorkens, 2000).

Calcium concentrations also oscillated during the observation period. Specifically, the values recorded in June were consistently higher than those observed in August, and the 2022 values were higher those recorded in 2021. Following the initial irrigation in June 2021, the treatment site exhibited the highest values (12 mg L^{-1}), which were approximately twice those observed in the brook and one-third higher than the control. In June 2022, there was an increase to 16 mg L^{-1} for the treatment and control sites and to 12 mg L^{-1} for the brook. In general, *M. margaritifera* is found in areas on bedrock, where calcium concentrations are naturally low. However, the calcium concentrations measured in pearl mussel streams are typically highly variable, which is why a benchmark is absent from the CEN standard (Boon et al., 2019). Instead, monitoring is necessary when there are pronounced fluctuations that can be attributed to anthropogenic impacts in the catchments. In this case, the variations were similar at all sample sites indicating that they were not explicitly influenced by the treatment.

Electrical conductivity is the ability of a material to conduct electricity. In pure water it is close to zero and increases with the concentration of dissolved ions and salts. Accordingly, it is correlated with nitrate and calcium levels and follows a similar pattern, with high levels in June and lower levels in August. The measured values were found to be similar across all sites, with a range of 180 to 160 µS cm^{-1}. In general, values below 100 µS cm^{-1} would be favorable for pearl mussel streams (Skinner et al., 2003). Sites with functional pearl mussel populations exhibited values of up to 150 µS cm^{-1}, with a mean of $<$ 100 µS cm^{-1} (Geist and Auerswald, 2007). The literature suggests values between 70 and 80 µS cm^{-1} (Jungbluth et al., 1985; Bauer, 1988; Hruska, 1995). and between 100 and 200 µS cm^{-1} (Moog, 1993; Moorkens, 2000). The values measured here are therefore not optimal and reach the upper edge of a tolerable range for *M. margaritifera.* However, when considered in conjunction with a sufficient oxygen supply, they do not necessarily represent an adverse condition. This is true also for pH values, which showed a similar pattern of higher values in June and lower values in August, but ranged within the interval acceptable for pearl mussel streams of 6.1 to 8. Within this range, there is no evidence for preferences of *M. margaritifera* (Sachteleben et al., 2004).

Temperature was constantly low in the treatment site for the first three irrigation periods whereas it varied in the control and the brook, which exhibited higher values for the June measurements. In August 2022, however, temperature rose in all sites, although considerably only in the ditch (treatment and control sites). The temperature curves in the stream and the control match the weather conditions during the observation period. In 2021, Bavaria experienced the third warmest June since 1881 with an average temperature of 18.9 °C. This was followed by the first August since 2014 with water temperatures being statistically too low (mean 16.1 °C, DWD). In 2022, the summer started like the year before, with warm average temperatures of 18.9 °C in June, but developed into an extremely warm and dry summer with 19.8 °C on average in August. Given that the treatment and control sites are only a few metres apart on the same water, the consistently low temperatures after the first three irrigation periods indicate a temperature drop due to irrigation. The practice of flood pulse irrigation has been demonstrated to elevate the groundwater table (Leibundgut and Kohn, 2014b). If this were to occur at the treatment site, it could potentially have resulted in a cooling effect by facilitating water exchange between the surface and groundwater systems, buffering temperature fluctuations in the environment. Such a cooling effect would improve habitat quality by increasing oxygen solubility. In addition, algae from temperate headwater streams are likely to contain more long-chain PUFAs than algae from warm waters (Grunicke et al., 2022), improving food quality for *M. margaritifera*. Especially in the context of global warming, such a buffering effect would be valuable for the protection of pristine aquatic habitats. However, this effect disappeared in the fourth irrigation period in August 2022, when high temperatures and a severe drought affected a large part of the country. This may have resulted in the buffering effect being reached at its limits if it had been accompanied by a decline in groundwater levels, which no longer exerted an influence on the temperature at the treatment site.

5.2.4.3 Effects of flood irrigation on sediment quality of the irrigation drain

Ordinary flood meadows naturally trap sediment. Irrigation treatment was found to reduce sediment loads by 74 % through 5 m of grass and by 99.9 % through 15 m of grass (Cook et al., 2017). In this experiment, deposited sediment was compared between the carrier (brook) and the drain with (treatment) and without (control) the influence of meadow irrigation. Sediment traps showed the highest proportion of fine sediments at the control site, an intermediate position at treatment site and the coarsest sediment texture in the brook. The difference between treatment and control increased after the second irrigation period.

Penetration resistance was also highest in the brook, with mean values of 1.17 kg cm^{-2}. Much lower values were measured in the drain, but still significantly higher mean values in the treatment (0.13 kg cm^{-2}) than in the control (0.04 kg cm^{-2}). Geist and Auerswald (2007) measured penetration resistances of 0.04 – 0.39 kg cm^{-2} (mean = 0.16 kg cm^{-2}) at sites with functioning mussel populations. Sites where no reproduction occurred for at least 30 years (non-functioning) showed values between <0.01 and 4 kg cm^{-2} and a mean of 0.18 kg cm^{-2}. This shows, that neither too soft nor too hard sediments are suitable for pearl mussel populations. In this experiment, the higher proportion of fine sediments at the control site resulted in a soft bed with low penetration resistance. This condition was improved at the treatment site with a lower proportion of fine sediments and a significantly higher penetration resistance. Compared to the results of Geist and Auerswald (2007), the sediments of the brook seem to be too hard and those of the control site too soft. The treatment site still had a lower mean penetration resistance than that measured in the streams with functioning populations, but of all the sites compared, it had the closest values, indicating an improvement in sediment conditions due to the irrigation treatment.

5.2.4.4 Effects of flood irrigation on growth and survival of juvenile freshwater pearl mussels

The impetus for these investigations was the assumption that flood pulse irrigation could add valuable detritus from hay meadows to the waterscape, thereby providing a food resource for filter feeders such as the freshwater pearl mussel. The results of the bioindication experiment indicate the potential for the irrigation treatment to enhance mussel survival. In the first year of the experiment, in situ growth and survival rates were improved at the treatment site compared to the brook and the control. In 2022, the treatment site continued to demonstrate the highest survival rate, although growth rates exhibited variability. Also the ex-situ analysis revealed an enhanced survival rate of 87 % on average in the treatment site as compared to 81 % in the brook and 73 % in the control site. Stable isotope analyses demonstrated that the diet of freshwater pearl mussels comprises both riparian and aquatic particles (Brauns et al., 2021). It is therefore recommended, that detritus collection for mussel culture be conducted in a water with a connection to its riparian zone. Grunicke et al. (2022) found that juvenile FPM exhibited higher growth rates when fed with aquatic detritus containing a mixture of terrestrial and aquatic particles, as compared to those fed with riparian detritus only. Mixtures of detritus and algae are commonly used in pearl mussel hatcheries. It was proposed that algae induce higher growth rates due to their high PUFA content, and that detritus is required as a ‘healthy' food source that provides essential trace elements such as calcium, buffers nitrite concentrations and leads to better survival rates (Eybe et al., 2013). It is hypothesized that detritus adheres to clay particles, which have been found to be mobilised in irrigated meadows (Cook et al., 2017). In this experiment, the main difference between treatment and control was not growth but survival rate. This indicates that the algal fraction, which is believed to mediate growth, is of aquatic origin. It is possible that algae are enriched in the pool upstream of the control site, which would explain the better growth rates in the ditch as compared to the brook and greater diversity of algae species at the control site as revealed by eDNA analysis. However, the number of species belonging to the *Poaceae* family was more than twice as high at the treatment site, which supports the theory that these are provided by the irrigation treatment. Species from the family *Chironomidae* were almost seven times more diverse in the treatment site, although the role of *Chironomidae* in the diet of freshwater pearl mussels remains unclear. They are added to feed mixtures in pearl mussel hatcheries as protein sources (Gum et al., 2011) but feeding experiments revealed no effect on growth or survival of *M. margaritifera* (Eybe et al., 2013).

5.2.4.5 Conclusion

The planning and implementation of this experiment was a complex process that involved numerous actors, including stakeholders, water authorities, the Huschermühle hatchery, which is operated by the NGO Bund Naturschutz in Hof and the Technical University of Munich (TUM) who conducted sediment and water chemistry measurements. The prevailing weather conditions also had an impact on the work, with the summer of 2021 being exceptionally wet and the summer of 2022 being characterised by high temperatures and low precipitation. As a result, the experimental design was irregular, which made it challenging to provide robust statistical evidence for the findings. Nevertheless, to the authors knowledge, this is the first study to investigate functional relationships between *M. margaritifera* habitat parameters and the historic meadow management technique of conceptual flood pulse irrigation. The investigation of the effects of flood pulse irrigation using a variety of methods has yielded indications of improvements in the economic value of extensively managed grasslands. This may encourage landowners to consider the application of this practice. Moreover, an enhanced water and sediment quality, coupled with the incorporation of meadow detritus, could potentially result in an improved habitat quality in the drains for *M margaritifera*. These aspects warrant further investigation. The concept of conceptual flood pulse irrigation, with its favourable impact on groundwater levels, soil composition and water and sediment quality, also represents a promising tool for landscape management, particularly in the context of climate change.

General discussion

The habitat of the freshwater pearl mussel *M. margaritifera* is frequently described as “pristine”, as the species demands a high water quality with a stable hydrological regime and low sedimentation rates. However, when taken literally, the concept of pristine habitats is today more of a relic than a reality, as human influence has extended even to the most remote environments (Rockström et al., 2009; Steffen et al., 2015). In densely populated regions like Central Europe, human interaction with landscapes and ecosystems has a long tradition and is likely to be an integral part of many ecosystems (Hobbs et al., 2009). The critical question that arises is how much human impact these ecosystems can tolerate before they are irrevocably damaged (Vörösmarty et al., 2010).

Historical land use has left an indelible mark also on aquatic habitats, demonstrating that many of these environments have undergone significant changes over time (Jackson et al., 2001). In the Czech Republic, the decline of pearl mussel distribution has been linked to a number of factors, including river regulation, pollution, land abandonment, intensification of arable land use, and modification of forest use and diversity (Simonab et al., 2015). These alterations in land use have influenced the temperature regime, erosion patterns and water chemistry, resulting in a scarcity of detritus particles with sufficient nutrient and calcium content (Tichá et al., 2012). This is believed to be the primary factor impeding the reproduction and survival of juvenile *M. margaritifera* (Hruska, 1999).

The study of the evolution of land use presented in Chapter 1 of this thesis has revealed similar outcomes to those, described by Simonab et al. (2015). Significant alterations to pivotal landscape components, including wetlands and floodplains, agricultural land, and river morphology, have been identified across the study area. These changes have also been present in the remote border regions between Bavaria and Bohemia, where pearl mussel populations still exist and mark a degradation of habitat quality. The survival of *M. margaritifera* in this region may thus be attributed to the long-term reduction of human activities in these areas during the period of Cold War. Today, strong efforts of nature conservation prevent the extinction of the species, but the habitats that would support the existence of self-sufficient and reproducing populations have been lost (Stöckl et al., 2020).

The human-induced disruption to ecosystem equilibrium has resulted in the necessity for costly conservation and restoration efforts, which often fall short of reversing the environmental decline and merely delaying extinction in some cases (Hobbs et al., 2009). In the case of *M. margaritifera*, these endeavours entail the ex-situ breeding of mussels and the release of juveniles into selected locations with favourable habitat conditions. However, the efficacy of these measures is uncertain, given that funding for such projects is often limited to a few years, and the maturation process for juvenile pearl mussels can take up to 15 years (Geist et al., 2023). These conservation measures represent crucial emergency procedures designed to address imminent threats to species extinction. Nevertheless, the success of these efforts is contingent upon the restoration of the species' natural habitats and should aim to explore sustainable human practices that can foster environmental health, rather than further degradation (Barnosky et al., 2012). *M. margaritifera* serves as an indicator species, not only of oligotrophic streams but also of oligotrophic catchments (Geist, 2010). In order to inform holistic conservation approaches that begin at the landscape level, it can help to gain an understanding of the landscape constitution from a time when stable pearl mussel populations existed. The loss of valuable landscape structures commenced over two centuries ago, and the diverse landscapes of that period were largely defined by human land management practices. Although the farming practices of that time allowed for a high level of biodiversity, from a contemporary perspective, they are so uneconomical that they are now only practised for the purposes of landscape conservation or species protection, with the assistance of state subsidies or voluntary work. Consequently, if biodiverse landscapes are no longer a by-product of human economic interests, they must be actively conserved for their own sake. However, to address the current environmental crises in this way, nature conservation would have to become a central part of our societies worldwide (IPBES, 2019). Another goal for the future would be to reconcile the conflicting interests of biodiversity and modern society, and to develop ways of managing land that are both economically attractive and sustainable.

The analysis of the influence of conceptual meadow irrigation on the freshwater pearl mussel in Chapter 5 of this thesis has revealed strong indication for this very traditional land use concept to support the habitat of *M. margaritifera*. This presents a compelling case for the ecological value of historical land management techniques. Traditional flood pulse irrigation has been shown to support the mussel's habitat by oxygenating water and interstitial space and providing detritus, which serves as food. Combined with its benefits on the natural balance, such as water retention and ground water elevation and for the economic use of grasslands through irrigation, fertilisation and pest control (Leibundgut, 2003), it might be a nature-based solution (NBS) for land use in pearl mussel populated areas. As such, it incorporates socio-economic development, promotes community involvement and is thought to be more resilient and sustainable than classical nature conservation by creating win-win outcomes for both people and nature (Seddon et al., 2021). While the traditional method of conceptual meadow irrigation may not be easily integrated into modern management practices, certain technological adaptations, such as the automation of irrigation wires, could make it viable in today's context.

Novel technologies also present new opportunities for nature conservation in general. Environmental DNA (eDNA), remote sensing or artificial intelligence offer powerful tools that can enhance conservation effectiveness. These innovations allow for faster, more comprehensive assessments of ecosystems, enabling conservationists to monitor biodiversity on a large scale and detect changes that might otherwise go unnoticed with classical methods (Deiner et al., 2017).

The combination of environmental metabarcoding with species distribution models such as sjSDM, as presented in Chapter 2, offers exciting possibilities for conservation planning. This approach can help to uncover hidden community interactions and predict shifts in species distributions due to environmental changes, providing valuable insights for habitat management (Carraro et al., 2018). As reference databases expand and sequencing technology advances, many of the limitations of metabarcoding, such as taxonomic resolution and the ability to quantify biomass, are expected to improve (Deiner et al., 2017). Moreover, the increasing accessibility of high-throughput sequencing technologies will continue to make metabarcoding more cost-effective for widespread use in conservation projects. However, further processing of these data by experts seems to remain inevitable, as a correct interpretation of the results depends on a profound understanding of the various limitations of this technique (Beng and Corlett, 2020). For instance, eDNA can only provide a snapshot of the organisms present at a given time, and it is often difficult to estimate population sizes or distinguish between living and dead organisms from eDNA alone (Cristescu, 2014).

The extensive utilisation of environmental metabarcoding for scientific purposes or within the context of conservation strategies has resulted in the generation of a substantial volume of data, facilitating an unparalleled understanding of species compositions within ecosystems. As a result, fortuitous discoveries of endangered or even lost species are also made, as evidenced in the present study (see also Table A 8). This makes environmental metabarcoding highly valuable for fields of nature conservation, where rapid and comprehensive biodiversity assessments are necessary (Bista et al., 2017). However, the added value of this approach can only be utilised for nature conservation if the relevant data is collected via an accessible and corresponding infrastructure, which is currently lacking. The immense potential offered by novel technologies such as environmental metabarcoding, for deepening our understanding of ecosystem dynamics, also underscores the complexity of ecological restoration and the limitations that even the most advanced technologies face in fully addressing habitat degradation (Pascher et al., 2022).

The multifaceted nature of environmental stressors was highlighted in this thesis by the outcomes of the study of *Myriophyllum alterniflorum* habitat parameters (see Chapter 3). The degradation observed in certain running waters was not the result of single factors like nitrogen enrichment, but rather a combination of nutrient loading, sedimentation, and altered water flow regimes (Sattler and Poschlod, 2023). This reinforces the need for a holistic approach to conservation, one that considers multiple stressors and promotes integrated land and water management practices (Dudgeon, 2019).

In conclusion, while pristine aquatic habitats may be a thing of the past, we can still learn from historical practices and embrace modern technologies to develop more sustainable ways of interacting with these ecosystems. To protect species like the freshwater pearl mussel, it is necessary to strengthen the resilience of landscapes and revitalise their ability to harbour an interconnected biodiversity. This requires a broad approach including landscape structure and catchment land use, as well as a clear societal and political intent. Environmental metabarcoding revealed in this study how multifactorial a species existence is, being dependant on abiotic and biotic filtering. This may give impetus for novel and inclusive concepts in nature conservation that broaden the scope and aim to a self-sustenance of ecosystems and their residents.

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Appendix

Table A 1 resource of maps used for each country, time layer and square in the historical landscape analyses. BV refers to Bavarian squares, BH to those from Bohemia.

Year ID	**year**	**plot**	**Map series**	**Publisher**	**Digital Copyright holder**
A – BV	1836	2	Historical parcel maps (Uraufnahmeblätter) 1 : 5.000	Tax Surveying Commission	Bavarian Surveying Administration
	1841				
	1852	1,2,5,7			
B – BV	1911	1	Topographical Atlas of the Kingdom of Bavaria 1 : 50.000	Military Topographical Bureau	Bavarian Surveying Administration
	1912	4			
	1913	1			
	1919	5,7			
	1921	2			
C – BV	1945	1,5,7	Topographic map of Bavaria 1 : 25.000	Bavarian Surveying Office	Bavarian Surveying Administration
	1952	2			
	1948	4	Aerial photographs (black-white)		
D - BV	1970	1,4,5	Topographic map 1 : 25.000	Bavarian State Surveying Office Munich	Bavarian Surveying Administration
	1983	2,7			
	1987		Aerial photographs (black-white)		
E – BV	2019		Digital Orthophoto (20 cm)	State Office for Digitisation, Broadband and Surveying	Bavarian Surveying Administration
	2020	5			
	2020	5	Digital Topographic map 1 : 25.000		
	2021	1,7			
E – BV	1987-2016		Biotope mapping	Bavarian State Office for the Environment	
A – BH	1838	2,3,8	Stable Cadastre 1:2880	Central Archive of Cadastre of Lands in Vienna	Land Survey Office
	1841	4,6			
	1845		II. Military Survey	Generalquartiermeisterstab	CENIA

Continuation of Table A 1: resource of maps used for each country, time layer and square in the historical landscape analyses. BV refers to Bavarian squares, BH to those from Bohemia.

Year ID	year	plot	Map series	Publisher	Digital Copyright holder
B – BH	1921	2	Topographic Section 1:25 000 of the third military mapping	K. u. k. Militär-geographisches Institut	Land Survey Office
	1936	8			
	1925	2,3,4			
	1926	6			
C – BH	1947	3	Topographic Maps of the General Staff of the Czechoslovak Army 1 : 25 000	Ministry of Defence	CENIA and GEODIS BRNO
	1848	4			
	1950	2			
	1949	8	Aerial photo 1 : 5.000		Land Survey Office
	1952	2			
	1950	6			
D – BH	1976	2	Topographic Maps of the General Staff of the Czechoslovak Army 1 : 25 000	Ministry of Defence	Geographical Library, Faculty of Science, Charles University
	1987	4,6,8			
	1983	2	Aerial photo		Land Survey Office
	1987	6			
	1988	3			
E - BH	2017		Base Map of the Czech Republic 1 : 25 000 (ZM 25)	Land Survey Office	Land Survey Office
	2019	8	Orthophoto (20 cm)		
	2021	2,3			
E – BH	2007-2017		Natura 2000	Nature Conservation Agency of the Czech Republic	
E - BH	2020/2021	2,3,6	Field mapping	INTERREG Project 293 „Historische Landnutzung“	

Appendix

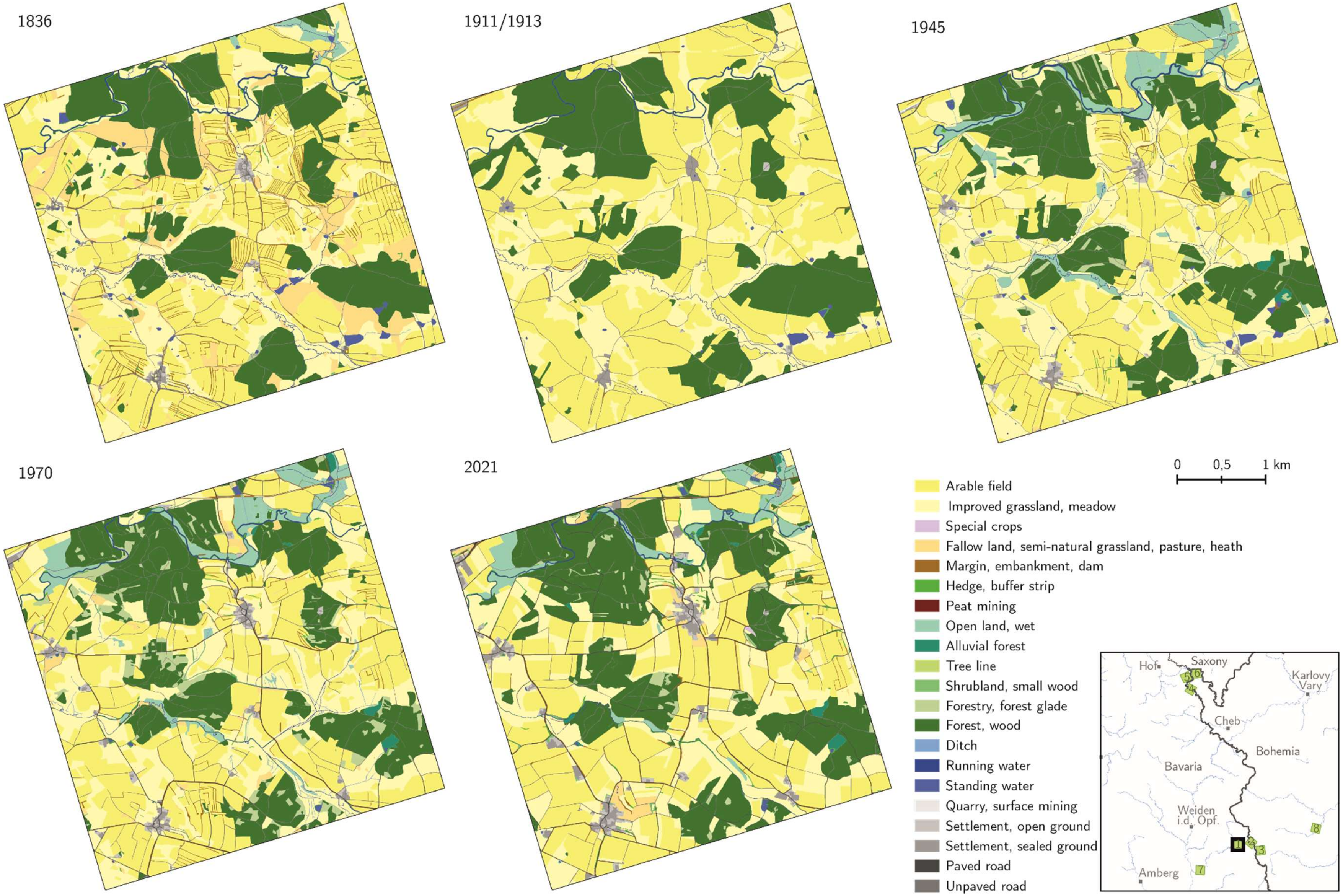

Figure A 1: Development of landuse from 1836 to 2021 in the Bavarian plot 1. References of all map resources are provided in Table A 1.

Appendix

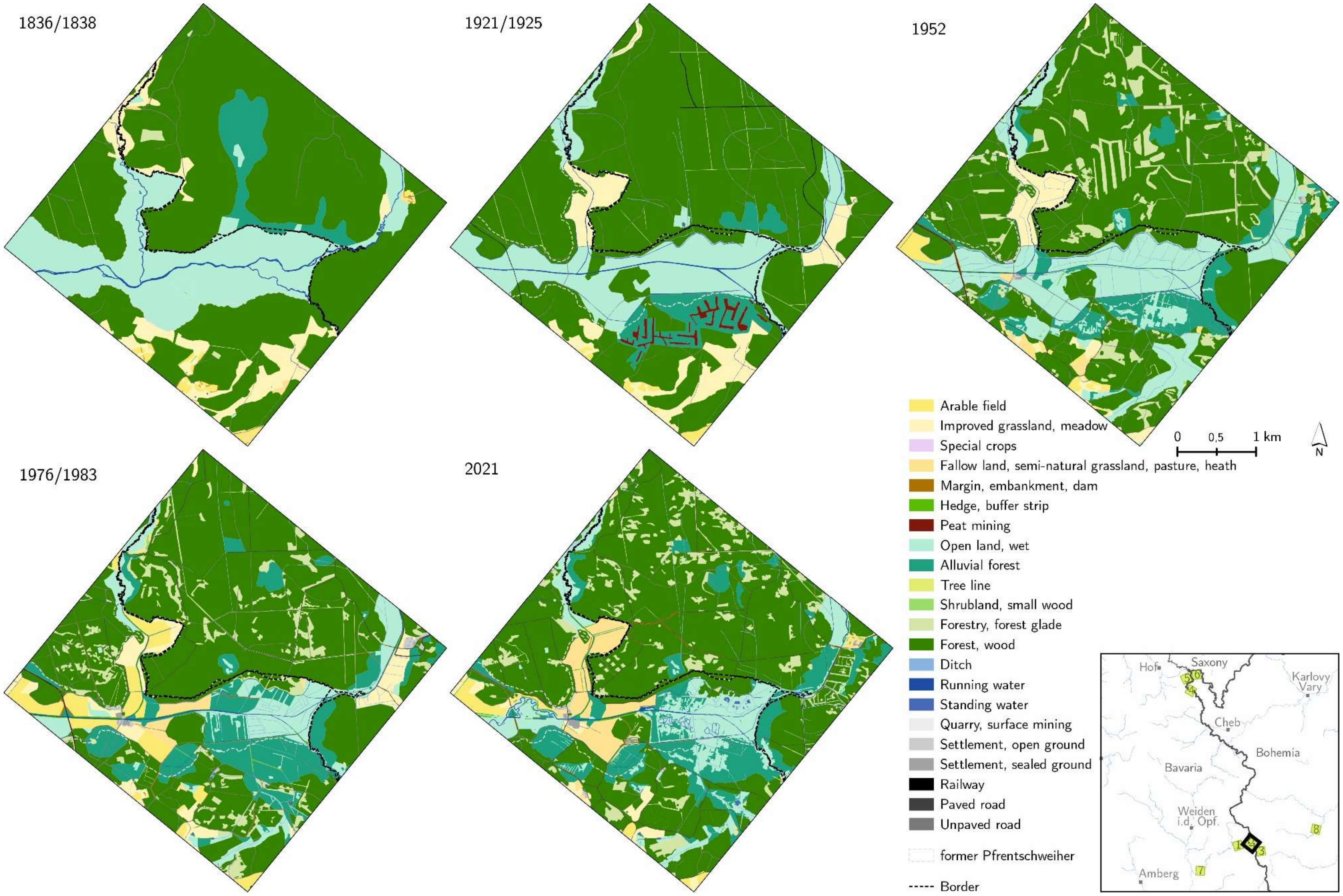

Figure A 2: Development of landuse from 1836 to 2021 in the cross-border plot 2. References of all map resources are provided in Table A 1.

Appendix

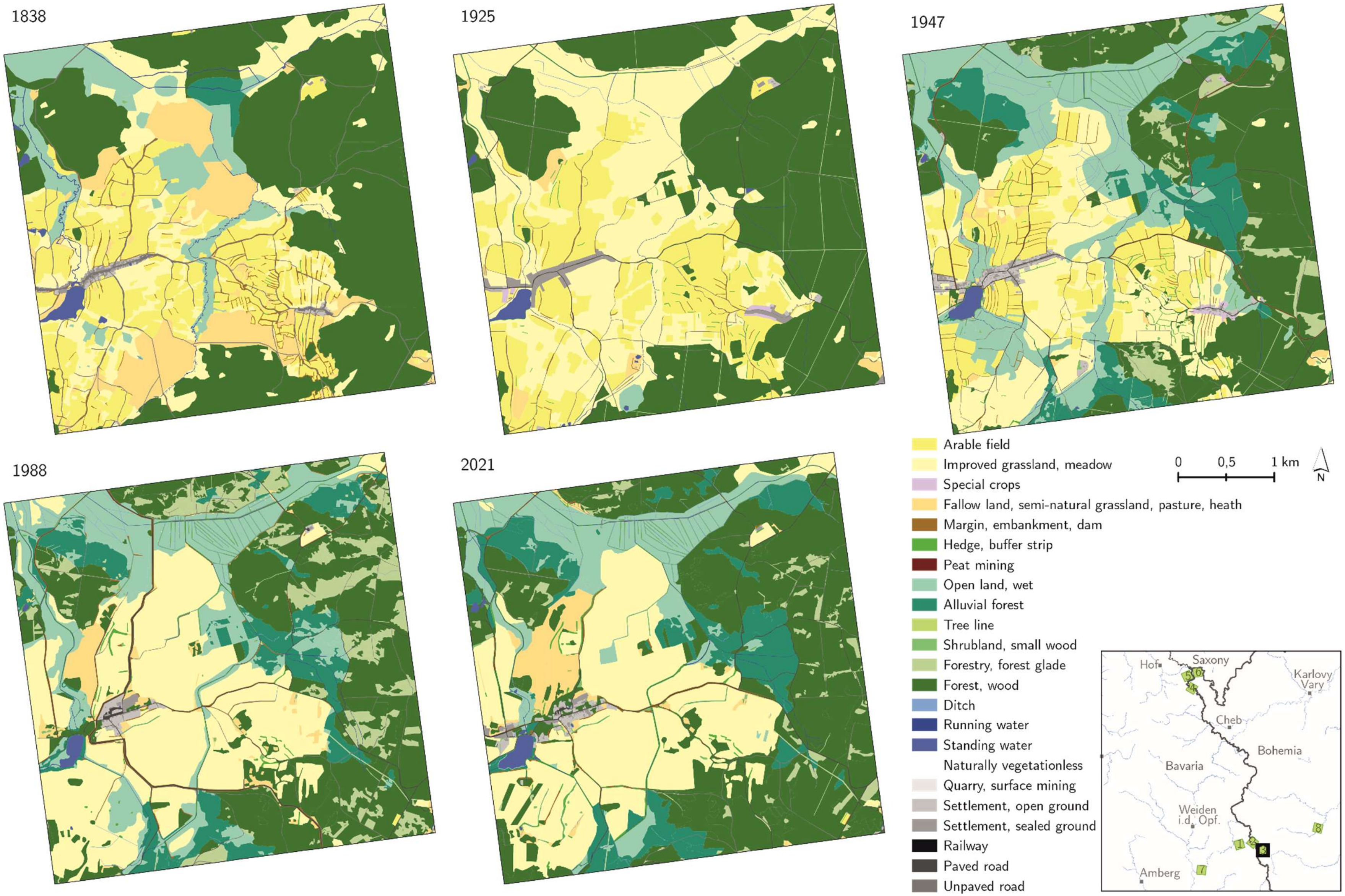

Figure A 3: Development of landuse from 1838 to 2021 in the Bohemian plot 3. References of all map resources are provided in Table A 1.

Appendix

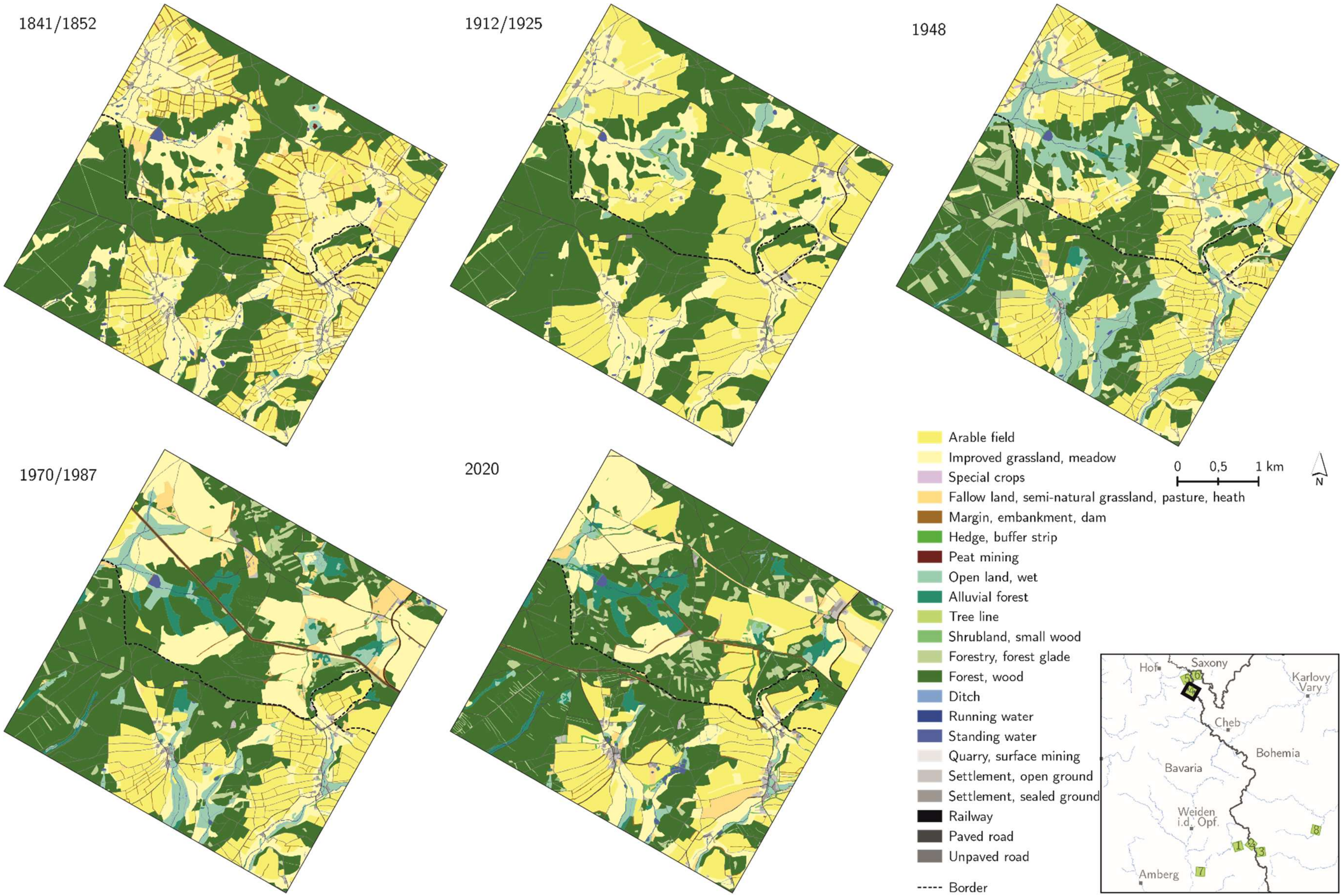

Figure A 4: Development of landuse from 1841 to 2020 in the cross-border plot 4. References of all map resources are provided in Table A 1.

Appendix

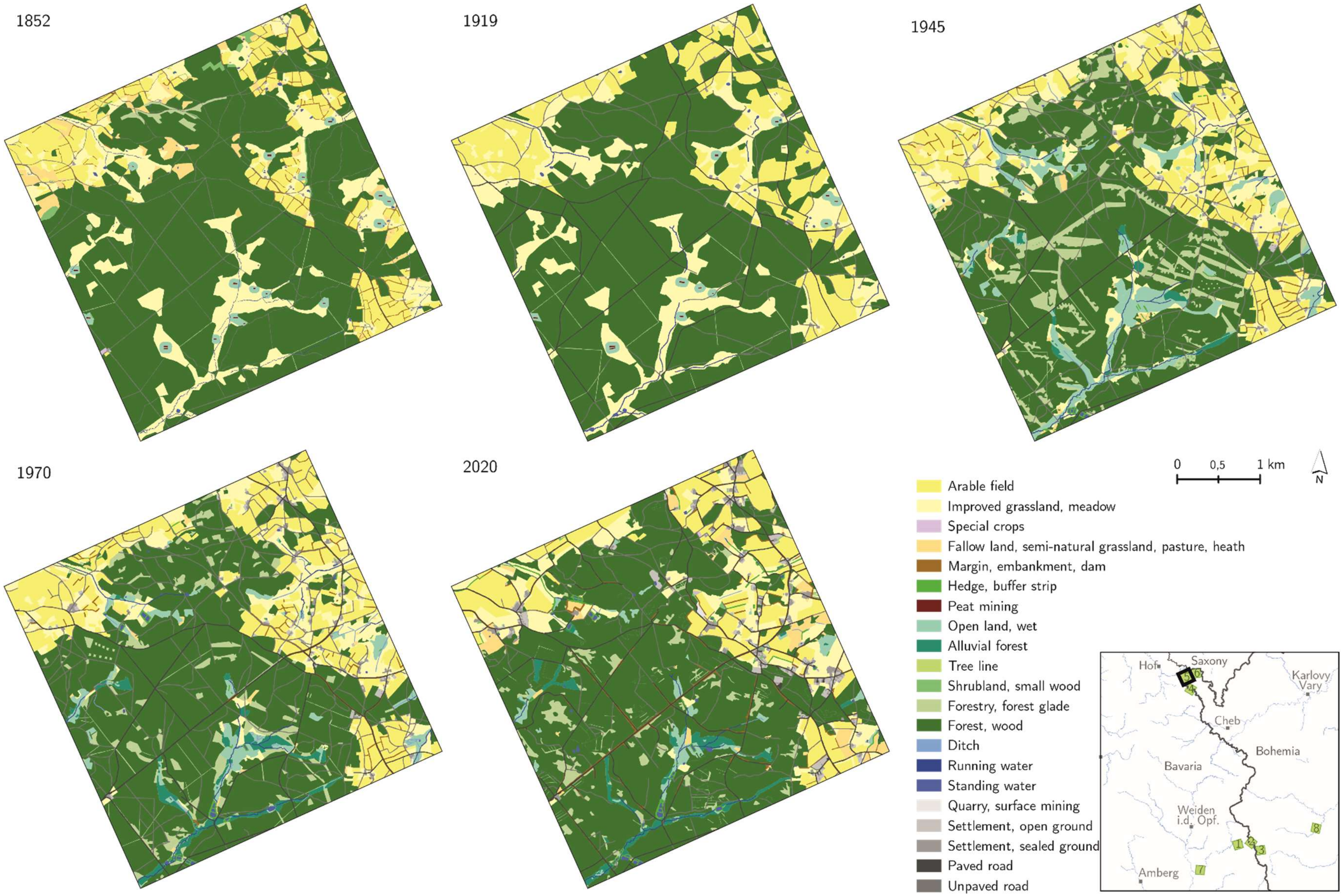

Figure A 5: Development of landuse from 1852 to 2020 in the Bavarian plot 5. References of all map resources are provided in Table A 1.

Appendix

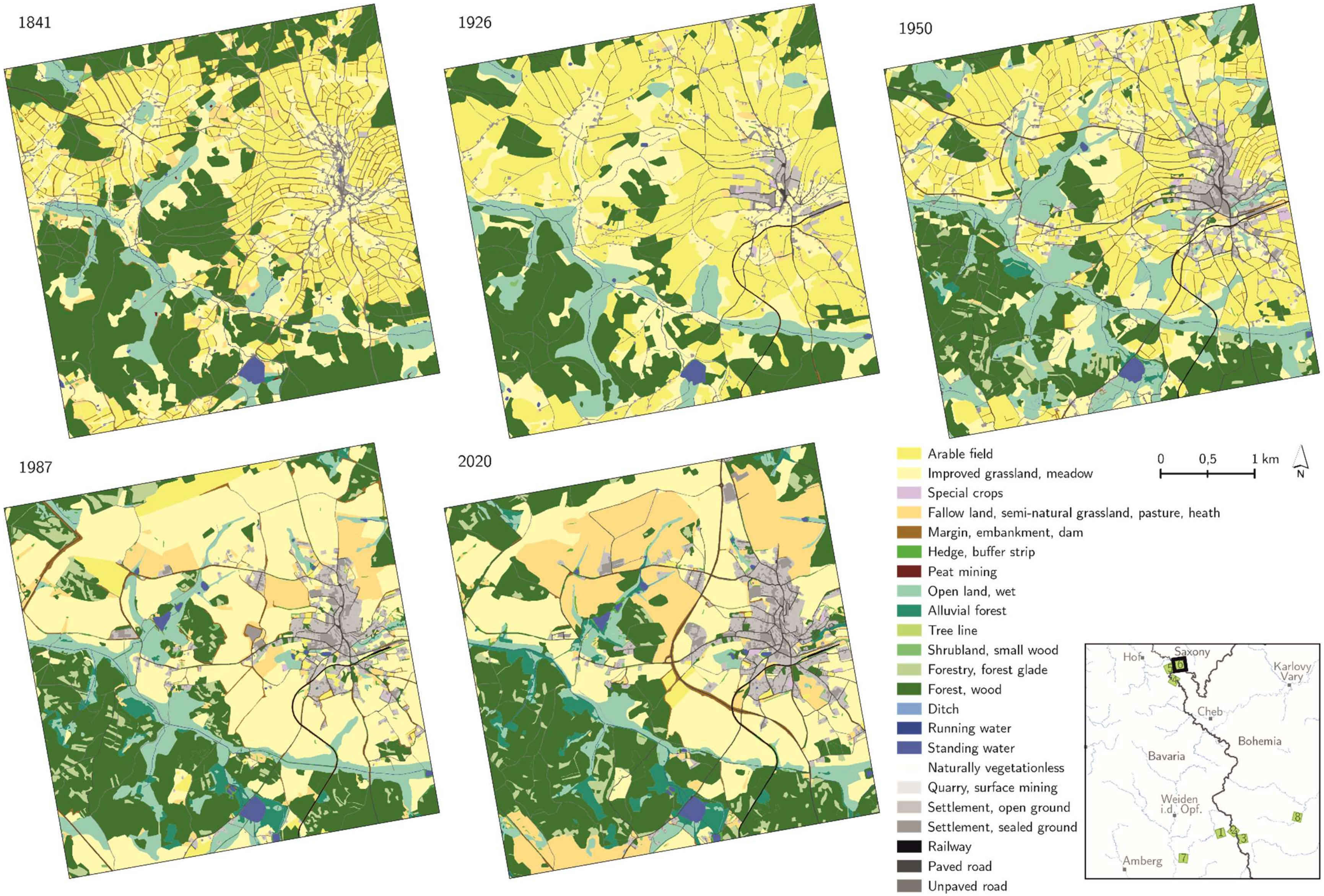

Figure A 6: Development of landuse from 1841 to 2020 in the Bohemian plot 6. References of all map resources are provided in Table A 1.

Appendix

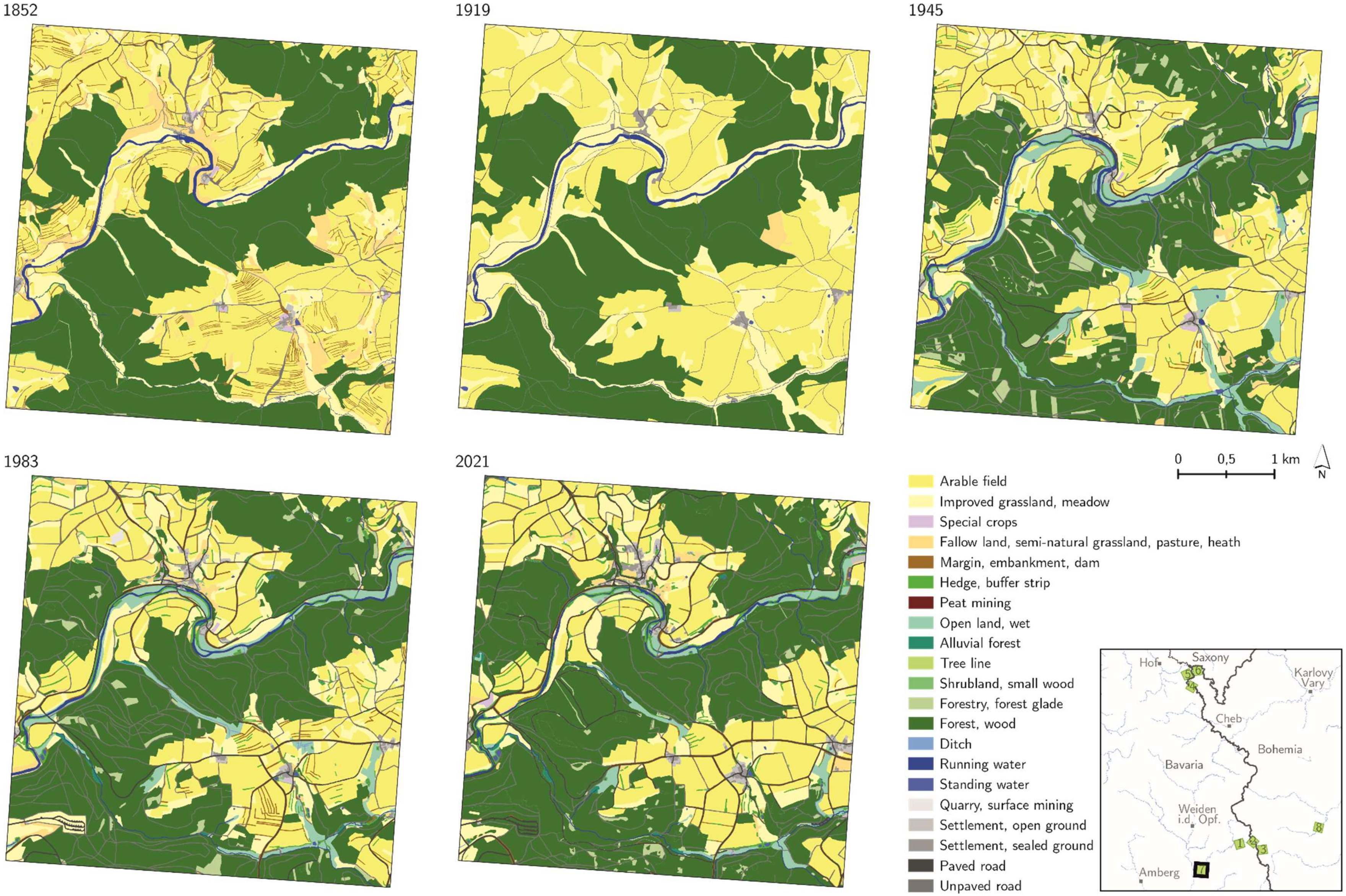

Figure A 7: Development of landuse from 1852 to 2021 in the Bavarian reference plot 8. References of all map resources are provided in Table A 1.

Appendix

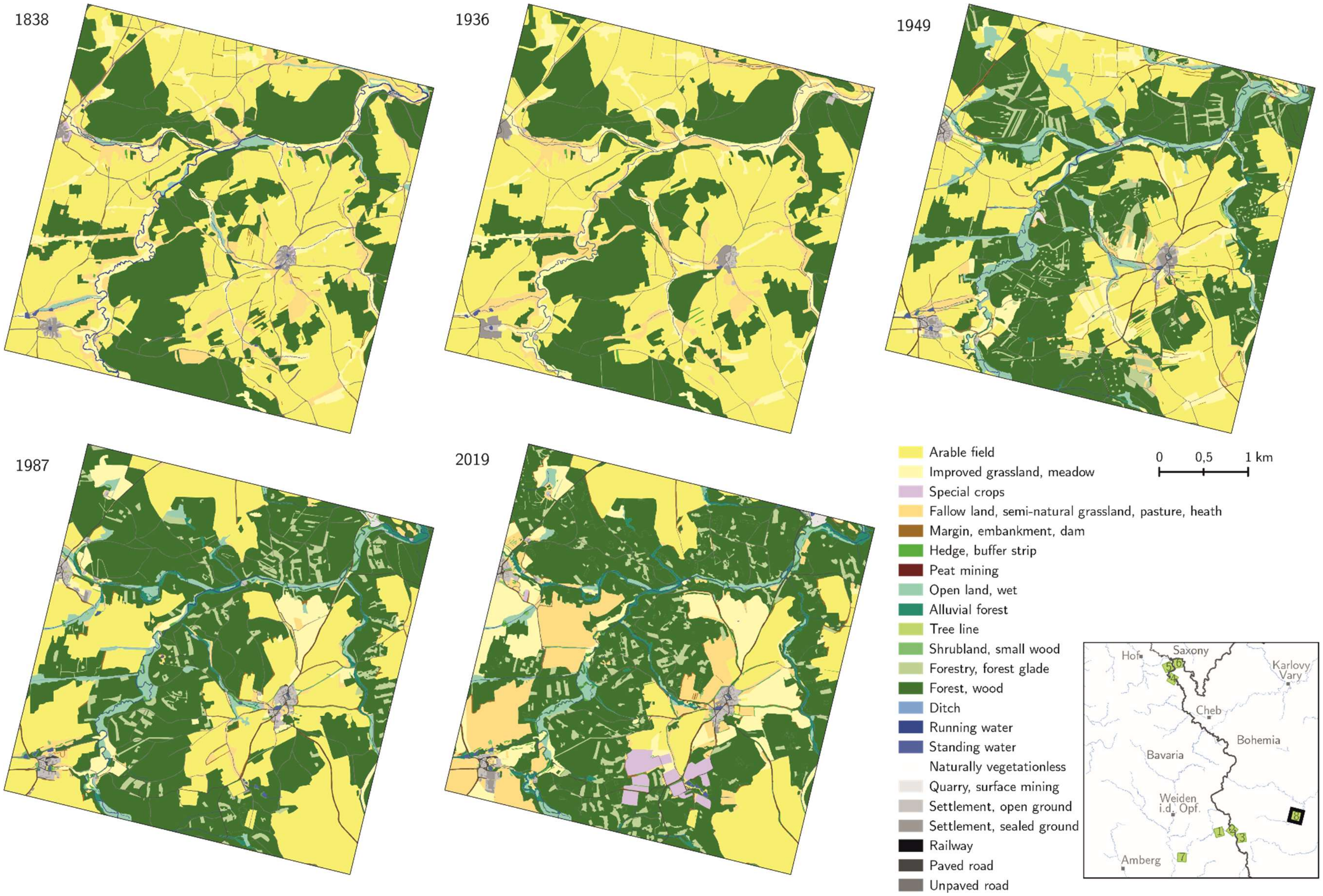

Figure A 8: Development of landuse from 1838 to 2019 in the Bohemian reference plot 8. References of all map resources are provided in Table A 1.

Table A 2: Differences in medians and p-values from post-hoc Dunn-test of changes in river sinuosity over the years A: ± 1850, C ± 1950 and E: ± 2020 as shown in Figure 7. BV = Bavaria, BH = Bohemia, r = reference, n = north, s = south.

	A vs. C			C vs. E			A vs. E		
plot	δ median	δ mean	p	δ median	δ mean	p	δ median	δ mean	p
1	7.75	-3.73	1.0	-2.83	0.27	1.00	4.70	-3.47	1.00
2	9.99	-3.80	0.21	-8.93	1.31	0.21	0.17	-2.54	1.00
3	-11.36	-15.93	0.14	-3.04	-1.16	0.58	-14.06	-16.91	0.02
4	-10.04	-10.37	0.00	3.02	2.03	0.84	-7.33	-8.55	0.02
5	-10.37	-9.06	0.11	-3.86	-5.11	0.07	-13.84	-13.71	0.00
6	-3.04	-7.07	0.05	-3.27	-0.72	0.87	-6.22	-7.74	0.01
BV r	-7.56	-7.01	0.55	1.82	-0.13	1.00	-5.88	-7.13	0.05
BH r	1.94	0.25	1.00	-5.59	-4.74	0.25	-3.75	-4.50	0.34
BH n	-4.17	-7.89	0.00	-1.23	1.38	1.00	-5.35	-6.62	0.02
BH s	-12.20	-17.05	0.01	-0.46	1.96	1.00	-12.60	-15.43	0.01
BV n	-10.65	-9.93	0.01	-3.88	-3.63	0.22	-14.11	-13.20	0.00
BV s	-0.35	-1.95	1.00	4.81	-0.07	1.00	4.45	-2.03	1.00

Table A 3: P-values of the alteration of arable field size over the years A: ± 1850, C ± 1950 and E: ± 2020 as shown in Figure 8.

	1	2	3	4	5	6	BV r	BH r	BV n	BV s	BH n	BH s
A vs. C	0.00	0.64	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
C vs. E	0.07	0.31	0.11	0.00	0.00	0.14	0.00	0.12	0.00	0.00	0.01	0.11
A vs. E	0.00	0.27	0.40	0.00	0.00	0.86	0.00	0.04	0.00	0.03	0.00	0.40

Table A 4: P-values of differences in *M. alterniflorum* coverage between the different classes of each environmental variable as derived from pairwise Wilcoxon test. A = sediment, B = depth, C = shading, D = flow velocity, E = turbidity.

	R^2	**p-value**	**NMDS1**	**NMDS2**
sediment	0.29	0.00	0.53	0.07
depth	0.26	0.00	0.50	-0.09
flow velocity	0.36	0.00	-0.59	-0.10
turbidity	0.44	0.00	0.66	-0.07
nitrate	0.32	0.00	-0.52	0.21
Callitriche cophocarpa	0.28	0.00	-0.04	-0.52
Callitriche hamulata	0.36	0.00	-0.16	-0.58
Lemna minor	0.34	0.00	0.58	-0.05
Myriophyllum alterniflorum	0.39	0.00	-0.55	0.31
Ranunculus spp.	0.22	0.00	-0.42	-0.21

Table A 5: Scores from NMDS of the sampled sections in the Pfreimd in 2012 as shown in Figure 2. Determination coefficient R^2 and p-value for the correlation of environmental vectors and coordinates along the NMDS1 and NMDS2 axis.

A	1	2	3	4	5	**D**	1	2	3	4
2	1.00	-	-	-	-	2	1.00	-	-	-
3	0.87	1.00	-	-	-	3	0.06	0.00	-	-
4	0.16	0.25	1.00	-	-	4	0.23	0.56	1.00	-
5	0.01	0.01	1.00	1.00	-					
6	0.16	0.25	1.00	1.00	-					
B						**E**				
2	1.00	-	-	-	-	2	0.03	-	-	-
3	0.04	0.19	-	-	-	3	0.00	1.00	-	-
4	0.02	0.07	1.00	-	-	4	0.00	0.53	1.00	-
5	1.00	1.00	1.00	1.00	-					
6	0.01	0.04	1.00	1.00	-					
C										
2	1.00	-	-	-						
3	0.49	1.00	-	-						
4	1.00	1.00	1.00	-						
5	0.78	0.19	0.03	0.82						

Table A 6: Scores from NMDS of the sampled sections in all five brooks as shown in Figure 4. Determination coefficient R^2 and p-value for the correlation of environmental vectors and coordinates along the NMDS1 and NMDS2 axis.

	R^2	p-value	NMDS1	NMDS2
conductivity	0.21	0.00	0.44	0.12
ammonium	0.27	0.00	-0.22	0.48
calcium	0.24	0.00	0.48	-0.05
nitrate	0.75	0.00	0.80	-0.34
phosphate	0.06	0.07	0.19	-0.14
pH	0.18	0.00	0.39	0.15
depth	0.73	0.00	-0.41	-0.75
turbidity	0.48	0.00	0.43	0.54
flow velocity	0.77	0.00	-0.73	-0.48
shading	0.18	0.00	-0.20	0.37
sediment	0.58	0.00	-0.40	-0.65

Table A 7: Information of calcium analysis with atomic absorption spectrometer M Serie 602147 v1,20

machine setting	setting description
general parameters	technic: flame, sample changer: CETAC, rack code: C60, dilution: none, segmented flow injection: none
spectrometer parameters	element: Ca, operating mode: extinction, wavelength: 422.7 nm, bandwidth: 0.5 nm, lamp current: 100 %, underground correction: off, high resolution: off, spectr. Parameter optimization: none, signal type: continuous, repetitions: fast, number of repetitions: 3, RDS test: no
flame parameters	flame type: N_2O-C_2H_2, fuel gas volume: 4.2 L/min, auxiliary gas: off, atomisers: 4 s, burner stabilisation time: 0 min, optimise fuel gas quantity: no, burner height: 7.4 mm, optimise burner height: no
sample changer parameters	sample changer: CETAC, automatic retrieval: no, sample preparation: no, standard preparation: manually, standard additions: none, sinks: off, flushing time; 4 s
calibration parameters	calibration mode: normal, customisation: curve segment, use saved calibration; no, unit: mg/L, scaled unit: mg/L, scaling factor: 1, quality limit: -10 % to 40 %, rescaling deviation: 10 %, action on error: mark and continue measuring

Table A 8: Species found in DNA metabarcoding with records in the Red List of endangered species for Bavaria (**BV**, **0**: extinct or vanished, **0***: extinct or vanished but found after 1979, **1**: threatened by extinction, **2**: strongly endangered, **3**: endangered, **R**: extremely rare with geographic restriction, **R*** extremely rare but actually not threatened, **V**: preliminary warning list, **G**: threat assumed, but status unknown, Scheuerer and Ahlmer (2003)) and for the Czech Republic (**CZ**, **A1**: extinct or vanished, **A3**: uncertain cases of extinct, vanished or missing taxa, **C1** critically threatened, **C2**: endangered, **C3**: vulnerable, **C4a** lower risk – near threatened, **C4b**: lower risk – data deficient, Grulich (2012)).

taxon	**BV**	**CZ**	**taxon**	**BV**	**CZ**
Ceratocephala falcata	0	-	*Quercus pubescens*	-	C3
Deschampsia setacea	0*	-	*Ranunculus aconitifolius*	-	C3
Plantago altissima	-	A1	*Taraxacum boekmanii*	-	C3
Urtica kioviensis	-	C1 b	*Taraxacum maricum*	-	C3
Veronica scardica	-	C1 b	*Cirsium heterophyllum*	3	-
Populus nigra	-	C1 t	*Poa chaixii*	3	-
Tephroseris longifolia	-	C1 t	*Populus alba*	3	-
Ventenata dubia	1	C1 t	*Ranunculus aquatilis*	3	-
Tephroseris integrifolia	1	C2 b	*Ranunculus circinatus*	3	-
Lysimachia punctata	1	-	*Ranunculus fluitans*	3	-
Ranunculus parnassiifolius	1	-	*Ranunculus peltatus*	3	-
Ranunculus polyanthemos	1	-	*Ribes rubrum*	3	-
Ajuga pyramidalis	-	C2	*Rubus fasciculatus*	3	-
Alnus alnobetula	-	C2	*Tephroseris helenitis*	3	-
Cerastium fontanum	-	C2	*Epilobium palustre*	-	C4a
Betula nana	2	C1	*Nuphar lutea*	-	C4a
Myriophyllum alterniflorum	2	C2	*Salix pentandra*	-	C4a
Potamogeton polygonifolius	2	C2	*Fraxinus angustifolia*	-	C4a
Prunus cerasifera	2	-	*Helictochloa pratensis*	-	C4a
Montia fontana	3	C1	*Epilobium obscurum*	G	C3
Ranunculus sardous	3	C2	*Callitriche cophocarpa*	G	-
Potamogeton alpinus	3	C2	*Callitriche hamulata*	G	-
Rubus vestitus	3	C2	*Myosotis laxa*	G	-
Achillea pannonica	3	C3	*Alchemilla glomerulans*	R	-
Lysimachia thyrsiflora	3	C3	*Rubus hypomalacus*	R*	A3
Phleum alpinum	3	C3	*Calamagrostis canescens*	V	-
Rosa gallica	3	C3	*Circaea alpina*	V	-
Chrysosplenium oppositifolium	3	C4a	*Festuca heterophylla*	V	-
Comarum palustre	3	C4a	*Petasites albus*	V	-
Limosella aquatica	3	C4a	*Rosa dumalis*	V	-
Utricularia australis	3	C4a	*Rumex thyrsiflorus*	V	-
Ribes nigrum	3	C4b	*Sagittaria sagittifolia*	V	-
Achillea setacea	-	C3			

German Federal State	**Full Source**
Baden - Wuerttemberg (BW)	LUBW (2021): Artenschutzprogramm Baden-Württemberg, Räumliches Informations- und Planungssystem (RIPS). (BW) Nagel, K.O., Mildner, M., Günter, C.P., Pfeiffer, M (2019): Die Flussperlmuschel (*Margaritifera margaritifera*) in Baden-Württemberg.
Bavaria (BV)	Fachinformationssystem Naturschutz (FIS-Natur). LfU, ¨OKON GmbH (1995): Muscheln. LfU, TUM-Muschelkoordinationsstelle (2011): Merkblatt Artenschutz - Flussperlmuschel (*Margaritifera margaritifera*)
Hesse (HE)	LNUG - Hessisches Naturschutzinformationssystem (2022): www.natureg.hessen.de. Hessen-Forst FENA, Nagel, K.O. (2003): Artensteckbrief - Flussperlmuschel (*Margaritifera margaritifera*).
Lower Saxony (NI)	Niedersaechsischer Landesbetrieb für Wasserwirtschaft, Küsten- und Naturschutz. NLWK (2011): Vollzugshinweise zum Schutz von Wirbellosenarten in Niedersachsen – Flussperlmuschel (*Margaritifera margaritifera*).
North Rhine - Westphalia (NW)	LANUV, Land NRW (2021): Datenlizenz Deutschland –Version 2.0: www.govdata.de/dl-de/by-2–0. Natur- und Umweltschutz-Akademie des Landes Nordrhein-Westfalen (2006): Schutz und Erhalt der Flussperlmuschel in Nordrhein-Westfalen. NUA-Heft 20. Feldhaus, G., Selheim, H., Steinberg, L. (2017): Schutz und Erhalt der Flussperlmuschel in NRW. Natur in NRW 2/2017.
Rhineland - Palatinate (RP)	LfU (2014): Darstellung auf der Grundlage von Daten des LfU Rheinland-Pfalz. (RP) Fischer, K. 1908: Die Flußperlmuschel (Margaritana margaritifera) in den B¨achen des Hochwaldes. In: Verhandlungen des Naturhistorischen Vereins der preußischen Rheinlande und Westfalens. 64. Jg, 1907. Bonn. LfU (2006): Flussperlmuschel (Margaritifera margaritifera) - Verbreitung in Rheinland-Pfalz.
Saarland (SL)	LUA - Ministerium für Umwelt und Verbraucherschutz/Zentrum für Biodokumentation (MUV/ZfB).
Saxony (SN)	LfU Zentrale Artdatenbank Sachsen im LfULG, interdisziplinaere Daten und Auswerungen (iDA): www.umwelt.sachsen.de.

Table A 9: References of the *M. margaritifera* distribution records shown in Figure 23.

Table A 10: Coefficients of linear regression analyzes of Ellenberg indicator values (EIV) for nitrate (N) and moisture (F), compared between type of site (ctr = control, trt = treatment) and year (2021, 2022) of vegetation mapping with 18 degrees of freedom for each model.

	Estimate	**Std. error**	**t-value**	**p value**
$\mathrm{EivN_{trt}}$~year	0.19	0.28	0.70	0.49
intercept	2.16	0.20	11.10	1.76 e^{-9}
model	Resid. Std. error: 0.61, adjusted R^2: -0.03, F-statistic: 0.50, p-value: 0.49			
$\mathrm{EivN_{ctl}}$~year	-0.11	0.32	-0.33	0.74
intercept	2.43	0.23	10.61	3.5 e^{-9}
model	Resid. Std. error: 0.62, adjusted R^2: -0.03, F-statistic: 0.50, p-value: 0.49			
$\mathrm{EivN_{2021}}$~type	-0.27	0.25	-1.11	0.28
intercept	2.43	0.17	14.06	3.78 e^{-11}
model	Resid. Std. error: 0.55, adjusted R^2: 0.01, F-statistic: 1.23, p-value: 0.28			
$\mathrm{EivN_{2022}}$~type	0.03	0.35	0.09	0.93
intercept	2.33	0.25	9.44	2.14 e^{-8}
model	Resid. Std. error: 0.78, adjusted R^2: -0.05, F-statistic: 0.01, p-value: 0.93			
$\mathrm{EivF_{trt}}$~year	0.91	0.29	3.10	0.00615
intercept	2.89	0.21	13.89	4.75 e^{-11}
model	Resid. Std. error: 0.66, adjusted R^2: 0.31, F-statistic: 9.62, p-value: 0.01			
$\mathrm{EivF_{ctl}}$~year	0.43	0.32	1.36	0.19
intercept	3.02	0.22	13.55	6.97 e^{-11}
model	Resid. Std. error: 0.71, adj. R^2: 0.04, F-statistic: 1.84, p-value: 0.19			
$\mathrm{EivF_{2021}}$~type	-0.14	0.27	-0.50	0.62
intercept	3.02	0.19	15.64	6.39 e^{-12}
model	Resid. Std. error: 0.61, adjusted R^2: -0.04, F-statistic: 0.25, p-value: 0.62			
$\mathrm{EivF_{2022}}$~type	0.35	0.33	1.04	0,31
intercept	3.45	0.24	14.62	1.98 e^{-11}
model	Resid. Std. error: 0.75, adjusted R^2: 0.01, F-statistic: 1.09, p-value: 0.31			

Table A 11: P values of soil chemical parameters active pH ($\mathrm{pH}_{\mathrm{act}}$), potential pH ($\mathrm{pH}_{\mathrm{pot}}$), conductivity (cond.), phosphorus (P), potassium (K) and C-N ration (C/N) at control (C) and treatment (T) site before (0) after first (1) and after second (2) irrigation, obtained with pairwise Wilcoxon test with Bonferroni continuity. Means and standard deviations (SD) of each site.

value		**C0**	**C1**	**C2**	**T1**	**T2**	**mean**	**SD**
$\mathrm{pH}_{\mathrm{act}}$	C0	--	--	--	--	--	6.31	0.27
	C1	1.00	--	--	--	--	6.16	0.47
	C2	0.02	0.14	--	--	--	5.38	0.70
	T0	0.00	0.00	0.00	--	--	6.70	0.05
	T1	0.00	0.00	0.00	1.00	--	6.67	0.07
	T2	1.00	1.00	0.00	0.00	0.00	6.19	0.12
$\mathrm{pH}_{\mathrm{pot}}$	C0	--	--	--	--	--	5.66	0.54
	C1	1.00	--	--	--	--	5.50	0.57
	C2	1.00	1.00	--	--	--	5.38	0.70
	T0	0.01	0.00	0.00	--	--	6.28	0.08
	T1	0.02	0.00	0.00	0.06	--	6.16	0.08
	T2	0.03	0.00	0.00	0.08	1.00	6.15	0.07
cond.	C0	--	--	--	--	--	46.93	6.87
	C1	1.00	--	--	--	--	42.69	5.47
	C2	0.39	1.00	--	--	--	38.91	8.28
	T0	0.68	0.09	0.37	--	--	54.87	10.03
	T1	1.00	1.00	0.96	0.68	--	47.30	6.96
	T2	1.00	1.00	0.68	0.35	1.00	46.15	5.30
P	C0	--	--	--	--	--	9.91	2.67
	C1	1.00	--	--	--	--	8.72	3.07
	C2	1.00	1.00	--	--	--	8.79	3.59
	T0	1.00	1.00	1.00	--	--	8.07	2.99
	T1	0.23	1.00	1.00	1.00	--	6.53	3.04
	T2	0.68	1.00	1.00	1.00	1.00	6.83	4.41
K	C0	--	--	--	--	--	0.22	0.05
	C1	1.00	--	--	--	--	0.24	0.08
	C2	1.00	1.00	--	--	--	0.27	0.07
	T0	1.00	1.00	1.00	--	--	0.23	0.04
	T1	1.00	1.00	0.81	1.00	--	0.21	0.04
	T2	1.00	1.00	1.00	1.00	1.00	0.22	0.03
C/N	C0	--	--	--	--	--	11.38	0.55
	C1	1.00	--	--	--	--	11.35	0.27
	C2	1.00	1.00	--	--	--	11.27	0.53
	T0	1.00	1.00	1.00	--	--	11.63	0.41
	T1	1.00	1.00	1.00	0.81	--	11.06	0.55
	T2	1.00	1.00	1.00	1.00	1.00	11.46	0.48

Table A 12: Means and standard deviations of chemical parameters measured after irrigations in 2022 in free water and interstitial at 6 sampling sites: brook upstream (us), brook middle (m), brook downstream (ds), ditch us (control 1), ditch m (control 2) and ditch ds (treatment). Oxygen (O_2) in mg l^{-1}, temperature (T) in °C, conductivity (con) in µS cm^{-1}, pH, redox potential (Eh) in mV.

parameter n = 6		**ditch_us (treatment)**	**ditch_m (control)**	**ditch_ds (control)**	**brook_us**	**brook _M**	**brook _ds**
O_2 FW	mean	7.44	7.74	7.41	9.09	9.09	9.11
	SD	0.53	0.74	0.60	0.43	0.43	0.41
O_2 INT	mean	2.48	3.08	6.07	7.73	7.90	7.57
	SD	1.35	1.45	0.90	0.85	1.29	1.86
Δ O_2	mean	4.96	4.66	1.35	1.36	1.19	1.54
	SD	1.46	1.54	0.59	0.78	0.99	1.96
T FW	mean	19.45	19.35	17.10	16.85	16.35	16.25
	SD	3.01	3.12	2.85	0.38	1.15	1.37
T INT	mean	23.87	21.28	20.00	17.55	17.58	17.57
	SD	2.40	2.20	2.41	1.12	1.52	1.51
Δ T	mean	-4.42	-1.93	-2.90	-0.70	-1.23	-1.32
	SD	2.32	1.21	3.81	0.74	0.39	0.25
con FW	mean	137.50	136.00	133.00	108.00	108.00	107.50
	SD	14.79	15.34	19.72	1.10	1.10	1.64
con INT	mean	155.33	145.67	136.83	109.33	108.67	111.00
	SD	46.80	19.50	21.90	1.75	2.34	3.35
Δ con	mean	-17.83	-9.67	-3.83	-1.33	-0.67	-3.50
	SD	38.61	8.82	7.41	1.03	2.07	2.35
pH FW	mean	7.10	6.80	6.65	7.25	7.35	7.30
	SD	0.11	0.11	0.60	0.05	0.16	0.11
pH INT	mean	6.57	6.68	6.27	6.95	6.88	7.02
	SD	0.05	0.08	0.15	0.08	0.10	0.16
Δ pH	mean	0.53	0.12	0.38	0.30	0.47	0.28
	SD	0.15	0.08	0.46	0.09	0.23	0.13
Eh FW	mean	533.50	558.50	555.50	549.50	554.50	545.00
	SD	18.07	1.64	3.83	8.22	7.12	2.19
Eh INT	mean	419.17	383.67	539.00	502.00	432.00	509.83
	SD	77.48	49.38	13.31	38.84	57.77	25.01
Δ Eh	mean	114.33	174.83	16.50	47.50	122.50	35.17

Table A 13: P values of growth rates of in-situ and ex-situ bioindication experiments obtained with pairwise Wilcoxon test with Bonferroni continuity and means and standard deviations (SD) of each period at brook (b), control (ctl) and treatment (trt) site.

period	**site**	**b**	**ctl**		**brook**	**control**	**treatment**
in-situ, 2021 June	ctl	0.887		mean	1.10	1.10	2.37
	trt	0.090	0.087	SD	2.39	2.39	2.89
in-situ, 2021 August	ctl	1.000		mean	1.08	1.08	1.25
	trt	1.000	1.000	SD	1.78	1.78	1.55
in-situ, 2022 June	ctl	1.000		mean	0.65	0.65	0.77
	trt	0.160	0.350	SD	1.13	1.13	1.44
ex-situ, 2021 June	ctl	0.148		mean	17.14	24.18	25.21
	trt	0.077	1.000	SD	9.07	4.56	6.84
ex-situ, 2021 August	ctl	1.000		mean	9.83	10.48	11.17
	trt	1.000	1.000	SD	6.91	7.35	7.05
ex-situ, 2022 June	ctl	1.000		mean	5.24	6.48	6.09
	trt	1.000	1.000	SD	7.41	11.67	6.25
ex-situ, 2022 August	ctl	1.000		mean	10.65	11.95	12.56
	trt	1.000	1.000	SD	11.33	11.87	13.22

Table A 14: Coefficients of survival rate from in-situ and ex-situ experiment obtained with logistic regression and compared between the sample sites. Null-dev. = null-deviance, DOF = degrees of freedom, resid.-dev. = residual deviance, AIC = Akaike information criterion.

In-situ	**estimate**	**std. error**	**z-value**	**p-value**
brook vs. control	0.06714	0.6648	0.183	0.855
brook vs. treatment	0.20294	0.36815	0.551	0.581
control vs. treatment	0.1358	0.3687	0.368	0.713
	Null-dev.: 247.31 (DOF: 179), resid.-dev.: 246.99 (DOF: 177), AIC: 252.99			
ex-situ				
brook vs. control	-0.2877	0.5381	-0.535	0.59292
brook vs. treatment	0.3483	0.5936	0.587	0.557340
control vs. treatment	0.6360	0.5739	1.108	0.26782
	Null-dev.: 120.10 (DOF: 119), resid.-dev.: 118.84 (DOF: 117), AIC: 224.84			

Table A 15: P-values obtained with pairwise Wilcoxon test and Bonferroni adjustment for chemical components calcium (Ca), phosphorus (P), magnesium (Mg), natrium (Na), potassium (K) and loss on ignition (L.O.I.) of detritus collected at three sample sites during irrigation periods. Means and standard deviations are given for each site.

variable	**site**	**brook**	**control**		**brook**	**control**	**treatment**
L.O.I	control	1.00		mean	12.31	11.02	14.70
	treatment	0.63	0.28	SD	2.68	2.70	2.59
Ca	control	0.89		mean	42.40	37.03	56.43
	treatment	1.00	0.63	SD	8.11	9.78	14.78
P	control	1.00		mean	16.03	16.09	20.84
	treatment	1.00	1.00	SD	13.14	14.15	18.44
Mg	control	1.00		mean	6.41	7.12	8.06
	treatment	1.00	1.00	SD	2.28	3.19	3.62
Na	control	1.00		mean	17.14	16.04	18.13
	treatment	1.00	1.00	SD	7.80	8.68	8.14
K	control	0.89		mean	5.77	7.77	6.55
	treatment	1.00	1.00	SD	3.07	1.41	1.80

Table A 16: Means, minima (min), maxima (max), standard deviations (SD) and p-values of penetration resistance measured in all three sites after each irrigation period on 20 spots within 1 m ^{2}. N(brook) = 120, n(control) = 80, n(treatment) = 40.

site	**mean**	**min**	**max**	**SD**	**p-value**
brook	1.17	0.40	1.43	0.27	0.00
control	0.04	0.00	0.24	0.06	0.00
treatment	0.13	0.00	0.24	0.08	0.00

Quantitative analysis of metabarcoding results

The four selected primers detected mostly uniquely the species groups they were targeted to. As shown in Figure A 9, the plant primer ITS2 detected only 15 % of all detected species, 14 % were only detected by ITS2-primers and 1 % was commonly detected by 18S-primers and ITS2-primers. With algae 18S-primers, 29 % of all species were detected. Small overlaps occurred with all other primers; 21 species (1 %) were in common with ITS2 and MZB-I respectively and one species was detected with 18S and MZB-II primer. MZB-I primers detected 23 % of the whole species set, 15 % were unique, 1 % in common with 18S and 7 % in common with MZB-II primers. The latter detected the greatest share of all species with 42 %, 35 % were uniquely detected with MZB-II, 7 % were commonly detected with MZB-I and -II and one species was also found with 18S-primers. There were no overlaps between more than two primer pairs.

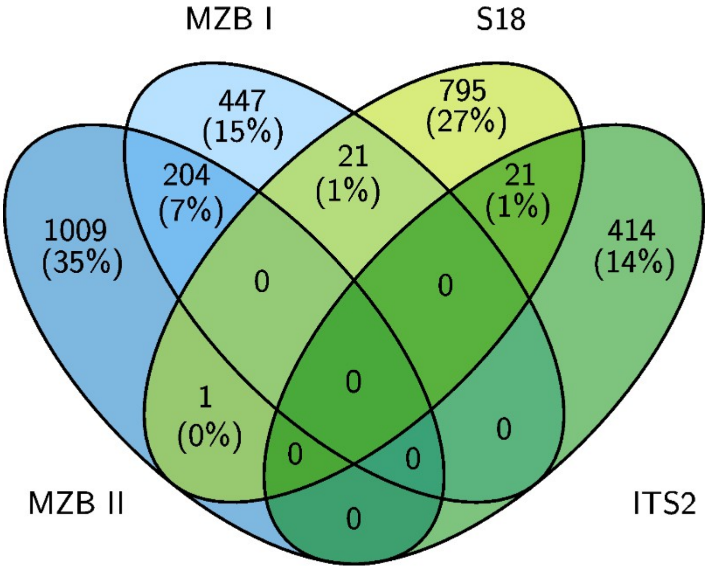

Figure A 9: Venn diagram on species numbers detected with each of the four selected primers: macrozoobenthos primers MZB I and MZB II, algae primer 18S and plant primer ITS2, and overlaps in identified taxa between different primers.

Regarding the types of sampling sites (Figure A 10b), 22 % of all species occurred commonly in all types of sites. 20 % occurred in active and extinct sites, 4 % in active and detritus sites and 5 % in extinct and detritus sites. The highest number of species was detected in extinct sites (1925), where 22 % of all species occurred exclusively. In active sites, a total of 1777 species was detected and 18 % of all detected species were only found here. In detritus sites the lowest number of species was detected (1137) and 10 % of all species were exclusively found in samples from these sites. Differences and overlaps in species sets identified in different sampling periods is shown in Figure A 10a. Here, a probe of 10 sites from types active, detritus and extinct was repeated also in August 2022 to check the impact of the time period on metabarcoding results. 11 % of all detected taxa at the selected sites were found in samples from every of the three sampling periods. In 2022 there was a total of 421 taxa and of these, 67 % were already detected in 2021, 41 % in both sampling months and 12 % overlap with June or August exclusively. The overlap between the different months of the same year was 23 % of all taxa and 25 % of all taxa from 2021. Herein, more taxa were detected in June (1053 or 74 % of 2021 taxa) than in August (916 or 64 % of 2021 taxa).

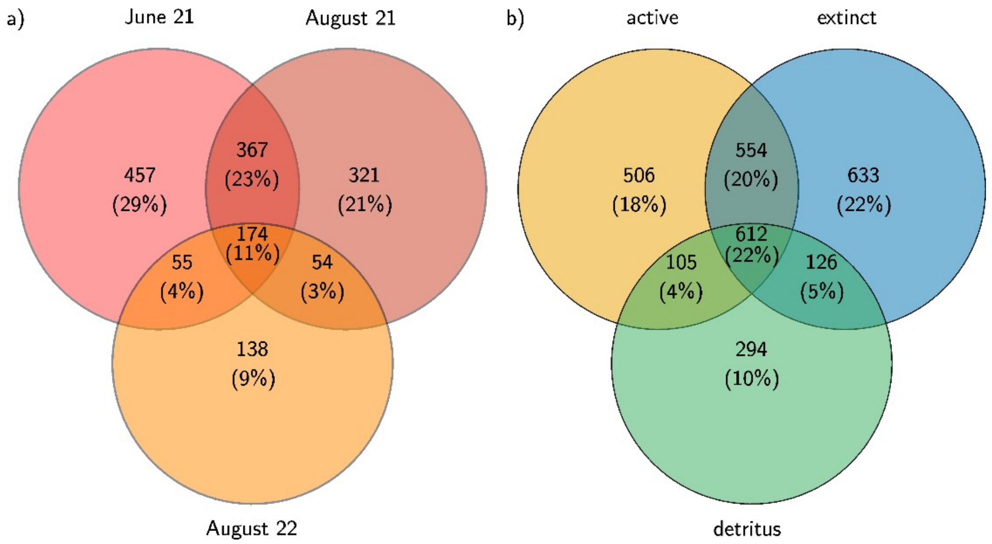

Figure A 10: Venn diagram of species numbers detected in different sampling times (June and August) and years (2021 and 2022, a), which was conducted in a probe of 10 sites for each time and different types of sample sites (active, extinct and detritus, b) which represent all sites sampled in June and August of 2021.

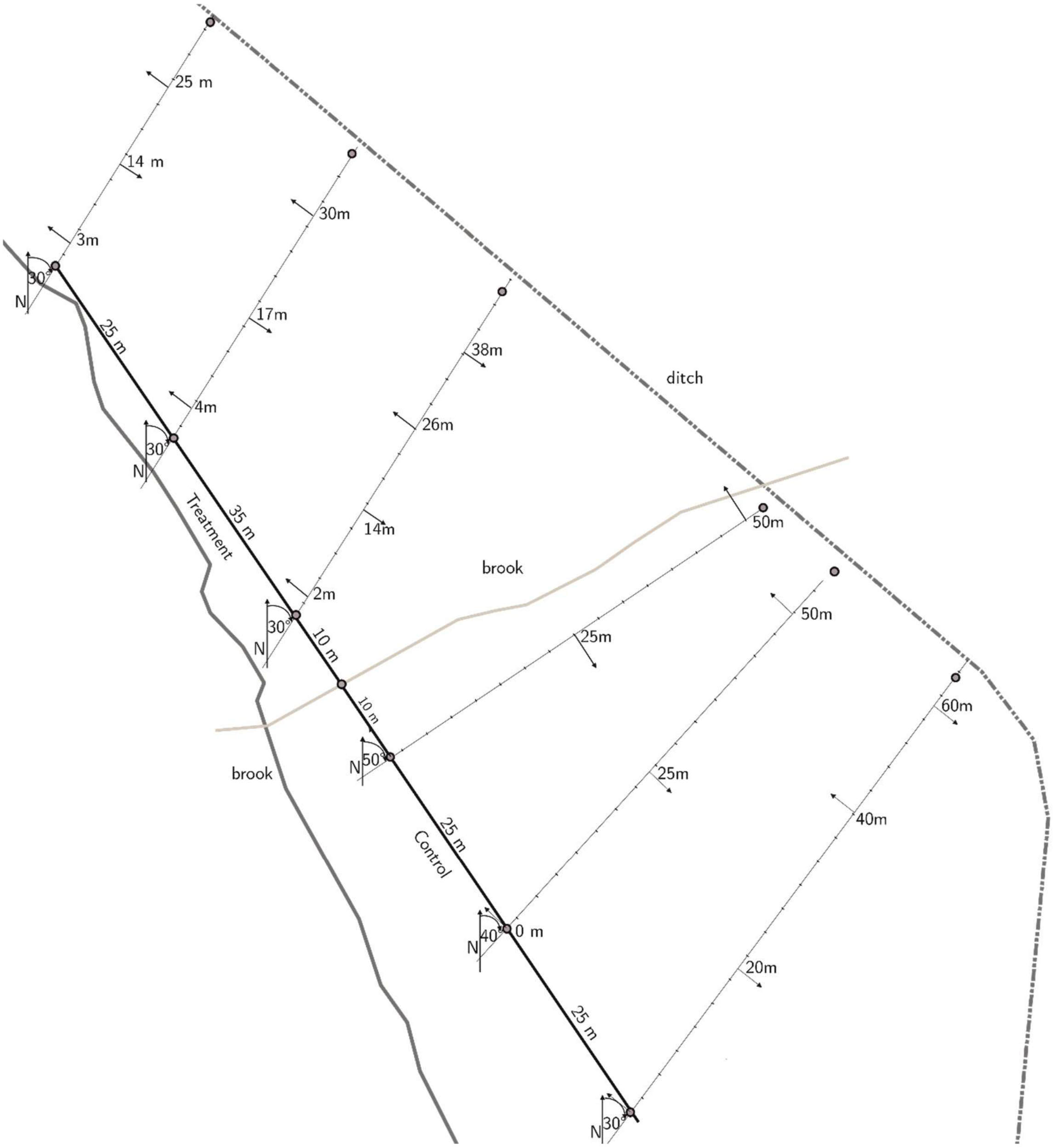

Figure A 11: Plan overview of the irrigation meadow with distribution of vegetation relevées on the treatment and control side of the meadow.

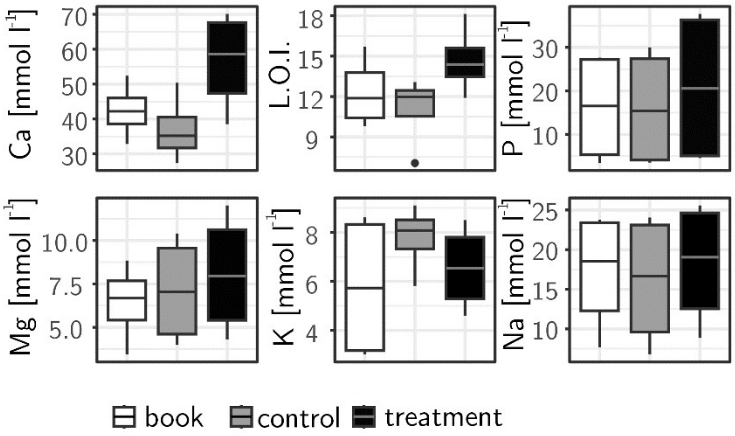

Figure A 12: Chemical analyses of detritus collected at the three sample sites over all observation periods. Ca = calcium, L.O.I. = loss of ignition, P = phosphorous, Mg = magnesia, K = potassium, Na = sodium.