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Communities of aquatic macroinvertebrates affected by human activities

Marek Let

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Společenstva vodních bezobratlých ovlivněná lidskými činnostmi

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CHAPTER 1

GENERAL INTRODUCTION

1. Role of aquatic macroinvertebrates

Macroinvertebrates represent an essential part of aquatic biota and mediate ecosystem functions including sediment mixing, nutrient recycling and energy flow through food webs (Wallace and Webster, 1996; Covich et al., 1999; Balcombe et al., 2005; Sundar et al., 2020). From a pure economic perspective, aquatic macroinvertebrates are necessary as a food source for the production of fish in both running and still waters, although some groups such as black flies (Simuliidae) or biting midges (Culicidae) can bring a strong negative impact on the health of people and livestock from the medical point of view (Resh and Rosenberg, 2015). The positive role of macroinvertebrates in maintaining good quality water prevails over occasional troubles in water management, e.g. bioturbation of sediments by benthic macroinvertebrates in surface water resources (Burton and Johnston, 2010; Welch, 2014). Much flowing water passes through subsurface hyporheic and alluvial zones, where benthic communities (macro-, meio- and micro-fauna) continuously mediate priceless ecological services by preventing the accumulation of organics in little gaps of mineral sediments (increasing porosity) and the formation of anaerobic bacterial processes in bottom waters (purification). Hence, their contribution is also necessary for maintaining stable and quality resources of especially shallow groundwaters (Covich et al., 1999; Mermillod Blondin et al., 2003; Štěrba, 2008; Hunting et al., 2012). With the debasement of many originally drinking water sources caused by severe pollution from municipalities and agriculture, their importance in this field has become evident (Nolan and Hitt, 2006). Last but not least is their influence on culture and recreation (Macadam and Stockan, 2015), and utilisation of their community metrics as indices of environmental quality, i.e. bioindication (e.g. Birk et al., 2012).

Benthic macroinvertebrate communities can substantially accelerate detrital decomposing; an estimated 20–73% of riparian leaf litter is processed by zoobenthos in headwater streams (Graça, 2001). These feeding activities are followed by releasing bound nutrients into solution, excretion, and burrowing into sediments. Supporting algae, bacteria, fungi, protozoans, and aquatic macrophytes with these nutrients increases their growth. Increased growth of these sedentary organisms is mainly utilized by the macrozoobenthic functional feeding group of "grazers and scrapers". The group of "predators" controls the numbers, distribution, and size of their prey. Aquatic and aerial stages of macroinvertebrates represented the high-quality food sources consumed by aquatic and terrestrial vertebrates (Covich et al., 1999; Mermillod Blondin et al., 2003). Knowledge about species-specific ecological and biological traits of macroinvertebrates may help to understand and predict ecosystem dynamics related to the change of environmental conditions from collected datasets, that usually consist of multiple-species plots. Phylogenetic similarities/dissimilarities and species traits, such as habitat specialisations, the body shape, reproductive potential, or variable requirement for oxygen, can be potential reasons for the abundance or absence of particular organisms in communities faced with specific conditions and having variable within-community species interactions (McGill et al., 2006; Šmilauer and Lepš, 2014).

1.2. Functional feeding groups

Generalisation of the ecological function of benthic macroinvertebrate communities in lotic ecosystem was summarised within the River Continuum Concept (RCC; Vannote et al., 1980) which has been still widely accepted. The classification of benthic invertebrate species according to their feeding strategies resulted in the formation of several functional feeding groups (also referred to as feeding guilds or feeding strategies) – shredders (mainly

processing leaf litter – coarse particulate organic matter; CPOM), grazers and scrapers (mainly processing living algae and periphyton), collector gatherers (collecting smaller-sized particle of sedimented organic matter – fine particulate organic matter; FPOM), collector filterers (collecting FPOM from water column) and predators (predate on other invertebrates). Their composition and abundances should correspond to the main available sources of food (e.g. sedimented leaf litter, periphyton, drifting fine particulates, prey organisms), from headwaters to potamal zones of rivers, including macro- and micro-habitats. Thus, given results provide indirect information about sources of food in prospected site. RCC was later enhanced by theoretical explanations of other ecological trends occurring in that environment and adjacent ecosystems, such as Nutrient Spiralling in Streams (Newbold et al., 1982), RCC for Very Large Rivers (Sedell et al., 1989), Flood Pulse Concept in River-Floodplain System (Junk et al., 1989), Hyporheic Corridor Concept (Stanford and Ward, 1993), Riverine Productivity Model (Thorp and Delong, 1994). The Serial Discontinuity Concept reflects natural and especially human-made disturbances in the lotic ecosystems (Ward and Stanford, 1983; Stanford and Ward, 2001). Impoundments on streams and rivers have served as a formal model example causing biological discontinuities, i.e. changes in natural distribution of species, community structures and functions, abundance, biomass, and species richness (Hauer et al., 1989; Stanford and Ward, 1989; Ward and Stanford, 1990; Munn and Brusven, 1991; Helešic et al., 2014b).

Although the classification into functional feeding groups is a useful tool, it represents a strongly generalised system that dismisses, for instance (i) differences between species (e.g. different species can act with different efficiency in food processing; (Lodge et al., 1994; Covich et al., 1999; Crowl et al., 2001), (ii) differences within single species (e.g. early stages may feed in a different way than later stages) or (iii) within-species plasticity in feeding behaviour under specific conditions (e.g. some stoneflies exposed to acidic water can rather prefer "grazing and scraping" periphyton, algae and mosses instead of "shredding" leaf litter; Dangles, 2002). Mouth appendages in arthropods are usually adapted to hold more than one function; therefore, the classification of single species to a proportion of multiple categories may smooth a possible error in a certain way (Covich et al., 1999; Crowl et al., 2001). Recently, additional feeding strategies, such as passive-filter feeders (net-spinning caddisflies and other filterers dependent on supplement of drifting food), active-filter feeders (organisms independent of water flow since having movable filter apparatus, e.g. bivalves or some chironomids) and xylophages (organisms feeding on decaying wood) are used in analyses of invertebrate communities. As already outlined in the introduction of the Serial Discontinuity Concept (Ward and Stanford, 1983), the shift in the composition of feeding strategies can reflect anthropogenic interferences to the environment (Boulton et al., 1992; Lima et al., 2022).

2. Anthropogenic disturbances and human-induced environmental gradients

Disturbance is a relatively short-term event that disrupts ecosystem function, communities, or population and can change resources and substrate availability, or other environmental conditions (White and Pickett, 1985). In addition, a disturbance in the ecological sense has to have some biological consequence different from those caused by common seasonal cycles (Stanley et al., 2010; Battisti et al., 2016). Disturbances can play a pivotal role in the regulation of species diversity in lotic ecosystems (Lake, 2000) and can change the rate of ecosystem processes, i.e. sediment mixing, nutrient cycling and energy flow for a while (Covich et al., 1999). The abnormal disturbance from the perspective of its severity or duration may leave an obvious "trace" after recovery by the extinction of some indigenous species and colonisation

of nonindigenous ones, whilst ecosystemic functions are expected to be recovered into the formal status (e.g. Marten, 2001).

Gradients in ecology are functions of single or multiple variables assumed or detected in natural systems (Müller, 1998). Environmental gradients and gradients of species composition change are commonly observed trends within ecological studies (Šmilauer and Lepš, 2014). Hence, gradients of disturbances may be considered an environmental gradient with usually a short-term duration.

2.1. Types of disturbances and biotic responses

Several attempts to characterise and generalise damaging events and their consequence towards aquatic ecosystems were published (Stanley et al., 2010). According to the concept of Lake (2000), so-called perturbances consist of two consecutive events: disturbance (disturbing forces causing damage) and the response of affected biota to the given damage. The disturbance and the response can have three basic forms – pulse, press and ramp – based on their duration and temporal dynamics (Figure 1). The "pulse disturbances" are rapid and short-term events, such as floods or contamination by a non-persistent toxicant. The "press disturbances" are rapid and long-term events, such as a sediment input or contamination by a persistent toxicant, and the "ramp disturbances" are gradually increasing long-term events (such as prolonged droughts). This concept describes abiotic events, even though it could be tested also on a biotic type of disturbances (such as introduction of nonindigenous highly invasive organisms). From the perspective of the aquatic invasions, the "ramp shape" of the invader abundance can be assumed at the beginning; however, subsequent organismal interactions may later produce decreasing abundance due to increasing experience with the "invader" and/or due to the depletion of resources.

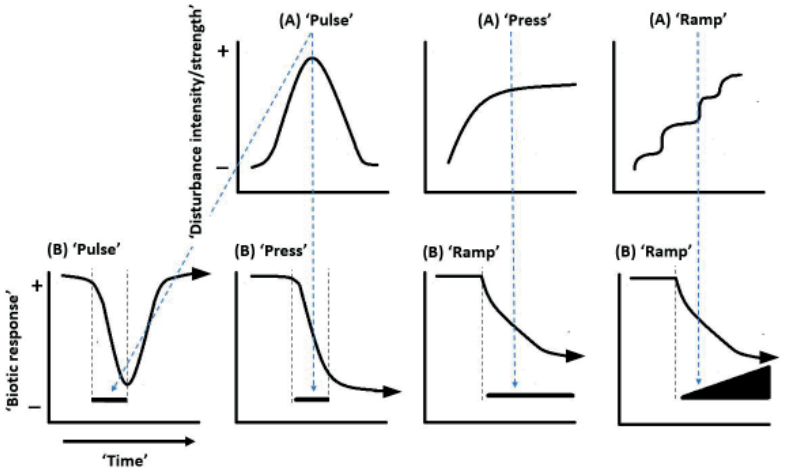

Figure 1. *Types of disturbances (**A**) and particular biotic responses (**B**) according to Lake (2000).*

In contrast to the work of Lake (2000), for example Pickett et al. (1989) do not generalise the response of invertebrate communities onto basic community metrics (e.g. abundance or richness); however, they rather proposed that an ecological unit of research interest with a hierarchical structure should be first determined, whereas the disturbance is a change of

its minimal (first-order) structure; i.e. the system of "entities", e.g. organisms, resources, ecological strengths, that interacting in such way they can persist in a time. Thereafter changes in the structures of the highest orders within the chosen ecological unit cannot be considered disturbance in right sense according to these authors. The authors suggest several types of ecological entities and their minimal structures in which a strength of potential disturbing process can be evaluated.

Some studies have reported negative relationships between species diversity and strengths of flood disturbance, while other studies have reported a unimodal curve of species richness in relation to frequency or intensity of floods. The second case supports the so-called intermediate disturbance hypothesis. This hypothesis assumes that both low and high levels of disturbance negatively influence diversity. The low level could hypothetically cause such intense competition between organisms that the given fauna finally consists of a few strongest competitors. The opposite extreme enables survival of only the species adapted to extreme conditions. Concurrently, the ecosystem's primary productivity can be negatively correlated to some disturbances like floods, whereas the relationship between productivity and diversity is often considered unimodal (Figure 2). This assumption also supports the validity of the intermediate disturbance hypothesis (Rosenzweig and Abramsky, 1993; Lake, 2000).

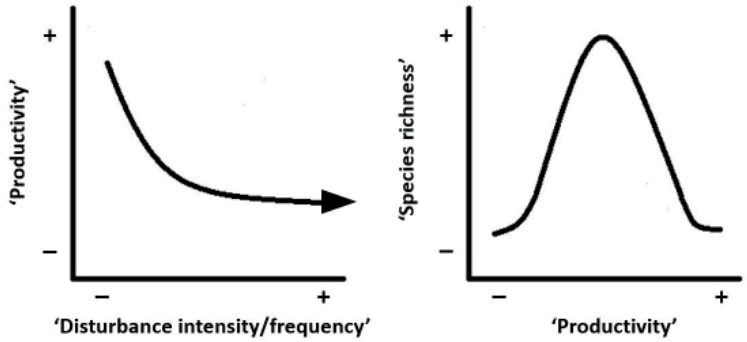

Figure 2. *Suggested relationships between disturbances, productivity and species richness supporting the "intermediate disturbance hypothesis" (Rosenzweig and Abramsky, 1993; Lake, 2000)*

Disturbance, interference, impact, perturbance, pressure, and stress are often perceived as synonyms in the ecological literature. For example, Battisti et al. (2016) proposed to define *interference* (or *pressure*) as a factor influencing organisms and can be identified as their response. *Stress* and *disturbance* are considered subtypes of *interferences* (or *pressures*): the *stress* is a softer form that does not substantially change populations and communities but has a specific negative effect (e.g. decreased appetite); if populations or communities are significantly changed from a formal status, the cause is called *disturbance*. The *impact* and *perturbance* can be understood as subtypes of disturbance: the *impact* is more predictable (e.g. impact of cultured fish to zooplankton), whilst the *perturbance* is less predictable (e.g. hurricane). A quite different hierarchy and definitions were suggested, e.g., by Rykiel (1985); thus, it points the terminological inconsistency out of this issue. This is probably given by a high relativeness of some characteristics occurring among individual attempts to define these terms.

It is also important to explain the concept of species *resistance* and *resilience*, which relates to biotic responses to a disturbance and is frequently used in ecological literature.

Both mechanisms enable individuals and/or populations to cope with adverse effects of disturbances and are often combined within one species. In theory, the species with high *resistance* have a higher chance of surviving a disturbance in a refuge than those with low *resistance*. However, if disturbance intensity or duration exceeds a threshold level specific to the *resistance* capability of that species, its *resistance* stops being efficient. On the other hand, the term *resilience* refers to the ability of species to quickly recolonise the ecosystem after a disturbance has subsided. In other words, although the species with low *resistance* but high *resilience* will show a rapid population decrease along with an increase in disturbance intensity or duration, its high *resilience* can ensure a fast population recovery. High reproductive and dispersal ability are often prerequisites for strong *resilience* (Hershkovitz and Gasith, 2013; Lake, 2013).

3. Human activities and aquatic macroinvertebrates

Human activities can interfere with communities of aquatic invertebrates in the following basic ways: (i) alterations in hydrological regimes, (ii) alterations in habitat and food resources, (iii) changes in quality of water, and (iv) spreading of nonindigenous species. All factors can act independently; however, their separate induction can often trigger others. Concurrently, the cumulation of multiple anthropogenic factors is expected for areas with a higher density of human population, which are typically represented by lowlands (Blann et al., 2009). For example, Bojková et al. (2012) reported dramatic reductions in stonefly populations at several phases, which corresponded to the initial periods of large-scale implementations of new interferences into the landscape in the Czech Republic during the 20th century. The populations in lower altitudes have never recovered, although those in near-natural mountain habitats remained almost intact. The greatest changes were found at the sites affected by organic pollution and a mixture of organic pollution and channelisation or impoundment. The strong terrestrial-aquatic linkage can rapidly change both environments, even if only one environment goes through an anthropogenic alteration.

3.1. Alterations in hydrological regime

A hydrological regime is a basic assumption for the existence of aquatic organisms. Temporal drying or flooding of the environments can affect formal communities or completely exchange ecosystemic functions for a while (McElravy et al., 1989; Bradley and Ormerod, 2001). However, several key structures in riverine landscapes (e.g. alluvial sediment formation as underground water reservoir connected to watercourses) would not have existed without passages of massive floods (Štěrba, 2008). General adaptations and specialism to both extremes are evolved in aquatic macroinvertebrates since similar mechanisms are shared between the hydrological alterations of natural and or semi-natural origin. For example, discharges of the African watercourses, where Resh et al. (2004) analysed macrozoobenthic communities, fluctuated between 900,000 $l \cdot s^{-1}$ in the wet season and 15 $l \cdot s^{-1}$ in the dry season. Lotic chironomid and mayfly larvae can react to flood by downstream migration into flood refugia (pools), and contradictory, some eruciform caddisflies react to increased turbidity and velocity (signs of flood) by moving from the tops of boulders to the sides or bottom (Cobb et al., 1992; Brooks, 1997). The term "torrenticoles" refers to organisms having morphological adaptions to extremely fast current velocities like leeches (Hirudinea), heptageniid mayflies or blephariceridid dipterans (Boulton and Lake, 2008). These examples

represent rather resistance mechanisms; nevertheless, many species show also developed resilience at the level of individuals (e.g. diapausing eggs during unfavourable conditions in crustaceans and insects) or the resilience at the population level (e.g. high reproductive and dispersal ability in many chironomids). For example, Wagner et al. (2000) found that the mayfly *Baetis rhodani* was most abundant in years with continuously high discharge and with seasonally increased discharge; however, it did not well proliferate in years with low discharges and non-seasonal rapid flood events. Contradictory, *Baetis vernus* was the most abundant in the years with these non-seasonal flood events. The success of the latter species was likely caused by the ability of its eggs to persist the event in diapauses. On the other hand, some aquatic macroinvertebrates, e.g. aquatic true bugs and dytiscid beetles, can proliferate from deteriorating drought in running waters by predation on formal communities (Boulton and Lake, 2008), and some chironomids prefer temporal waters rather than the permanent ones (Vallenduuk and Pillot, 2007; Pillot, 2009).

The ecological importance of droughts in aquatic ecosystems has begun more apparent with predicted climate change, although intermittent aquatic ecosystems have been always ubiquitous and have a displaceable role in biota diversity and particular adaptations (Datry et al., 2017). The change from permanent to temporal surface flow can disturb the structures and functions of local macroinvertebrate communities via elimination of taxa sensitive to changed conditions (e.g. Boulton, 2003 or Matthaei et al., 2010). In spite of that, temporal drying of lotic ecosystems can pronounce in higher spatial and temporal taxonomic -diversity (i.e. extend of differences between individual sites of the same region in taxonomic diversities) – mainly due to the replacement of species – at least during the initial several years with summer stream drying (Crabot et al., 2021). According to Boulton (2003), there are several threshold stages of hydrological drought critical for stream macroinvertebrates:

(i.) Loss of lateral connectivity to riparian habitats (mainly fringe vegetation), which eliminates refuges from predators (fish), exit points for some emerging insect and habitats preferred by phytophilous sprawler and climbers, such as damselfly nymphs.

(ii.) Loss of longitudinal continuity (flow cessation), which eliminates many rheophilic taxa almost immediately, prevents a drift, and causes rapid deterioration of water quality (nevertheless, the watercourses with well-developed hyporheic zone consisting of porous gravel and sand can sustain longitudinal connectivity by subsurface flow).

(iii.) Total desiccation of riverbed, representing the most critical stage for the majority of aquatic macroinvertebrates. Dead or dying specimens serve as food for terrestrial organisms like ants. Wet refuges under accumulated organic material, stones, or crayfish burrows are used by survived epibenthic macroinvertebrates.

(iv.) Subsurface drought, representing a critical threshold for the organisms permanently inhabiting or capable of penetrating hyporheic zone – e.g. burrowing macroinvertebrates like nematodes, oligochaetes, some chironomids, or crayfishes, hyporheic crustaceans, sometimes true stygobionts, and possibly early or even latter stages of epibenthic macroinvertebrates (Clifford, 1996; Boulton, 2000; Ledger and Hildrew, 2000; Štěrba, 2008; Guo et al., 2019; Pařil et al., 2019; Pernecker et al., 2020).

The hyporheic zones are considered having pivotal role as refuge for surface macroinvertebrates during droughts (Boulton, 2003). Nevertheless, these refuges cannot sometimes serve well because of siltation of the interstices which is very common in drying

watercourses (Matthaei et al., 2010), or only lack of dissolved oxygen. Conditions in hyporheic zones can often deteriorate in the catchments affected by human activities (Gómez et al., 2017). According to Bertoncin et al. (2019) and Crabot et al. (2021), prolonged droughts can decrease resilience of aquatic macroinvertebrate communities thus it could increase their sensitivity to other stressors. Flow cessation in lotic environments predetermines their higher vulnerability to pollution (Goméz et al., 2017)

Intentional or unintentional human interferences to hydrological regimes are visible in the cultural landscape. For example, the reorganisation of the cultural landscape in the Czech Republic into a large block of arable lands by drying up residual peat bogs, wetlands, and wet meadows, and channelisation of the initial river network (artificial catchments consisting of surface channels, ditches and subsurface collectors) created suboptimal conditions for rich aquatic wildlife (Štěrba, 2008). Draining urban areas is an important measure; however, it has also adverse side effects on the hydrological regime (Bedient et al., 2008); e.g. Junk et al. (1989) reported that short and usually unpredictable flood pulses occur in heavily modified systems with floodplains that have been leveed and drained by man. Repetitive cycles of severe drought and flood periods can be at least deteriorated by these engineering interventions to landscapes regardless of climatic change (Chen et al., 2001). During the initial period, water drained from soil reservoirs, natural wetlands and springs may stabilise discharge in the rivers and enhance primary productivity; however, over time, it can reduce the capacity of the land to retain and absorb water and can pronounce in massive agricultural runoff. Therefore, these measures may strongly influence the aquatic macroinvertebrates in multiple ways (Blann et al., 2009). Reestablishment of, e.g. stormwater wetlands can mitigate some of these effects (Martin et al., 2012).

The building and operation of dams and water reservoirs also alter the hydrological regime by increasing evaporation from the reservoir surface, water abstraction for various purposes, influencing groundwaters, and manipulating surface discharge. Especially hydroelectric dams can alter hydrological regime depending on power plant type and operation (from highly dynamic discharge to artificially stabilised, i.e. with mitigated common seasonal hydrological events). The extreme discharge fluctuations can rise hydrological (physical) stress in aquatic macroinvertebrates which along with other co-occurring factors (habitat alterations and homogenisation, changes in food resources and altered thermal regime; see parts 3.2. *Alterations in habitat and food resources* and 3.3. *Changes in quality of water* below) can be responsible for the elimination of many originally presented species at the affected localities (Hauer et al., 1989; Stanford and Ward, 1989; Ward and Stanford, 1990; Munn and Brusven, 1991). Reservoirs intended for supplying drinking or production water decrease mean discharges of the downstream watercourses; however, extreme discharge fluctuations may not be typical. Water reservoirs have the potential to mitigate the effect of seasonal events like droughts and floods (Helešic et al., 2014b).

3.2. Alterations in habitat and food resources

Anthropogenic alteration of habitats can occur at several scales, from the smallest and most fragile structures (micro-habitats, e.g. change in substrata) to the largest and presumably most robust structures (macro-habitats, e.g. change from lotic to lentic ecosystem). Habitats of macroinvertebrates can be altered due to human-modified hydrological regime – from changes in dynamic and distribution of light depositions representing microhabitats and food to a loss of formal habitats given by manipulated discharge (Štěrba, 2008; Helešic et al., 2014b; Jaeger et al., 2017). Both high and low discharges destroy and generate habitat

patchiness and patchiness of the biota (Lake, 2000). Habitat unification given by small- or large-scale direct interferences into the river channels in cultural landscapes (fortification of riverbanks, excavation or paving of riverbeds, operation of dams, straightening and deepening river channels) can also result in unification and homogenisation of aquatic biota, including macroinvertebrates as well as their habitats (Rahel, 2002; Rasmussen et al., 2012; Skála et al., 2019). For example, built ponds, dams, and weirs can substantially alter natural deposition of riverine sediments. The frequent extreme discharges above large hydroelectric dams often lead to habitat alteration and homogenisation (e.g. prevalence of so-called "armoured" substrate; Munn and Brusven, 1991). Hypolimnial-release dams can substantially change available food resources. For example, Hauer et al. (1989) reported that increased size and availability of seston under a dam cascade probably resulted in increased abundance and biomass of net-spinning hydropsychids. The presence of filamentous green algae resulted in a great abundance of hydroptilid caddisfly *Ochrotrichia* sp. (algae cell piercer). In contrast, e.g. obligate scrapper *Glossosoma* sp. was present only in the upper sections where diatoms were considered a main primary food resource. However, even a virtually inconspicuous change in riverine management, such as removing dead tree trunks from riverbeds, can result in the extinction of microhabitat specialists. Furthermore, the intensification of aquaculture approaches in the Czech Republic likely caused the extinction of one of the biggest aquatic beetles *Dytiscus latissimus*, although the South Bohemia region had represented one of the most important localities for this species worldwide till the beginning of the 20th century (Kolář et al., 2018). Changes in surrounding terrestrial environments may consequently reflect in the changed aquatic environment. For example, reducing stream forest cover can higher solar radiation exposure that supports the production of photoautotrophic organisms. A succession of the functional feeding group of "grazers and scrapers" (e.g. aquatic gastropods, baetid mayflies) usually follows this trend, whilst the abundance of the group "shredders", dependent on the supply of leaf litter, will decline (Goedkoop and Johnson, 2001; Lima et al., 2022). There are also reported differences between tree species in leaf litter quality as a food source for macroinvertebrates. Compared to the evergreen coniferous plantations, deciduous riparian forests let sunlight through canopies of trees off-vegetation season (Friberg, 1997). Large-scale forest harvesting may lead to massive erosion, impairing formal microhabitats (sedimentation or siltation), local communities, and ecosystemic functions. For example, Minshall (2003) predicts that stream ecosystems in the catchment area impacted by severe forest fires will not stabilise for at least 10–15 years. A similar phenomenon happens by runoff from arable land and the input of sewage sludges from municipalities or industrial complexes to recipients. A so-called sewage-derived organic matter which leaks from wastewater treatment plants and sewer infrastructures may represent high-quality food (with a high ATP level given by high content of bacterial, fungal, and protozoa detritivores) for the organisms able to withstand deteriorated accompanying environmental conditions (DeBruyn and Rasmussen, 2002; Loomer et al., 2015). For example, many species of mayflies, caddisflies, and most stoneflies (EPT taxa) avoid of substrates formed by fine sediments, whilst e.g. some chironomids and naidid oligochaetes prefer it (Beermann et al., 2018). Nevertheless, applying phosphorus and sediments into experimental mesocosms resulted in significant antagonistic interaction mitigating the avoidance of sediments, e.g. in caddisflies *Micropterna* sp. and *Agapetus fulviceps* or the mayfly *Baetis muticus* (Davis et al., 2018). On the other hand, the interaction of low flow and sediment addition in New Zealand conditions acted agonistically with decreasing richness of EPT taxa and especially abundances of leptophlebiid mayfly *Deleatidum* sp. and sericostomatoid caddisfly *Pycnocentrodes* sp. (Matthaei et al., 2010).

3.3. Changes in quality of water

Basic physicochemical parameters of water (mainly temperature, pH, oxygen concentrations, and water hardness) and the content of chemical compounds in water are often modified by human activities. Changes in water quality can significantly shape species composition in macroinvertebrate communities and can shift ecosystemic functions (e.g. Burdon et al., 2019). The temperature regime belongs to the most fundamental factors responsible for functioning whole ecosystems. From the perspective of aquatic macroinvertebrates, there are species-specific limits given by the highest possible temperature gradient which they can withstand on the one side (connected to the decreased oxygen solubility) (Haidekker and Hering, 2008), and species-specific thermal demand (e.g. for egg and embryonic development) on the other side (Marten, 1990). Both decreased temperatures from the original status and increased temperatures may often arise in aquatic ecosystems due to human interferences. For example, harvesting riparian forests surrounding streams can increase temperatures by several degrees of Celsius, especially in summer. Leaving a riparian forest fringe with a width of about the height of trees on both riverbanks can mitigate the temperature increase (Moore et al., 2005). The presence of reservoirs can also change the downstream temperature regime in the recipients. Shallow reservoirs and ponds can increase the temperature in summer, while a discharge from the hypolimnion of deep reservoirs can cause lower temperatures in summer and higher temperatures in winter downstream of the dam (Kalff, 2002; Helešic et al., 2014b). Especially reservoirs in the middle and lower river zones (lower rhithral and potamal zones) cause significant biological discontinuities. The reservoirs in the upper zones (crenal and upper rhithral zones) can have a less significant effect if the thermal regime in the reach below the dam is not substantially different from the reach above (Hauer et al., 1989; Stanford and Ward, 1989; Ward and Stanford, 1990). In recent times, the increasing water temperatures in the Czech Republic pose a threat to the larvae of indigenous stoneflies because most of these taxa cannot long-term survive in temperatures higher than 22 °C (Pařil, 2011). On the other hand, the oligothermic stonefly *Protonemura intricata* or mayfly *Arthroplea congener* can avoid of high suboptimal summer temperature by thermic-controlled egg diapause (Landa, 1969; Marten and Zwick, 1989).

The anthropogenic acidification of inland waters is or has been usually caused by nitrogen and sulphur emission, and recovery to former pH values is often slow (Hardekopf et al., 2008). Nevertheless, coniferous plantation forests may also contribute to water acidification since they can acidify soil at a higher rate compared to deciduous ones, partly due to their rapid growth rates with increased ion exchange and partly due to slowly decomposing litter (e.g. Nilsson, 1993; Weatherley et al., 1993; Hughes et al., 1994; Stevens et al., 1994). Among macroinvertebrates, crustaceans, molluscs, and mayflies are generally sensitive to lower pH, whilst some insect taxa (caddisflies, dipterans, stoneflies) can be resistant or specialised to the acidic environment (Hardekopf et al., 2008; Skála et al., 2019).

Water hardness and high content of nutrients (carbon, nitrogen, phosphorus) have a positive effect on the production of macroinvertebrates if the ecosystem still provides niches for keystone species – this is usually limited by decreased oxygen concentrations and the presence of toxic compounds (Bunzel et al., 2013; Helešic et al., 2014a). Despite pollution of the communal-wastewater-origin has decreased in the European Union by implementing advanced wastewater treatment technologies, it represents a serious problem in many other countries (Soares, 2020). Furthermore, periodical loadings of non-treated sewage from many municipalities still reach water recipients, despite the modern technologies of wastewater treatment, often due to the involvement of historical sewer infrastructure in the recent wastewater management (Suchowska-Kisielewicz and Nowogoński, 2021). The shift in the

structure of macroinvertebrate communities caused by organic pollution was described in detail, e.g. by Sládeček (1973) who characterised the communities across several classes of increasing saprobity (a level of organic matter input and decomposition from xeno- to poly-saprobity).

Hydrological droughts can strongly influence a number of physicochemical parameters in in both small and bigger spatiotemporal scales, i.e. higher variation in these parameters can be expected in temporally flowing ecosystems compared to permanent ones. For example, hyporheic water in desert streams can be warmer than the remaining surface water at night. Since a drying ecosystem is often supplied by groundwaters and subsurface waters of different origins and biochemical treatments, the concentrations of oxygen, nutrients (e.g. nitrates, ammonia etc.), organic carbon, and ionic compositions can vary site by site. There can be a substantial difference between remaining pools over a distance shorter than several tens meters. The groundwater can dilute the total concentration of elements or increase water salinity as occurs in temporal Mediterranean streams or in streams supplied with groundwaters of cyclic marine origin. (Gómez et al., 2017). The seepage of groundwater from alluvial zones can improve water physicochemical parameters for surviving in a drying stream (Štěrba, 2008). Depending on particular small-scale spatial conditions, these aspects can either increase or decrease the chance for survival of aquatic macroinvertebrates relying on resistance over time. However, it can be stated, that usual anthropogenic activity in the catchments can significantly worsen the availability of well-serving refuges (Boulton, 2003).

The presence of plenty of toxic, biologically active, and ecosystem-disturbing compounds persists in aquatic environments situated in cultural landscapes (e.g. Richmond et al., 2017; Grabicová et al., 2020). Many of them cannot be eliminated by the current wastewater treatment technologies (Golovko et al., 2014). Moreover, agriculture chemicals involving mainly mineral fertilizers and toxic pesticides are periodically applied directly on a great proportion of all terrestrial land (Foley, 2011). Edge-of-field runoff from such treated lands may cause a highly toxic "pulse" disturbance for aquatic wildlife. Since these often extremely toxic waves can occur for only a few hours during a year (following typical application patterns), the resulted ecological perturbation may result in a "press" response (Figure 1) of highly sensible aquatic organisms without appropriate population resilience (Lake, 2000; Stehle and Schulz, 2015). Compound-specific mechanisms of biological effect, half-lives, polarity, and other properties of "how toxicants behave" in the environment (e.g., experimentally determined parameters as K_{OW}, K_{OC} or Kd) are important predictors of their impact on the ecosystems. It is expected that macroinvertebrate family richness can be reduced by approximately 30% at pesticide concentration not-exceeding accepted regulatory threshold levels (Stehle and Schulz, 2015), and according to Liess and Von Der Ohe (2005) the pesticide concentration equal to 1% acute 48-h LC_{50} for *Daphnia magna* can cause a long-term change of macroinvertebrate community composition. For predicting the magnitude of pesticide contamination from benthic macroinvertebrate communities, the classification of detected taxa into two groups – SPEAR ("species at risk") and SPEnotAR ("species not at risk") – was proposed by Liess and Von Der Ohe (2005), based on: (i) relative sensitivity of given species to toxicants (the "Toxic Unit" approach – TU; e.g. Wijngaarden et al., 2005); (ii) particular generation time (species with the requirement of more than 0.5 year for one generation cycle considered sensitive because of potentially slow recovery possibilities), (iii) given migration ability (low migration ability considered disadvantageous for colonising previously contaminated sites), and (iv) presence of its aquatic stages during the time of maximal pesticide application (usually the spring season). So-called $SPEAR_{pesticides}$ index should better negatively correlate to TU or concentrations of pesticides found in prospected sites than, e.g. commonly used "Shannon diversity index" (H') that reflects any nonspecific diversity disturbance (Becker and Liess, 2017;

Knillmann et al., 2018) and its predictive power was successfully tested by multiple studies out of the region of its origin (e.g. Hunt et al., 2017; Ganatra et al., 2021). Notwithstanding, the $SPEAR_{pesticides}$ old classification (software "SPEAR calculator", version 0.10.0) was revised by Knillmann et al. (2018). Since the stretches of headwaters with a good hydromorphology and natural riverbed situated in riparian forests can mitigate a negative impact of pesticides (Orlinskiy et al., 2015), a new category of $SPEAR_{refuge}$ – eleven originally $SPEAR_{pesticides}$ able to survive toxic waves in refuge areas and/or quickly recolonise previously contaminated stretches (a dispersal-based resilience) – were removed out of the old $SPEAR_{pesticides}$ classification list (Table 1; Knillmann et al., 2018).

Table 1. *The list of $SPEAR_{refuge}$ ("species at risk" sensible to pesticide contamination frequently detected in contaminated sites when natural refugial stretches with a good hydromorphology are nearby present) according to Knillmann et al. (2018).*

Order	Family	Taxon
Diptera	Tabanidae	Tabanidae fam. gen. sp.
Ephemeroptera	Ephemerellidae	*Serratella ignita*
	Ephemeridae	*Ephemera danica*
	Leptophlebiidae	*Habrophlebia fusca*
		Habrophlebia lauta
		Leptophlebia marginata
		Leptophlebia vespertina
		Paraleptophlebia submarginata
Plecoptera	Nemouridae	*Amphinemura sulcicollis*
		Nemoura cinerea
Seriata	Dendrocoelidae	*Dendrocoelum lacteum*
Trichoptera	Goeridae	*Silo nigricornis*
		Silo pallipes
		Silo piceus
	Limnephilidae	*Anabolia nervosa*
		Ironoquia dubia
	Leptoceridae	*Athripsodes aterrimus*
		Athripsodes cinereus
		Mystacides longicornis
	Sericostomatidae	*Sericostoma flavicorne/personatum*
		Notidobia ciliaris

Compared to pesticides, a detrimental effect of pharmaceutical active compounds (PhACs) on aquatic macroinvertebrates at environmentally relevant concentration levels is less known (Brausch et al., 2012). Since these compounds enter the recipients in a mixture of a non-specifiable number of xenobiotics (Embrandiri et al., 2016), evaluation of the effect of individual compounds on the natural community structure can be impossible. Practically, there is no reported precise evaluation of their mixture-specific effect on aquatic macroinvertebrate communities in natural non-manipulated environments. Commonly reported laboratory or mesocosm studies aimed at exposures of macroinvertebrates to single or several compounds; altered behaviour, rate of development, or metabolomes compared to the control specimens were the most usual significant endpoints reached at the environmentally relevant concentrations (Fong and Ford, 2014; Kubec et al., 2019; Ložek et al., 2019, 2020; Hossain et al., 2021). According to these results, it can be concluded that

low concentrations of single or several PhACs administrated for a short time could cause a disturbance at the levels of individuals. Nevertheless, the studies with vertebrates, algae, bacteria, and humans report harmful effects (sometimes even at the population level) that cannot be underestimated (Brooks et al., 2012; Lee et al., 2016; Richmond et al., 2017; Santos et al., 2020; Srain et al., 2021). The authors of scarce observational studies in the natural environment concluded that pharmaceuticals show likely a much lower capability to disturb communities of macroinvertebrates than the alterations in hydrological conditions, physicochemical parameters of water, and rather lower capabilities than pollution by nutrients, organics and pesticides (Damásio et al., 2011; Arenas-Sánchez et al., 2016; Burdon et al., 2019). These studies faced the general problem that pharmaceuticals' variance and effect could be hardly partialled out of co-occurring stressors. However, it should be mentioned that communities exposed to serious loads of PhACs usually inhabit environments modified by human-induced interferences for at least decades (the localities situated in and under urban areas). Assuming these communities consist of a regional "pool" of taxa adapted to anthropogenic environmental conditions (and there are reports about possible adaptations of macroinvertebrates to toxicants; e.g. Siddique et al., 2020), PhACs may not have to show their full disturbing power, when such sites with variable pharmaceutical loads, however, sharing common long-term multiple-stressor influences, are compared. A manipulative multi-factorial mesocosm experiment using communities from multiple sites (including sites with low and high anthropogenic impact) can provide more relevant data.

Besides pesticides and PhACs, many hazardous contaminants are produced and/or dispersed unintentionally into the aquatic environment, such as microplastics (Ivleva et al., 2017), heavy metals (Baby et al., 2010), polycyclic aromatic hydrocarbons – PAHs (Honda and Suzuki, 2020), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs and PCDFs) (Fletcher and McKay, 1993), phthalates (Gani et al., 2017), or radionuclides (Kumblad et al., 2006). A wide range of hazardous compounds mainly occurs in those aquatic environments receiving municipal or industrial wastewaters and/or connected to the lands where the compounds are handled (pathway via soil or precipitation runoff) (Li, 2014; Kodešová et al., 2015). For example, parking-lot pavement sealants were recognised as a major PAH source in Texas's urban stream sediments. Decreased richness and density of chironomids and increased density of oligochaetes were related to the increased PAH concentrations in sediment. The taxa preferring pools responded more strongly than the taxa preferring riffles (Scoggins et al., 2007). Decreased abundances of sediment-burrowing amphipod *Diporeia* spp. at marine sites contaminated by PAHs (Cox and Clements, 2013) or significantly decreased relative richness of mayflies with increasing probable effect concentration of total PAHs were also reported (Moran et al., 2020). Nevertheless, many toxicants are emitted into the atmosphere during combustion and dispersed in the environment via condensation along with atmospheric flows, whereas highly volatile substances can spread spontaneously (Wania and Mackay, 1996; Zhang and Wong, 2007). Persistent organic pollutants (POPs) are considered the most hazardous substances and their production and use are banned (Annex A) or limited for special occasions (DDT; Annex B) in the countries that signed to comply with the Stockholm Convention (2001) (Ashraf, 2017). For example, significantly decreased relative richness of stoneflies, coleopterans and the relative abundance of intolerant taxa along with the increasing probable effect concentration of chlordane (Annex A) was reported in USA streams in 2014 (Moran et al., 2020).

3.4. Spreading of nonindigenous species

The colonisation of aquatic environments by nonindigenous species, either as a result of direct introductions by humans or as a less human-controlled entropy-like process, is considered one of the major threats to the biodiversity and resilience of aquatic macroinvertebrate communities (Jackson and Füreder, 2006). Rahel (2002) called the resulting phenomenon a "biotic homogenisation", where (i) the introductions of nonindigenous species are one of the three suggested interacting causes, including (ii) extirpation of native species (iii) and environmental alterations that can facilitate this process. It can be expected that the colonisation of ecosystems by nonindigenous species will be followed by a loss of populations of one or more indigenous species (Lodge et al., 1998) which share a similar niche with the nonindigenous one and/or are dependent on disrupted or disappeared ecological strengths. Additionally, establishing the population of nonindigenous species may alter energy flow and exchange nutrient cycling (Covich et al., 1999). For example, the introduction of rusty crayfish *Faxonius rusticus* into shallow North American lakes resulted in removing entire macrophyte beds in their littoral zones, expelled the indigenous virile (northern) crayfish *Faxonius virilis*, caused a shift in macroinvertebrate community composition, and impacted the fish stock by removing the macrophyte habitat (Lodge et al., 1994). Another example is the introduction of opossum shrimp *Mysis relicta* into many North American lakes which resulted in a fisheries decline instead of their flourishing. The opossum shrimp predated on juvenile zooplankton and decreases food availability for salmonids. Along with a decrease in fish stock, fewer bald eagles and bears were observed close to the lakes (Martinez and Bergersen, 1989; Spencer et al., 1991). Global invasive spreading of freshwater molluscs, like the zebra mussel *Dreissena polymorpha*, Asian clam *Corbicula fluminea*, Chinese pond mussel *Sinanodonta woodiana*, and the New Zealand mud snail *Potamopyrgus antipodarum*, into the freshwater ecosystems out of their origin has been usually reported to be responsible for weakeing or extinction of formal species populations via competing for food and space and by altering habitats (e.g Alonso and Castro-Díez, 2012; Schmidlin et al., 2012). On the other hand, there are also reports suggesting zero effect or even a positive effect of nonindigenous species on some indigenous macroinvertebrates (e.g. Richardson, 2020). For example, the occurrence of *D. polymorpha* is positively related to higher densities of "grazer and scraper" and "predator" functional feeding groups of benthic macroinvertebrates in general (particularly leeches, flatworms – Platyhelminthes, and mayflies), small-sized gastropods and gammarids. Conversely, its presence is associated with falls in populations of large-sized gastropods, sphaeriid clams, *Diporeia* spp., and large filter-feeding taxa. According to the published studies, a similar effect on macroinvertebrate communities can be expected for other introduced attaching mussels (Ward and Ricciardi, 2007). A negative effect of *D. polymorpha* on the abundance of zooplankton and phytoplankton suggests in rapid change in ecosystemic function (energy and nutrient flows can be displaced from water column nearer to the bottom) with possible consequences (Adlerstein et al., 2013). An initial occurrence of nonindigenous species out of the range of their origin is sometimes called "biological invasions"; however, the nonindigenous species can often colonise free niches in the localities which were/have been under a long-term anthropogenic or natural perturbation process. These engaging, however, sometimes misleading conclusions, may still serve as pretext for using mindless hard power against invasive spread (e.g. by releasing toxic compounds) to demonstrate management strength in most of the human societies. In addition, interactions between biotic and abiotic parameters in the "invaded" environments that can arise along the time may result in satisfactory conditions by using rational management. According to Jackson and Füreder (2006), the long-term studies considering both ecosystem structure and functions will have an important role in interpreting the impact of these introductions.

4. Experimental approaches and particular trade-offs

The community ecology still balances four basic different experimental approaches how to test or support their hypotheses (Table 2) – the reason is the goal of applying its conclusions to real natural systems that are very complex:

Table 2. *Comparison of the strengths and weaknesses of different types of experiments in ecology (according to Diamond, 1986). LE = laboratory experiment – manipulative experiment conducted with communities held in controlled laboratory conditions; FE = field experiment – manipulative experiment conducted with communities in natural conditions; NTE = natural trajectory experiment – experiment conducted in nature using community sampling across a longer time; NSE = natural snapshot experiment – experiment conducted in nature using one-time sampling.*

	Type of experiment			
	LE	**FE**	**NTE**	**NSE**
Regulation of independent variables	highest	medium/low	none	none
Site matching	highest	medium	medium/low	lowest
Ability to follow trajectory	yes	yes	yes	no
Maximum temporal scale	lowest	lowest	highest	highest
Maximum spatial scale	lowest	low	highest	highest
Scope (range of manipulations)	lowest	medium/low	medium/high	highest
Realism	none/low	high	highest	highest
Generality	none	low	high	high

Manipulative experiments in controlled or uncontrolled (field) conditions (*laboratory* or *field experiments*, respectively, see Table 2) are a promising approach that enables partial out the individual responses of macroinvertebrate communities to every single stressor and interactions of multiple stressors (e.g. Beermann et al., 2018; Davis et al., 2018). However, the given responses are affected by high spatial and temporal constraints. For example, artificial conditions in *laboratory experiments* can select out the organisms that did not profit well in the artificial environment and limit or prevent e.g. interactions between macroinvertebrates and larger animals, migration, presence of refuge, and several other aspects. On the other hand, experiments in controlled conditions allow for testing casual effects compared to natural experiments (*natural trajectory* or *snapshot experiments*; see Table 2). The latter ones only allow, in principle, to test and/or point out the correlations within/between community metrics and environmental variables and the tests of interactions of multiple stressors are usually impossible. Nevertheless, the interactions of two stressors led significantly more to the synergic or antagonistic effects toward on the freshwater biota than the additive ones as was shown by, e.g. Piggott et al. (2015) or Jackson et al. (2016). This conclusion calls for greater restraint in interpreting the results of experiments in uncontrolled or semi-controlled environmental conditions (*natural trajectory/snapshot experiments* or *field experiment*, respectively; Table 2).

5. Bioindication using aquatic macroinvertebrates

A shift in aquatic macroinvertebrate communities can reflect a change in environmental conditions including the effect of anthropogenic disturbances, whereas some authors were aware of this knowledge at least since the 19th century (e.g. Kolenati, 1848). A number of approaches have been applied or developed to access the change in the quality of the aquatic environment using multivariable data of macroinvertebrate community compositions. Since the importance of human pressure on aquatic ecosystems was increasing, some of these methods have been adopted by water management administration and their mandatory application was incorporated into the legislation of individual states or state groups (e.g. Birk et al., 2012; Helešic et al., 2014a). Compared to chemical and biochemical analyses of environmental parameters, which are more or less limited by sampling time, the results of analyses of macroinvertebrate communities can provide a piece of evidence about already passed disturbances that can be still hardly detectable by any other current methods. While the initial methods might indicate the gradient of organic pollution in lotic waters using macroinvertebrates (Sandin and Hering, 2004), the modern approaches – like those following European Union Water Framework Directive 2000/60/EC (European Parliament and the Council, 2000) – WFD – often strive to access the ecological status in general. Since aquatic macroinvertebrates provide valuable socioeconomic ecosystem services, the achievement of a high or good ecological status of macroinvertebrate fauna according to the parameters of WFD is one of the assigned aims for the countries of the European Union (Borja et al., 2008, Birk et al., 2012).

The approaches for accessing the quality of the environment and the ecological status of macroinvertebrate communities within water management involve: (i) diversity indices, (ii) biotic indices, (iii) similarity indices, (iv) relative abundance of disturbance-intolerant/tolerant taxa, (v) species traits, (vi) scores from multivariate statistical methods (ordination methods or regression forests), and (vii) comparisons to predicted reference communities – prediction systems (Kokeš and Vojtíšková, 1999; Johnson et al., 2012; Helešic et al., 2014a):

(i.) Diversity indices like Margalef's and Gleason's diversity indices (both applying logarithm), and Menhinick's index (applying square roots) express diversity through the proportion between richness and total abundance – the index values are high (better ecological status) if the total richness is high and abundance is low. The most used H' (applying a logarithmic function) or Simpson index (applying a quadratic function), calculated as a sum of proportions between the abundance of each taxon and total abundance, reflect also equilibrity between abundances of individual species – except for high richness within low abundance, also a high equilibrity should indicate better ecological status. However, the equilibrity is better indicated by "species evenness" which is calculated using H' as:

$$J\text{-evenness} = H'/\ln(\text{total richness})$$

or

$$E\text{-evenness} = \exp(H')/\text{total richness}$$

The diversity indices can be useful during the comparison of two or multiple sites of interest. They are non-specific – each species (or higher taxonomic unit) have the same weight. Therefore, these indices reflect a non-specific type of disturbance. It is ideal to calculate these indices from the data processed into the deepest possible taxonomic level (Kokeš and Vojtíšková, 1999); nevertheless, they are sometimes calculated using

the abundances of individual families (e.g. H' in Oliveira et al., 2011). Using the biomass instead of abundances is also possible (Kokeš and Vojtíšková, 1999).

(ii.) The biotic indices are introduced by empirical (expert-based) or statistically supported values reflecting sensitivities of individual taxa to disturbances, especially organic (Sládeček, 1973) and pesticide pollution (Liess and Von Der Ohe, 2005). However, they serve also well for the evaluation of general ecological status. The resulted values are compared to predicted classes of environmental quality which correspond to the concentration of dissolved oxygen and BOD (Biochemical Oxygen Demand corresponding to organic pollution) or TUs. Thus, only one sampling site (e.g. one sample from multihabitat kick-sampling) may be enough to evaluate ecological or environmental status. There are averaged biotic indices like the saprobic index (used for many decades in Central Europe and requiring deeper taxonomic identification – ideally into the species level; Sandin and Hering, 2004; Helešic et al., 2014a), ASPT (Average Score Per Taxon) index calculated from BMWP (Biological Monitoring Working Party) score (developed in the United Kingdom, requiring less deep taxonomic identification – especially only at the family level – and reflecting neither abundance but only the presence/absence of systematic units; Helešic et al., 2014a), or above mentioned $SPEAR_{pesticides}$ and $SPEAR_{refuge}$ indices (Liess and Von Der Ohe, 2005, Knillmann et al., 2018). On the other hand, the biotic indices developed in Western Europe like Belgian biotic index (BBI) (requiring less deep taxonomic identification – mostly only genus/families – and reflecting neither abundance but only the presence/absence of systematic units), Extended Biotic Index (ETBI; a similar principle as BBI), Index Biologique Global Normalisé (IBGN) (indicating organic pollution and disrupted physical conditions using systematic units in shallow running waters) use two resulted values for match into a reference table of environmental quality instead of averaging (Kokeš and Vojtíšková, 1999, Helešic et al., 2014a). Nevertheless, the principle is similar. The disadvantage of biotic indices is their rather empirically estimated indicator value for each systematic unit. Their specific intent to indicate some type of pollution is undoubtedly burdened by frequent overlap with other types of co-occurring disturbances.

(iii.) Similarity indices serve to compare two (or more) communities from the perspective of similarity in the presented taxonomic composition. Jaccard's and Sörensen's indices count only with presence/absence of each species, whilst indices like Czekanowski's, Sokal's, Canbera's, Morisit's, Horn's, or Bray-Curtis take into the account also individual species abundance. The similarity can be also measured by the ordination methods, e.g. using Euclidean distance (Kokeš and Vojtíšková, 1999; Helešic et al., 2014a).

(iv.) Values of relative abundance (or richness) of disturbance-intolerant/disturbance-tolerant taxa provide simple and useful bioindication which is often used for evaluation of ecological experiments (e.g. Davis et al., 2018); however, it is also useful for monitoring within water management (Moran et al., 2020). For example, relative abundances of Ephemeroptera-Plecoptera-Trichoptera (EPT), which are considered pollution-sensitive, or relative abundance of Oligochaeta, considered as saprobionts and pollution-resistant, can provide information similar to, e.g. saprobic index.

(v.) Proportions of species traits calculated from species abundances or biomass can be advantageous by comparisons of communities sampled in different ecological regions. It is based on the assumption that change in species traits remains conserved within a greater area compared to the distribution of taxa which is region-specific (Kokeš

and Vojtíšková, 1999). The shift in the composition of species traits compared to a reference status can also indicate the change in the ecosystem's functioning. On the other hand, the calculations require data about species traits which generalise and can introduce errors (see the second paragraph in part *1.2. Functional feeding groups* above). Functional diversities can be also calculated as single-value indices, e.g. using the Rao coefficient (Lepš et al., 2006).

(vi.) The sample and species scores yielded from multivariate statistical methods like, e.g. Principal component analysis (PCA), Correspondence analysis (CA), Non-metric multidimensional scaling (nMDS), or regression forests can serve for analyses of greater datasets and for identifying new potential trends and community patterns (e.g. Verdonschot and Nijboer, 2004 or Sandin and Hering, 2004).

(vii.) The systems like PERLA (Czech Republic) or RIVPAC (River invertebrate prediction and classification system, the United Kingdom) should predict community composition based on the reference database including environmental and topological parameters (e.g. altitude, stream order, ecoregion, discharge etc.). The comparison between the predicted and analysed communities can indicate the ecological quality of the sampled site (Kokeš and Vojtíšková, 1999). Systems predicting a natural shift in aquatic macroinvertebrate compositions based on a natural environmental gradient have been yet designed a long time ago. For example, Zelinka (1953) published a system of mayfly (Ephemeroptera) taxonomic representation along with the stream zonation (from the Ameletid zone to Ephemerid zone).

In conclusion, it must be noted that community indices are a useful tool for the management of aquatic ecosystems; however, their application strongly generalises and creates their own environments constrained by mathematical and empirical algorithms. The keystone parameters for the indication of ecological status are taxonomic identities, their abundances and eventually their biomass (e.g. Birk et al., 2012), which enable to reconstruct conditions more precisely. Furthermore, detailed taxonomic identification, which is not often necessary for the calculation of some indices, could prevent a decline of rare species and develop novel knowledge (Nijboer and Schmidt-Kloiber, 2004).

6. Objectives and main hypotheses of Ph.D. thesis

The present thesis was mainly aimed at finding potential relationships between environmental data and compositions of stream macroinvertebrate communities. The main objectives were to evaluate the influence of pollution from i) wastewater treatment plants and ii) agriculture on macroinvertebrate communities and to evaluate the influence of iii) invasive species on macroinvertebrates inhabiting the invaded ecosystem.

Main hypotheses of the thesis:

- Are these human-induced conditions able to alter macroinvertebrate taxonomic compositions and abundance in the prospected sites?
- Are these conditions able to shape compositions of species traits?
- What is their effect size compared to other natural or modified environmental variables?
- What are the relationships between taxa, species traits and these environmental parameters?

Since this thesis is primarily based on sampling in natural environments, multiple anthropogenic factors that usually co-occur together in impaired environments had to be reflected. A range of natural and anthropogenic interferences which resulted especially in habitat alterations, severe droughts, the presence of an invasive species, and the contamination by insecticides, trace metals and communal wastewaters were assessed. Additionally, several laboratory ecotoxicological experiments aimed at testing of chosen effects of municipal wastewater, pharmaceutical active compounds citalopram and methamphetamine, and a pesticide/pharmaceutical niclosamide on aquatic macroinvertebrates (mayflies, caddisflies, crayfish, dragonflies, and nonbiting midges) were also performed. However, yielded data were not included in this thesis either they have not been fully analysed yet, or they were considered to have little coherence with the presented topic aimed at the ecology of aquatic macroinvertebrate communities.

7. Used methods

Presented experiments composed of samplings of macroinvertebrates, recordings of environmental conditions, laboratory analyses, and statistical analyses. Macroinvertebrates were sampled by the multi-habitat kick-sampling method, the Surber sampler method, or by catching host crayfish. Some environmental parameters were measured or empirically estimated *in situ* (e.g. physicochemical parameters measured by a terrain multi-meter). Sampled waters and sediments were analysed mostly for pollutants using chromatography and mass spectrometry in the Laboratory of Environmental Chemistry and Biochemistry of the Faculty of Fisheries and Protection of Waters, hereinafter only "FFPW", and in the laboratories of Povodí Labe and Povodí Vltavy, State Enterprises. Macroinvertebrates were identified to the deepest possible level in the Laboratory of Freshwater Ecosystems of the FFPW and a laboratory of Povodí Labe, State Enterprise using microscopes and stereomicroscopes, scientific literature, and, in one case, using molecular methods. Abundances of taxa in each sample were recorded. Data were analysed using software R-studio (written by RStudio team; Boston, MA, United States) and Canoco 5 (written by Cajo J.F. ter Braak and Petr Šmilauer; Wageningen, the Netherlands, and P. Šmilauer, Czech Republic). Linear models (LMs), Generalised linear models (GLMs), Generalised mixed-effect models (GLMMs), Principal component analyses (PCA), Redundancy analyses (RDA), and Canonical correspondence analyses (CCA) were employed for the analysis of data. A more specific description of used materials and methods can be found in Chapters 2, 3 and 4.

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CHAPTER 2

INSECTICIDES AND DROUGHT AS A FATAL COMBINATION FOR A STREAM MACROINVERTEBRATE ASSEMBLAGE IN A CATCHMENT AREA EXPLOITED BY LARGE-SCALE AGRICULTURE

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Insecticides and Drought as a Fatal Combination for a Stream Macroinvertebrate Assemblage in a Catchment Area Exploited by Large-Scale Agriculture

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Abstract: This case study documents responses in a headwater macroinvertebrate assemblage to insecticide pollution and hydrological drought. In 2014, the Doubravka brook (Czech Republic) was damaged by a large overflow of a mixture of chlorpyrifos (CPS) and cypermethrin (CP). In 2016–2017, this brook was then affected by severe drought that sometimes led to an almost complete absence of surface water. We found significant relationships between the strength of both these disturbances and the deeper taxonomic levels of both the overall macroinvertebrate assemblage (classes) and the arthropod assemblage alone (orders and dipteran families), as well as the functional feeding groups (FFGs). The CPS-CP contamination was mostly negatively correlated to arthropod and non-arthropod taxa and was positively correlated only with FFG collector-gatherers; on the other hand, the drought was negatively correlated to Simuliidae, Ephemeroptera, Trichoptera, and the FFG of grazer-scrapers and passive filterers. Drought conditions correlated most positively with Isopoda, Ostracoda, Heteroptera, adult Coleoptera, and predator and active filterer FFGs. The chosen eco-indicators ($SPEAR_{pesticides}$, $SPEAR_{refuge}$, BMWP, and EPT) used as support information reveal the poor ecological status of the whole assemblage, including the control site, the cause of which is most likely to be the exploitation of the adjacent catchment area by large-scale agriculture. This type of agricultural exploitation will undoubtedly affect macroinvertebrate assemblages as a result of agrochemical and soil inputs during run-off events and will also exacerbate the effect of droughts when precipitation levels drop.

Keywords: headwaters; benthic species; chlorpyrifos; cypermethrin; organophosphate insecticide; pyrethroid insecticide; hydrological droughts; functional feeding groups; contamination

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1. Introduction

Macroinvertebrate assemblages inhabiting headwaters in a cultural landscape have to cope with exposure to anthropogenic activities and their implications [1–5]. The vast majority of published studies generally associate human activities with negative impacts on stream invertebrate biota and their ecological functioning; that is, they provoke a fall in diversity, abundance, biomass, and organic matter processing [6–12]. Nevertheless, human-triggered effects can result in greater biomass of specific aquatic organisms [13–15] or, occasionally, in higher species richness [16,17], and some ostensibly heavily affected sites may sometimes even act as refuges for endangered species [18–20].

Over the last few years, much of Europe has suffered from severe droughts in combination with unusually high temperatures [21,22], and climatological prognoses suggest that these events will become more frequent in the future [23,24]. Combined with intensive

land use and its effects on functioning water regimes, climate change could cause huge ecological and economic damage [25–28]. Affected headwaters will dry up more often and more quickly, thereby multiplying the impact of pollution by agricultural or urban wastewater [29–31]. Aside from the plethora of pollutants that occur in surface waters, pesticides (e.g., insecticides, herbicides, and fungicides) are of concern due to the negative effect they have, in particular, on aquatic biota and, in general, on whole ecosystem functioning [32–35]. The main reason is their massive use worldwide and their typically high specific toxicity for non-target aquatic organisms [5,36–38]. Pesticides are an integral part of conventional agriculture and enter the water at much higher concentrations after short-term runoff events than those that are usually detected by standard monitoring sampling methods [39,40]. Therefore, pesticides or their residues may have acute or chronic effects on aquatic organisms [41,42]. Furthermore, toxicants with great adsorption to soil or organic matter may persist in sediments; hence, they can threat sediment dwelling organism for a prolonged time [10,43]. The presence of pesticides in aquatic ecosystems is often accompanied by a wide range of anthropogenic influences, such as habitat degradation, drainage [44,45], artificial siltation and sedimentation [46,47], increased nutrient input, and the occurrence of more frequent extreme hydrological events (droughts and floods) at interconnected sites [44,48]. These factors often exacerbate the negative effects of pollutants on aquatic biota, generally due to the losses they induce in many kinds of resources in an ecological sense (e.g., microhabitats, refuges, food, and the strength of interactions) [49–51].

Invertebrate populations should be able to withstand specific disturbances whose impact will presumptively be reversible. Nevertheless, certain regularly recurring processes in combination with other biotic or abiotic conditions will undoubtedly damage exposed assemblages by eliminating the most susceptible species (e.g., *Margaritifera margaritifera* or *Prosopistoma pennigerum*) or even entire deeper taxonomic groups (e.g., heptageniid mayflies, perlid stoneflies, and goerid caddisflies), thereby eventually negating ecological traits [4,49,52–57]. For this reason, laboratory and field experiments such as those defined by Diamond [58] that manipulate the effects of xenobiotics under various experimental settings (simulation of diverse possible natural scenarios) are necessary [11]. However, experiments carried out in natural scenarios (natural experiments) are also essential as they can verify obtained knowledge in real environments [59].

The aim of this study was thus to assess the effects of serious insecticide contamination on a headwater macroinvertebrate assemblage and observe the ability of species to recolonize affected stretches of the damaged water course. We used a dataset that was not created purely for research purposes; rather, it was used by the state environmental protection authorities as part of their legal investigation into the accident. Thus, it lacks some of the information needed for a thorough assessment of certain aspects of the macroinvertebrate assemblage. Our study reveals the response of this macroinvertebrate assemblage to an extreme event, a situation that is rarely observed, probably because—unlike chronic effects—these accidents only occur rarely. In terms of climate change and evolving strategies in agriculture, our results reflect a situation that will increasingly threaten organisms in watercourses in agriculture landscapes, as well as all other lifeforms that are dependent on these damaged water bodies.

2. Materials and Methods

2.1. Locality Description

The Doubravka brook is situated in central Bohemia (Czech Republic, Central Europe, spring to confluence: N 49.7775686, E 15.5793436–N 49.8619361, E 15.4977614; Figure 1). This small, third-order stream (the Stahler order used) is 13.8 km long, has a catchment area of 21.6 km^2 and average discharge of up to 9 L s^{-1}. It rises at an altitude of 460 m a.s.l. and flows into a fifth-order stream at 251 m a.s.l. The riverbed of the brook is natural and it is surrounded by typical alluvial vegetation, together with part of a cultural coniferous forest (in all, an approximately 150–600 m wide bio-corridor). This sector of the brook has been preserved due to the steep terrain. Most of its catchment area is occupied by

uniform blocks of fields (each with an average area of 30 ha) where rapeseed, corn, and cereals are cultivated. The fields are drained by underground water collectors connected to straight channels, as well as to artificial and/or originally temporary or permanent tributaries. The brook ecosystem harbors microhabitats with shallow riffles with macro-, meso-, micro-lithal, and pools anchored by the root systems of alder trees (*Alnus glutinosa*, *A. incana*). In these microhabitats detritus, particulate organic matter (POM), smaller-sized pieces of gravel and xylal accumulate. However, in recent years this locality has been affected by severe droughts leading to zero surface discharge, a phenomenon that was often observed during the study period, mainly during extremely hot summers. The riffles often disappeared and the bottom of the isolated pools became almost completely covered by silt and organic matter.

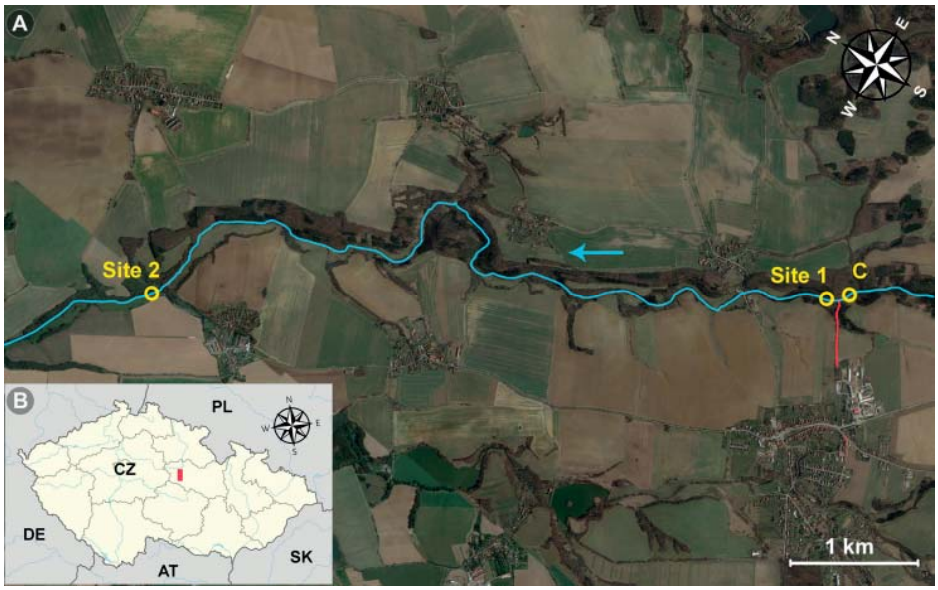

Figure 1. Map of the study area: (**A**) Locations of the sampling sites along the longitudinal profile of the Doubravka brook (in blue; flow direction indicated by the blue arrow). The contaminated drainage channel is highlighted in red. The sampling sites are shown as yellow circles: C and Site 1 = Transect 1; Site 2 = Transect 2. (**B**) Study area shown as the red rectangle in the Czech Republic.

2.2. Insecticide Contamination

In March 2014, the brook was accidentally contaminated by a commercial insecticide product containing organophosphate chlorpyrifos (CPS) and pyrethroid cypermethrin (CP) (at a ratio of 10:1, respectively). It flowed down a straight drainage channel receiving wastewater and runoff-water from a local agricultural company that had illegally stored unused insecticide. The concentration of CPS in the sediment dry weight (d.w.) was 13 mg kg^{-1} at the confluence of the brook and the drainage channel (Site 1, Figure 1) five days after the accident. Mass mortality of over 10,000 individuals of the critically endangered noble crayfish *Astacus astacus*, macrozoobenthos, and fish (brown trout *Salmo trutta* and stone loach *Barbatula barbatula*) was observed for over 6 km downstream from Site 1. *A. astacus* and *B. barbatula* samples were analysed for CPS (77 and 31,000 µg of CPS kg^{-1} of body weight, respectively). CPS sediment concentrations decreased rapidly

over the following months (Table 1). The bottom of the contaminated stretch of the brook also became abnormally overgrown by filamentous algae and periphyton during the first months after the accident.

Table 1. Chlorpyrifos content in the sediments analysed in the samples from Sites C (control = unaffected, Transect 1), 1 (affected, Transect 1) and 2 (affected, Transect 2) during the sampling period 2014–2017. The asterisks indicate macroinvertebrate sampling. LOQ = 20 μg kg^{-1} (d.w.).

Sampling Date	Site C (μg kg^{-1} d.w.)	Site 1 (μg kg^{-1} d.w.)	Site 2 (μg kg^{-1} d.w.)	Time Passed after Accident (Days)
4th IV 2014	<LOQ	13,000	350	5
20th VI 2014 *	<LOQ	128	51	83
17th IV 2015 *	<LOQ	87	<LOQ	384
26th VII 2016 *	<LOQ	33	<LOQ	850
19th IX 2017 *	<LOQ	25	<LOQ	1476

2.3. Sediment Sampling

Sediment was sampled according to the standardized accredited methods (ČSN EN ISO 5667-1, ČSN EN ISO 5667-3, ČSN ISO 5667-12, ČSN ISO 5667-14, and ČSN ISO 5667-15) by a telescopic sampler with a wide-neck stainless steel container. A layer of the bottom sediment was scrapped using the edge of the container. Regarding the character of the riverbed containing a small amount of fine sediment, sediment was sampled in several places where it was possible within one sampling site. These individual sub-samples were homogenized into one mixed sample, put inside polyethylene sampling bottles, and stored in a polystyrene thermo box filled with ice during transport prior to the laboratory analysis.

2.4. Analysis for Pesticides

2.4.1. Reagents and Materials

The analytical standards of chlorpyrifos (chlorpyrifos-ethyl, CAS 2921-88-2) and deuterated chlorpyrifos-D10 (CAS 285138-81-0) (Dr. Ehrenstorfer; Augsburg, Germany) were of 99.49% and 99.1% purity, respectively. Methanol and mobile phase additive ammonium acetate were LC-MS purity (Merck; Darmstadt, Germany). An SG Water Ultra Clear TWF UV plus TM with TOC-Monitoring water system from SG Water (Hamburg, Germany) was used throughout the study to obtain the LC-MS grade water.

2.4.2. Sample Processing and Analyses

The ultrasonic assisted extraction with methanol solvent was applied for the preparation of sediment samples as it was described by Ferenčík and Schovánková [60]. The samples were dried at 20 °C using a lyophilizer Christ Alpha 1–4 (Osterode, Germany), and sifted through a 2 mm Retsch sieve (stainless steel). The 10 mL aliquot of each sample was homogenized by grinding using a mixer mill MM 200 from Retsch (Haan, Germany) and zirconium oxide 25 mL mixing jars with balls. 5 μL of the internal standard solution (chlorpyrifos-D10, 100 pg mL^{-1}) were spiked into 0.5 g homogenized subsamples, then extracted using 5 mL of methanol in 50 mL glass Erlenmeyer flasks in ultrasonic bath Binder Electronic (Berlin, Germany) for 60 min at 40 °C (maximum ultrasonic power). The extracts were centrifuged by centrifuge Jouan B4I (Thermo Electron Industries, Chateau-Gontier, France) at 2400 g. The 100 μL aliquot of extract was diluted with 900 μL of 100 mmol L^{-1} ammonium acetate in a 2 mL sample vial used for LC-MS/MS measurement. A smaller aliquot of a sample or higher extraction volume was selected for the more contaminated samples.

2.4.3. LC-MS/MS Conditions

Based on published literature [61], development and optimization of the LC-MS/MS method was done. The chromatographic separations were conducted using a Waters Acquity UPLC HSS T3 column (100.0 × 2.1 mm, 1.8 μm particle size) and guard col-

umn 2.1 × 5.0 mm with the same chemistry, thermostated at 40 °C. 5% methanol and 5 mmol L^{-1} ammonium acetate in water (A) and 5 mmol L^{-1} ammonium acetate in methanol (B) were used as mobile phases. Gradient elution program was set up from 0.1% B to 99.9% B, the method duration was 18.5 min. The injection volume was 250 µL.

The LC-MS/MS analysis was carried out using triple quadrupole Waters Premier XE (Manchester, UK) connected with ultra-high-performance liquid chromatograph Waters Acquity UPLC® (Milford, MA, USA). The electrospray ionization source heated at 120 °C in the positive ionization mode (3.5 kV) was used. For acquisition and processing of data, the Waters MassLynx software was used.

Chlorpyrifos SRM transitions (precursor > product) were as follows: quantifying 349.8 > 197.7 (cone voltage—25 V and collision energy—19 V), qualifying 349.8 > 96.7 (cone voltage—25 V and collision energy—34 V). In addition, internal standard chlorpyrifos-D10 transitions were as follows: quantifying 359.8 > 198.7 (cone voltage—25 V and collision energy—19 V).

2.4.4. Validation of the Analytical Procedure

Six points' calibration curve was linear in the range of 10–500 ng mL^{-1}. Internal standard and matrix matching standard methods were used for quantification of chlorpyrifos. Recoveries of chlorpyrifos in sediments were calculated by spiking the matrix at two concentration levels—50 and 500 ng g^{-1}—in triplicates. The range of recoveries of the spiked samples was 78–121%. The repeatability of the method was determined as relative standard deviation (RSD) of repeating analysis of spiked samples and was lower than 20%. The method detection and quantitation limits of chlorpyrifos was expressed based on S/N ratio of 3 and 10, respectively [61]. The limit of quantitation (LOQ) was calculated to 20 µg kg^{-1} (d.w.) with a measurement uncertainty of 30% covering the whole sample preparation and measurement procedure assessed using control chart (2 s) constructed at a concentration level 50 µg kg^{-1}.

2.5. Macrozoobenthos Sampling and Analysis

2.5.1. Sampling and Determination

Monitoring took place in 2014–2017. Three sites along the brook were chosen (Figure 1). Site C (control) was not contaminated by the insecticides. Sampling of macrozoobenthos was performed using kick sampler (25 × 25 cm net frame dimensions, 500 µm mesh) by 3 min-long sampling multihabitat method, following rules of European Water Framework Directive (WFD) [62]. Briefly, this method is based on an a priori evaluation of presence and spatial proportions of various microhabitats at the chosen sampling site. Subsequently, an interval taken from the total time (3 min) is allotted for each sampling at particular chosen micro-habitats regarding to their spatial proportionality compared with the other ones. The sub-samples of micro-habitats were always pooled into only one sample, processed using a round steel sieve (40 cm diameter, 500 µm mesh) and preserved in 70% technical ethanol. Organisms were determined to the highest possible taxonomic level; their frequencies in the samples were recorded (Table S6). Before determination, single portions of material were individually separated from smaller particles using a small plastic sieve (500 µm mesh) and tap water.

The first sampling was performed in June 2014 (approximately 2.5 months after the accident). The others in 2015–2017 were restricted between April and September (Table 1). The sampling was initiated by the state monitoring authorities investigating the accident. Therefore, the experimental design lacked replicates of macrozoobenthos samples at individual Sites and timepoints and relied instead on a multihabitat sampling approach.

2.5.2. Estimation of Taxa Traits

A dataset containing the ecological and plasticity traits of all detected taxa was created (Table S7). The categories of these ecological traits are represented by the following five subsets: *Functional feeding groups* (Collector-gatherers, Shredders, Xylophages, Active filter-

ers, Passive filterers, Predators, and Grazer-scrapers,) *Macro-habitat preference* (Limnophiles, Rheophiles, and Torrenticoles), *Meso-habitat preference* (Epibenthos, Endobenthos, and Hyponeuston), *Micro-habitat preference* (Xylal—tree trunks, branches, and roots; Akal—fine to medium-sized gravel; POM—deposits of fine particulate organic matter; Pelal—mud and sludge; Detritus—deposits of coarse particulate organic matter;, Psammal—sand; Lithal—coarse gravel, cobbles and blocks; Phytal—algae, mosses, and plants) and *Reproductive ability* (<0.5 generation per year, 0.5 generation per year, 1 generation per year, 2 generations per year, and >2 generations per year). The plasticity trait used was *Body length* of adult or final larval instar (<4 mm, 4–7 mm, 8–12 mm, 13–18 mm, 19–25 mm, and >26 mm). The classification was empirically encoded by the proportion of a particular type of trait for detected taxa within each subset category.

2.5.3. Calculating Eco-Indicator Parameters

The base parameters (total richness, total abundance, Shannon–Wiener index) commonly used to describe a macroinvertebrate assemblage status were generated in R studio software using the *BiodiversityR* package [63] for all samples. Three types of 'Species At Risk' (SPEAR) classifications (coded by dummy variables) were used to reveal the proportions and sums of the abundances of the most susceptible species, as well as and their richness, as follows: (i) '$SPEAR_{pesticides}$ (old)' were classified taking into account certain lethal concentration levels and species-specific ecological traits that could be critical for the organism exposed to the pesticides [40]; (ii) the '$SPEAR_{refuge}$' concept represents the adjustment of the original $SPEAR_{pesticides}$ list with a category of sensitive species with a strong potential for successfully recolonising contaminated stretches of streams and brooks [49]; and (iii) '$SPEAR_{pesticides}$' are a subset of the '$SPEAR_{pesticides}$ (old)' that do not belong to the '$SPEAR_{refuge}$'. SPEAR indices were calculated according to the following adjusted equation:

$$\text{SPEAR index} = \frac{\sum_{i=1}^{n} \log(4xi + 1) \times y}{\sum_{i=1}^{n} \log(4xi + 1)} \quad (1)$$

where n is the total number of taxa, xi is the abundance of taxon i, and y is a binary code representing taxon i thus: 1 if it belongs to the SPEAR, otherwise 0. The log $(4x + 1)$ transformation of the abundances aims to decrease the influence of populations with mass development [49].

EPT (Ephemeroptera–Plecoptera–Trichoptera) richness, abundance, and relative abundance (%) were calculated because many species groups belonging to these three orders are often very sensitive to several types of disturbances including insecticide pollution. EPT-derived parameters are frequently used as a measure of the quality of the environment and/or the seriousness of a disturbance effect by comparing cases. The EPT abundance usually correlates with the SPEAR abundance.

The original *biological monitoring working party* (BMWP) score and its *average score per taxon* (ASPT), which are based on family presence rather than on abundance, was also derived [64]. The aim of the BMWP score is to indicate the status of organic pollution. Families sensitive to organics are usually more susceptible to insecticides.

2.6. Classification of Hydrological Status

The hydrological status semi-quantitative scoring system for implementation in the ordination analysis was designed following Boulton and Lake [53] (Table 2).The prior hydrological status at each sampling point was based on our observations (Figures S3–S5), precipitation and temperature data from previous months for the particular area, and discharge data in the river Doubrava—a second-order river receiving water of the Doubravka brook (data taken from the website of the Czech hydrometeorological institute, Prague, Czech Republic, Table S4 and Figure S2). In 2015–2017, abnormally high temperatures in this area were accompanied by a lack of precipitation. The scores assigned to each sample are shown in Table S5.

Table 2. Hydrological status score.

Description:	Score:
Fast-flowing	1
Loss of fast-flowing habitats	2
Loss of lateral connectivity to stream-edge habitats	3
Loss of longitudinal connectivity, flow stops, isolated pools form	4
Pools shrink water quality deteriorates	5
Total absence of water	6

2.7. Statistical Analysis

The dataset containing the abundance of individual taxa as response variables and two environmental predictor variables Chlorpyrifos-Cypermethrin contamination status (CPS-CP) and drought status (Drought), and one covariate (Transect), were analysed using the multivariate Canonical correspondence analysis (CCA) in CANOCO 5 software [65]. Similarly, the chosen deeper taxonomic levels for all detected taxa and arthropods (representing the most diverse and, simultaneously, the most sensitive groups to insecticides) were used as a response in the redundancy analysis (RDA)—classes and orders (dipterans divided into families), respectively. The variables CPS-CP and taxa abundance were log-transformed; the response variables were centered but not standardized. Additionally, the ecological and biological traits of the invertebrates (see Section 2.4.2. Estimation of species traits) were implemented as standardized composition-weighted trait averages (using the abundance of taxa in the samples) into independent RDA against the CPS-CP and Drought variables. The significance of the relationships between these environmental variables and individual compositions of taxa or traits was tested using particular canonical axes and their eigenvalues with a Monte Carlo permutation test (number of permutations: 1999). The cases were always shifted into two permutation blocks, defined by the *Transect* covariate, to maintain autocorrelation. Principal component analyses (PCA) were used for the trait compositions, which did not reveal any significant relationship with the explanatory variables.

3. Results

3.1. Recolonisation of Poisoned Stretches by Sensitive Insect Species

Eighty-three days after the accident, the sample from non-polluted Site C contained 24 insect taxa and one oligochaete taxon. At polluted Site 1, the species richness was reduced to eight taxa (two oligochaetes and six insects) (Table 3). Site 1 was recolonized by two species classified as $SPEAR_{pesticides}$ (*Ecdyonurus* sp. and *Rhyacophila nubila*) and three species considered as $SPEAR_{refuge}$ (*Ephemera danica*, *Halesus digitatus*, and *Hydropsyche instabilis*) after the poisoning. The two remaining sensitive insect taxa belonged to the Chrironomidae family, represented by an early Chironomidae gen. sp. instar and *Brillia bifida*.

Table 3. Summary of macroinvertebrate community parameters calculated for each sample within the sampling period (2014–2017). Abbreviations: Shannon–Wiener index (H'), number of species (N), Abundance (Abu). For meaning of parameters, see Section 2.4.3.

	2014			2015			2016			2017		
Parameter	**C**	**1**	**2**	**C**	**1**	**2**	**C**	**1**	**2**	**C**	**1**	**2**
Abundance	1142	228	2752	644	966	4472	2842	2313	7227	3308	776	1432
Richness	26	9	15	39	26	23	34	22	21	43	23	26
H'	2.40	1.44	1.51	3.10	2.06	1.69	2.05	2.44	1.53	2.99	2.17	2.77
$SPEAR_{pesticides}$ index	0.23	0.15	0.02	0.11	0.13	0.09	0.20	0.06	0.05	0.16	0	0.08
$SPEAR_{refuge}$ index	0.25	0.29	0.05	0.14	0.15	0.02	0.06	0	0	0.12	0.08	0.10
$SPEAR_{pesticides}$ index (old)	0.48	0.44	0.07	0.25	0.29	0.11	0.26	0.06	0.05	0.27	0.08	0.18
N $SPEAR_{pesticides}$	6	2	1	5	4	3	9	3	1	9	0	3
N $SPEAR_{refuge}$	7	3	1	6	5	1	2	0	0	5	2	3
N $SPEAR_{pesticides}$ (old)	13	5	2	11	9	4	11	3	1	14	2	6
Abu $SPEAR_{pesticides}$	306	8	1	40	30	26	94	9	68	136	0	24
Abu $SPEAR_{refuge}$	142	44	6	58	36	2	40	0	0	280	16	72
Abu $SPEAR_{pesticides}$ (old)	448	52	7	98	66	28	134	9	68	416	16	96
N EPT	14	5	2	12	10	4	7	0	1	11	2	4
Abu EPT	448	52	7	148	94	28	100	0	68	396	16	80
EPT%	42.73	22.81	0.25	22.98	9.73	0.63	3.52	0	0.94	11.97	2.06	5.59
Original BMWP score	100	42	26	107	77	42	102	49	38	141	65	72
ASPT index (±SEM)	6.67 ± 0.77	6.00 ± 1.35	3.71 ± 0.78	6.29 ± 0.78	5.50 ± 0.79	4.20 ± 0.63	5.10 ± 0.62	4.08 ± 0.53	3.80 ± 0.79	5.88 ± 0.63	4.64 ± 0.70	4.80 ± 0.76

At Site 2, 10 Insecta taxa, 3 Oligochaeta taxa and 2 Gastropoda taxa were detected. Two Diptera families (Chironomidae: Chironomidae gen. sp., *Chironomus riparius* gr., *Corynoneura* sp., *Micropsectra* sp., *Paratrichocladius rufiventris*, *Thienemannimyia* sp. and *Tvetenia verralli* and Simuliidae: *Simulium vernum*) dominated the insect assemblage. The remaining two detected insect taxa, both caddisflies classified as $SPEAR_{pesticides}$ (*Rhyacophyla* sp.) and $SPEAR_{refuge}$ (*Hydropsyche* sp.), were detected in very low numbers (one and eight individuals, respectively).

The $SPEAR_{pesticides}$ and $SPEAR_{refuge}$ detected at Site C but not in the poisoned stretches were *Ephemerella mucronata*, *Habrophlebia lauta*, *Serratella ignita*, *Leuctra* sp., *Nemoura* sp., *Hydropsyche siltalai*, *Polycentropus flavomaculatus* and *Potamophylax luctuosus*. The species not at risk ($SPEnotAR_{pesticides}$) that were not found to colonise at least one of the two contaminated Sites were *Microtendipes chloris* gr., *Orthocladius* sp., *Tanypodinae* gen. sp., *Tvetenia verralli*, Ceratopogonidae gen. sp., *Dicranota* sp., *Tipula maxima* and *Elmis* sp. lv.

3.2. Relationship between Disturbances and Macroinvertebrate Taxonomic Composition

A Monte-Carlo permutation test performed within independent RDA revealed the significant power of both variables (adjusted $p < 0.05$) for predicting the taxonomic compositions of the whole macroinvertebrate assemblage, which could be classified into seven deeper taxonomic levels (mostly classes) (Figure 2A), and the arthropod assemblage classified into 19 deeper taxonomic groups (mostly orders) (Figure 2B). Sediment contamination by insecticides (CPS-CP) negatively correlated with all the deeper taxonomic levels (in terms of their abundance) other than with Platyhelminthes (*Polycelis nigra*) (Figure 2A). Platyhelminthes, Crustacea, Bivalvia, Gastropoda and Insecta were the most reliably fitted groups. Hence, sensitivity to contamination, from the most to the least affected taxonomic groups, is as follows: Bivalvia (only *Pisidium casertanum* and *P. personatum*) and Gastropoda < Insecta and Crustacea (Ostracoda and Isopoda) < Platyhelminthes (*Polycelis nigra*) (no effect). The remaining two annelid taxa (Hirudinea and Oligochaeta) did not reach the same quality of fit; nevertheless, the Oligochaeta group does seem to be sensitive to CPS-CP contamination. The increasing intensity of drying up positively correlated with densities of all given taxa, but mostly with the Bivalvia species, Crustacea, Hirudinea, and Oligochaeta.

Predominantly lotic Heteroptera and adult Coleoptera were the commonest insect taxa in assemblages found at an advanced stage of drying up (Figure 2B). Conversely, the family Simuliidae was highly sensitive to drought; the EPT group was negatively correlated with both factors. This group showed relatively higher resilience to contamination compared to the other arthropods; however, it was almost completely lacking in samples taken under the worst hydrological conditions (Table 3).

The test performed using the CCA to ordinate the whole assemblage into the highest possible taxonomic level revealed the marginal effect of both predictors (adjusted $p = 0.055$ for the effect of both disturbances).

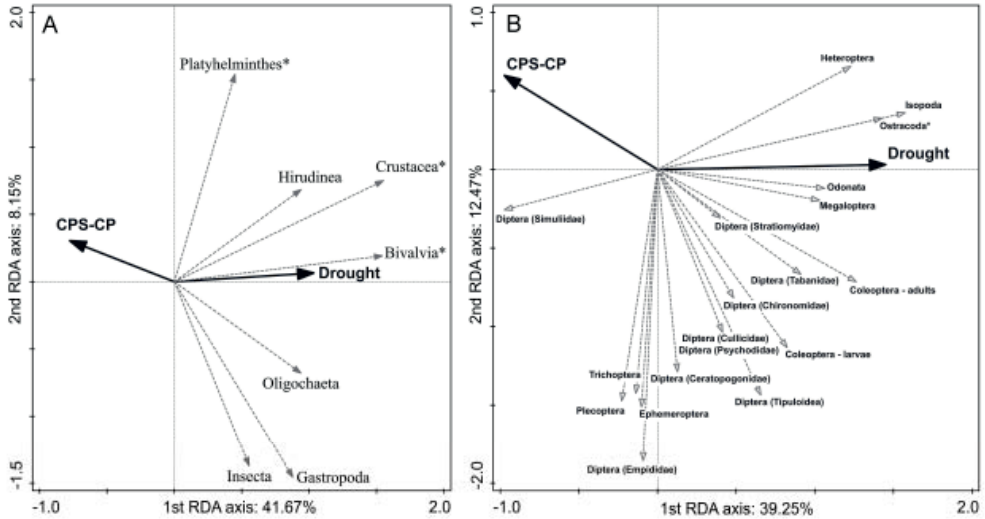

Figure 2. RDA ordination diagrams showing the relationships between disturbances (CPS-CP = chlorpyrifos-cypermethrin contamination derived from the chlorpyrifos sediment contamination, and Drought = drought status; see Materials and Methods) as solid black arrows, with taxa represented by grey arrowed lines. The power of the both disturbances to predict taxonomic compositions was significant (adjusted $p < 0.05$). The length of arrows corresponds to the quality of fit. (**A**) Relationships between disturbances and the composition of deeper taxonomic levels extrapolated from the abundances of all detected taxa (CPS-CP: explained variation 26.9%, pseudo-F = 3.3; Drought: explained variation 22.9%, pseudo-F = 3.7, respectively; adjusted variation explained by both predictors = 37.28%). Additional information for the labels marked by the asterisks: Platyhelminthes is represented by a single species—*Polycelis nigra*; Crustacea is represented by Ostracoda (not determined into a deeper taxonomic level) and *Asellus aquaticus*; Bivalvia is represented by only two species—*Pisidium casertanum* and *P. personatum*. (**B**) Relationships between disturbances and the composition of deeper taxonomic levels extrapolated from the abundances of arthropods. (CPS-CP: explained variation 24.7%, pseudo-F = 3.0; Drought: explained variation 27.0%, pseudo-F = 4.5, respectively; adjusted variation explained by both predictors = 39.65%). The asterisk marking Ostracoda indicates that this group was not determined into a deeper taxonomic level.

3.3. Effect of Disturbances on Ecological and Biological Species Traits

Of the ecological and plasticity traits described in Materials and Methods (see Section 2.4.2 *Estimation of Species Traits*), only the *Functional feeding groups* for all detected taxa (Figure 3) showed a significant relationship with the first ordination axis and both predictors (adjusted $p < 0.05$) within the partial RDA. The CPS-CP contamination negatively correlated with the abundance of *predators* and *active filterers*, both of which, however, prevailed over the other feeding strategies during drought conditions (Figure 3). This trend was caused by increased densities in, especially, chironomid, ostracod and bivalvian assemblages in isolated pools: namely, the facultative *active filterers* from the Tanytarsini tribe (Chironominae) such as *Micropsetra apposita* and obligatory *active filterers* represented by Ostracoda and Bivalvia (*Pisidium* sp.). Numerous *predators* or facultative *predators* from the Tanypodinae subfamily including *Macropelopia nebulosa*, *Procladius* (*Holotanypus*) sp., and *Thienemannimyia* sp. were followed by the nectonic heteropteran *Notonecta* sp. and the adults of the coleopterans *Platambus maculatus* and *Agabus didymus* during drying up conditions. On the other hand, contamination correlated positively with collector-gatherers for a short time period; nevertheless, this strategy was suppressed during the hydrological droughts, as occurred with passive filtration and grazing-scraping. The shredders

and xylophages were the least involved in material processing during both peaks of the perturbations (Table S3).

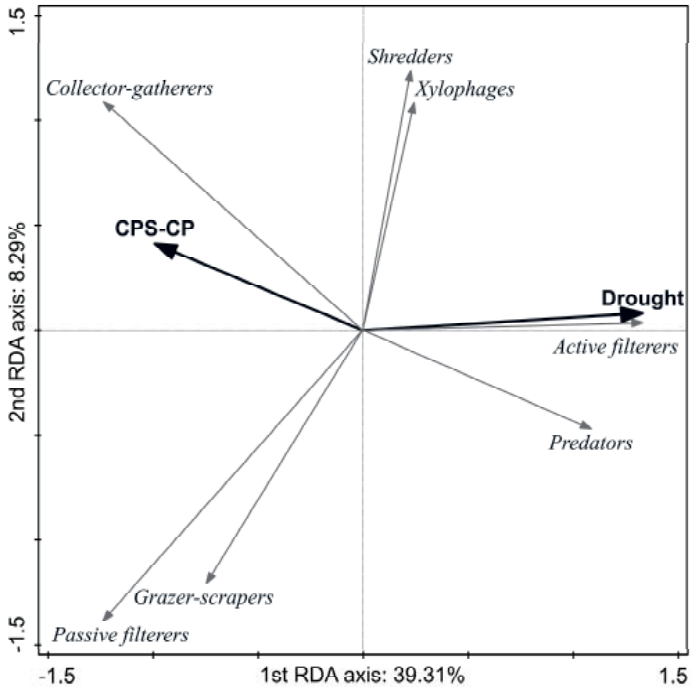

Figure 3. Ordination diagram showing the relationships between the average functional feeding groups (FFGs) represented by solid grey arrows and disturbances (CPS-CP = chlorpyrifos-cypermethrin contamination estimated from the chlorpyrifos sediment contamination and Drought = drought status; see Materials and Methods) represented by solid black arrows. Both disturbances have significant ($p < 0.05$) power to predict the FFG composition. Arrow length corresponds to the quality of fit. The FFG was extrapolated from abundances of all detected organisms (CPS-CP: explained variation 25.3%, pseudo-F = 3.1; Drought: explained variation 22.3%, pseudo-F = 3.4, respectively; adjusted variation explained by both predictors = 34.50%).

The remaining compositions of species traits extrapolated from taxa abundance did not significantly correspond with the variables. Nevertheless, relationships between disturbances and compositions of species traits (listed in the Section 2.4.2 *Estimation of Species Traits*) are shown in the diagrams resulting from the PCA (Figure S1).

4. Discussion

4.1. Response of Macroinvertebrate Taxa to the Disturbances

Macroinvertebrate species assemblages can substantially change after acute poisoning with insecticides and during prolonged periods of drought [53,66,67]. The significant relationship plotted in Figure 2A suggests that no groups of organisms prefer the CPS-CP contamination, and that only the Platyhelminthes (*Polycelis nigra*) tolerate it. Contrary to our prediction (Table S1) that the stream macroinvertebrate assemblage would shift from being dominated by arthropods to being dominated by non-arthropod species, the insect and crustacean groups, in general, did not seem to be the most affected groups [67]. The most likely cause of this effect is that the downstream part of the brook was colonized

by species from the upper non-affected part. In addition, the insect species with the ability to drift downstream or with winged imagines (ephemeropterans, plecopterans, and chironomids) are often perfectly adapted for colonizing new habitats, and so insects may in fact be very resilient to insecticides [34]. These adaptations may be much more advantageous than a resistance to a prolonged or severe disturbance [68,69]. However, if species populations are too weak or non-existing in adjacent brooks or catchments, they can become temporarily or permanently extinct [70].

Hydrological conditions—and especially droughts—heavily influenced the macroinvertebrate assemblage. The abundance of all groups correlated more or less positively with an increase in droughts (Figure 2A). Bivalves (*P. casertanum* and *P. personatum*), gastropods, oligochaetes, crustaceans (*Asellus aquaticus* and Ostracoda), as well as leeches, all showed greater correlation than insects (bivalves and gastropods may have increased their relative density due to a decrease in available space during droughts). Furthermore, the drought period was accompanied by increased sedimentation of organic matter caused by very low water discharge. A further meaningful biological factor that could be partially responsible for both the redundant accumulation of organic matter and assemblage shift is the total extinction of *A. astacus* in this brook. Before the accident, noble crayfish were found at a density of around one individual per m^2 at Sites 1 and 2, and its population did not recover at any point during this study (no individuals caught in traps) [71]. These bottom-dwellers shred and release leaf litter from under obstacles on riverbed into the current, dig burrows in riverbanks, and also feed on macrozoobenthos, presumably slow-movable macroinvertebrates [72].

The Crustacea group represented by the isopod *A. aquaticus* and the class Ostracoda (Figure 2A) appeared at all Sites during the dry period in 2016–2017, as did the Bivalvia. The relationship between CPS-CP and this group was similar to the relationship between CPS-CP and the insect group, which demonstrates the high specific toxicity of these compounds to arthropods in general [73,74]. Nevertheless, the drought had a generally positive effect on these two crustaceans (Figure 2A,B). The relationship between insecticide pollution and the abundance of *A. aquaticus* within ecosystems that are often affected by toxicants as well as organic pollution remains unclear [40]. Although *A. aquaticus* is very sensitive to acute exposure to insecticide, it can tolerate lower concentrations of various toxic compounds, including insecticides [75–77]. It is also able to avoid desiccation [78]; furthermore, Extence [79] reported a significant increase in the abundance of *A. aquaticus* in a lowland river during a drought period as a result of greater accumulation of organic matter. The response of *A. aquaticus* to these two types of disturbances can be summed as intolerance to acute poisoning but a general preference for an ecosystem disturbed by partial drying up and the increased deposition of organic material that can serve as a food resource.

The relationships between arthropod groups and disturbances shown in Figure 2B suggests that very few taxa were positively correlated with the CPS-CP contamination; however, the individual response to greater hydrological drought was more complex. Members of the family Simuliidae were the most negatively correlated with greater drought (and had a positive relationship with CPS-CP contamination), a logical finding given that, due to their feeding adaptations, they are dependent on flowing water current [80]. We believe that the positive relationship with increasing CPS-CP contamination is due to their ability to recolonize contaminated stretches of brook if favourable hydrological conditions are present (i.e., flowing water) [81]. The general intolerance of many species from the EPT group to pesticides, organic pollution, and a lack of water (factors that are typical in water courses affected by large-scale agriculture) has been well documented and is discussed in more detail below (Section 4.2) [82–84].

Taxonomic groups ordinated near the Chironomidae family were represented by the larvae of other dipteran species and coleopteran larvae and adults. This group of taxa seems to be very sensitive to acute pollution; however, it colonised the most contaminated Sites immediately after the mayflies and caddisflies and, unlike them, were tolerant to

drought. This also occurred in the families such as the Dytiscidae that colonising shrinking pools. Chironomids are the most complex family and individual species have very plastic ecological characteristics [85–88]. Their high susceptibility to CPS-CP and other insecticides under laboratory conditions does not correspond to the generally high resilience of many Chironomid species to various types of disturbances [89,90]. However, the preferences of chironomid larvae for certain hydrological conditions varies between species within subfamilies or even within a single genus. For instance, the subfamily Orthocladiinae decreased in abundance and richness during the dry period, whilst the Tanypodinae and Tanytarsini (*Micropsectra* sp.) dominated in the samples during drought conditions in 2016 (Table S2). The high densities of *Micropsectra* sp. could be a product of the stress caused by the drought, and numerous tanypodins would either feed on them or be similarly forced to move into refuges [84,91]. It is worth mentioning the fact that stream macroinvertebrates often concentrate in small wet areas during periods of drought, and that they can increase or decrease their densities depending on taxon-specific life histories [54]. This system may be much more at risk to different types of pollution and other interferences, and higher densities and weakening may favor the spread of disease within assemblages [92].

4.2. Response of Species-Trait Assemblages to Disturbances

Taxonomic and functional feeding group (FFG) assemblages often have intimately interrelated characteristics that change along environmental gradients and may react in different ways to disturbances [54]. Less time is usually required for the recovery of trophic structures than for the recovery of taxonomic assemblage structures [93]. Although shredders are considered to be the most susceptible group to insecticides, and despite the fact that they are often also sensitive to droughts, the approximal correlation between shredders and both drought and CPS-CP was close to zero [53,67]. Almost identical trends were estimated for xylophages. The relative abundance of shredders and xylophages was low at all Sites (average proportion to other FFGs: 6.96% and 1.39%, respectively), whilst collector-gatherers dominated in most cases (average proportion to other FFGs: 50.01%; Table S3). This could have been caused by the time of sampling (April–September) because shredder abundance commonly increases in autumn when more leaf litter is available [94]. However, if the whole assemblage were chronically influenced by insecticides at all Sites, an arthropod-shredder population, represented especially by caddisfly larvae (no gammarids were detected during the study), would be suppressed. Specifically, the shredder FFG was represented by the caddisfly *H. digitatus*, which prefers permanent waters, at the contaminated Sites after the accident, whilst the isopod *A. aquaticus*, which is considerably more tolerant to drought conditions, was the most abundant representative of the facultative shredding strategy during the period when the brook was drying up [78,95]. The exclusion of shredders from headwaters is thought to trigger ecological changes in overlapping assemblages due to the crucial role they play in the smooth cycling of nutrients by processing coarse particulate organic matter into smaller transportable particles (fecal pellets and orts). The headwater streams lacking shredders are colonized by saprotrophic microorganisms (bacteria, fungi, and protozoa), followed by microphagous collector-gatherers and filterer macroinvertebrates. This type of assemblage is not very resistant to the highly turbulent water flow found in headwaters after heavy precipitation [25,67,96,97].

The positive relationship between drying up and predator abundance has been described by Boulton and Lake [53], who named this phenomenon as 'predator soup'. It occurs as organism density increases in shrinking pools. Herbst et al. [98] reported an expansion of micro-predators (e.g., Tanypodinae, and Ceratopogonidae) that was partially the case in our study. The possible proliferation of an active filterer FFG assemblage in temporary standing water (e.g., Culicidae) was also suggested [53]. Nevertheless, a correct interpretation is more difficult because part of the active filtering organisms, e.g., Tanytarsini chironomids, would be forced to move into residual pools with negative consequences for their survival [84,91]. Passive filterers rely on water currents; thus, this feeding strategy may be useless when stream flow ceases. Nevertheless, within a species

this strategy is usually combined with other options for getting food. Similarly, the grazer-scrapers, being the second most dominant FFG after collector-gatherers, were negatively correlated with the drought variable. Despite the fact that this strategy could co-exist with others in a single species, many studies describe it as existing on its own without any other feeding mechanism, e.g., in certain heptagenid nymphs, gastropods, etc. [99]. Herbst et al. [98] reported a decrease in the relative abundance of grazers during a severe drought. According to our results, the abundance of grazers correlated negatively with the Drought variable, while its correlation with CPS-CP was close to zero.

4.3. Parameters of a Macroinvertebrate Assemblage at the Studied Localities

Stressor-specific eco-indicators are a useful tool for monitoring ecosystems because they reveal ecological effects and assess how single and combined stressors affect ecosystem structure and function [40,50,100]. The parameters calculated for each sampling Site (Table 3), which showed an obvious shift in a macroinvertebrate assemblage between localities, were not statistically supported. Although the multihabitat-sampling method used to assess the macrozoobenthos status is robust [62], this approach is often inadequate for a standard research analysis. However, in this particular study, the dataset was of use due to the very strong effect of the studied ecological disturbances. It is hard to interpret differences in total abundances from the data; nevertheless, fivefold lower macroinvertebrate abundance 2.5 months after poisoning in 2014 at Site 1 compared to Site C does suggest that macroinvertebrate density did not recover. Cuffney et al. [67] propose that approximately four months are needed for macroinvertebrate density to recover after an acute poisoning event (even though the biomass will remain significantly lower in the first year before recovering in the second [101]). However, in these authors' study the recolonization process from an upstream section did not occur, unlike in our study. The richness, BMWP score and ASPT index estimated for Site C were regularly higher than at Sites 1 and 2, which reveals the negative impact at both Sites during the monitoring period. The BMWP approach, which considers only the richness of specific family (not abundance), indicates the presence of a higher level of saprobity at these Sites [64].

Insecticide pollution chiefly affects the most susceptible stream insect assemblages [67]. The abundance (Abu) and richness (N) of the indicator species (SPEAR and EPT) decreased at the contaminated Sites; however, the indices calculated from the proportion of the sum of the sensitive species abundances vs. the sum of all species abundance (even if log-transformed) are intended for use in cases of chronical stress, not for events of massive spills of toxicants leading to the total extermination of all species [40]. Therefore, the conclusions resulting from these indices could be misinterpreted. For example, when comparing Site C with Site 1 in 2014, both samples had almost the same value for the $SPEAR_{pesticide}$ index (old), despite the huge contamination that had occurred there!

In terms of assemblage parameters (richness, H', SPEAR, EPT-derived values; see Table 3), site C was the least disturbed environment—despite its similar or even worse hydrological situation—when compared to Sites 1 and 2 in 2016 and 2017. Nevertheless, the values for the $SPEAR_{pesticides}$ index calculated for each sample, including these from Site C, reflect its 'poor' or 'bad' ecological status (according to the EU Water Framework Directive) [49,102]. These findings could be explained by the lack of any well-conserved refuge area. Sufficiently long stretches of uncontaminated headwaters situated in riparian forests are thought to act as significant functional refuges enhancing stream hydro-morphology and enabling the recolonization of a damaged downstream section [103]. However, pollution by an insecticide or any other kind of pesticides can often occur even in economically exploited forests [104].

Many authors consider that 'edge-of-field' runoff after heavy rain represents the main vector for pesticides entering water ecosystems [9,40,105,106]. According to Stehle and Schulz [52], the strength of their effect can be modulated by application patterns, geographical and meteorological conditions, the physicochemical properties of the insecticide (e.g., stronger sorption properties can reduce the impact) and its intrinsic toxicity. The basin of

the Doubravka brook including its source is exploited agriculturally. The excessive size of local fields (up to 100 ha) drained by surface and sometimes by sub-surface systems, combined with steep slopes, undoubtedly increases the risk of pollution by agricultural chemicals, of extreme hydrological situations and of soil erosion [44,45].

Immediately after the accident (2014), total invertebrate abundance and richness were substantially lower near the contamination source. The first pioneers to colonize Site 1 were the $SPEAR_{pesticides}$ (old) and EPT species, although there were also a number of $SPEAR_{refuge}$. Despite their high sensitivity to pesticides, these species are frequently detected in contaminated stretches near uncontaminated stream sections regardless of the pesticide pressure. The occurrence of $SPEAR_{refuge}$ in contaminated or disturbed streams can be caused by dispersal-based resilience and the ability of these organisms to disperse from uncontaminated to contaminated stream sections (via drift and adult dispersal) [49]. Given the spatial proximity between uncontaminated Site C situated upstream from Site 1 (Figure 1) and the time lapse between the accident and the sampling time (2.5 months), the drift of these insect species is the most likely explanation of their occurrence there. In addition, the proportion of stream organisms drifting downstream from stretches damaged by repeated insecticide pollution can be much higher than in undamaged stretches [67]. The situation at all sampling Sites (low values of $SPEAR_{pesticide}$ indices, agricultural exploitation of the basin, and the period of pesticide application between the accident and the first sampling) suggests chronic pollution by pesticides or another disturbance. Nevertheless, the lethality of short-term pollution and the level of macroinvertebrate drift from Site C is unknown; thus, recolonization via drift downstream from this Site could either be accelerated by a lower-effect disturbance, especially at the level of sensitive species richness, or inhibited by a higher-effect disturbance at this level and, especially, at a level of sensitive species abundance. It is worth mentioning that fresh generations of chironomids and Oligochaeta (the Naidinae subfamily) were detected at Site 1, although both groups are considered to be $SPEnotAR_{pesticides}$, and they could even have been reproducing at this Site after the accident. Since the bottom of contaminated stretches of brook became covered by large amounts of filamentous algae and periphyton during the first few months after the accident, these organisms could have prospered in this micro-habitat despite the persistence of the contamination in sediments (Figure S1B).

The distance between sampling Site C and sampling Site 2 (approximately 6 km) could represent the threshold distance needed for fast recolonisation by $SPEAR_{refuge}$ species [49]. This agrees with our findings because the detected abundance and richness of $SPEAR_{pesticides}$ and $SPEAR_{refuge}$ species were very low here compared to Site 1, although Site 2 was not damaged as much by the gradual flow of contaminated sediment from the drainage channel. On the other hand, the sensitive insect species ($SPEnotAR_{pesticides}$) represented by Diptera (Chironomidae and Simuliidae) might have recolonized this stretch given their reproductive potential. Since the detected dipteran larvae were mostly bi- or multi-voltine (and in a case of later instars, above all epibenthic), survival in the hyporheic zone is less likely. In the case of caddisflies, migration from the upstream section and/or from a possible small tributary refuge situated between Sites 1 and 2 cannot be excluded.

In 2016 and 2017, the almost total absence of $SPEAR_{pesticides}$ and EPT species at Sites 1 and 2 (Table 3) raises several possible questions about another pesticide contamination event (or another disturbance) during this period. This event was partially revealed by the increased concentration of cypermethrin recorded in sediments sampled in the drainage upstream from the confluence with the Doubravka brook. Despite this, a higher concentration of cypermethrin was not detected at the observed Sites. During this period, the area was affected by severe hydrological drought. Given that the drainage channel during the time period supplemented Sites 1 and 2 with muddy, pesticide-enriched water, this situation is a good example of the combined effects of drought, greater sedimentation and pesticide pollution ('ramp', 'press', and 'pulse' types of disturbance, respectively, according to Lake [107]), which led to partial absences of the EPT species, especially at Site 1 (Table 3). Despite the fact that locality C was affected by the same or even greater lack of

water than these two localities (Table S5), the EPT groups resisted here. Nevertheless, the redundant sedimentation caused by the lack of water current probably triggered the high development of chironomids and oligochaetes.

5. Conclusions

This case study attempts to describe how the macroinvertebrate assemblage responds to acute poisoning by insecticides (Chlorpyrifos and Cypermethrin) and severe hydrological droughts in a headwater stream that is chronically affected by agrochemicals and large-scale agriculture exploitation in its catchment area. In the case of the accidental insecticide contamination, the assemblage reacted with a drop in richness and abundance. The contaminated Sites were colonized by resilient taxa and the lotic insecticide-susceptible taxa were able to spread from non-contaminated refugial stretches situated upstream from the source of the contamination. In the case of hydrological droughts, lotic organisms declined and the assemblage shifted to favor taxa that prefer higher levels of organic pollution and lentic hypo-neustonic (nektonic) predatory insects.

A non-stable hydrological regime is a serious issue that many once-permanent aquatic ecosystems have to confront. Damage caused by agricultural pollution will be more serious for sensitive taxa if headwater ecosystems turn into semi-temporary systems. Nevertheless, the consequences of droughts are of greater concern to ecologists and administrative bodies. We are convinced that the rationale management of headwater basins is key in ensuring the proper ecological status of whole river networks, which are essential for human society in many aspects.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13101352/s1, Table S1: Presumed negative effect of chlorpyrifos and cypermethrin on chosen invertebrate taxonomic groups; Table S2: Abundance and richness of chironomid subfamilies and tribes in individual samples; Table S3: Percentage proportion of functional feeding groups in terms of abundance in individual samples; Figure S1: PCA ordination diagrams showing relationships within the composition of given taxa traits and between these traits and particular explanatory variables; Table S4: Meteorological data of the Vysočina Region (Czech Republic), containing the catchment area of the Doubravka brook, for individual years of the invertebrate sampling; Figure S2: The hydrologic situation during the sampling campaign 2014–2017 in the Doubrava river (Spačice, Czech Republic); Figure S3: Photographic documentation of different hydrologic situations through the year 2016 at the Site C (control = unaffected, Transect 1); Figure S4: Photographic documentation of different hydrologic situations through the year 2016 at the Site 1 (affected, Transect 1); Figure S5. Photographic documentation of different hydrologic situations through the year 2016 at the Site 2 (affected, Transect 2); Table S5: The hydrological status score assigned to each sample during the sampling period 2014–2017; Table S6: Detected taxa and their semi-quantitative abundances in all samples; Table S7: Detected taxa and their ecological and plasticity traits.

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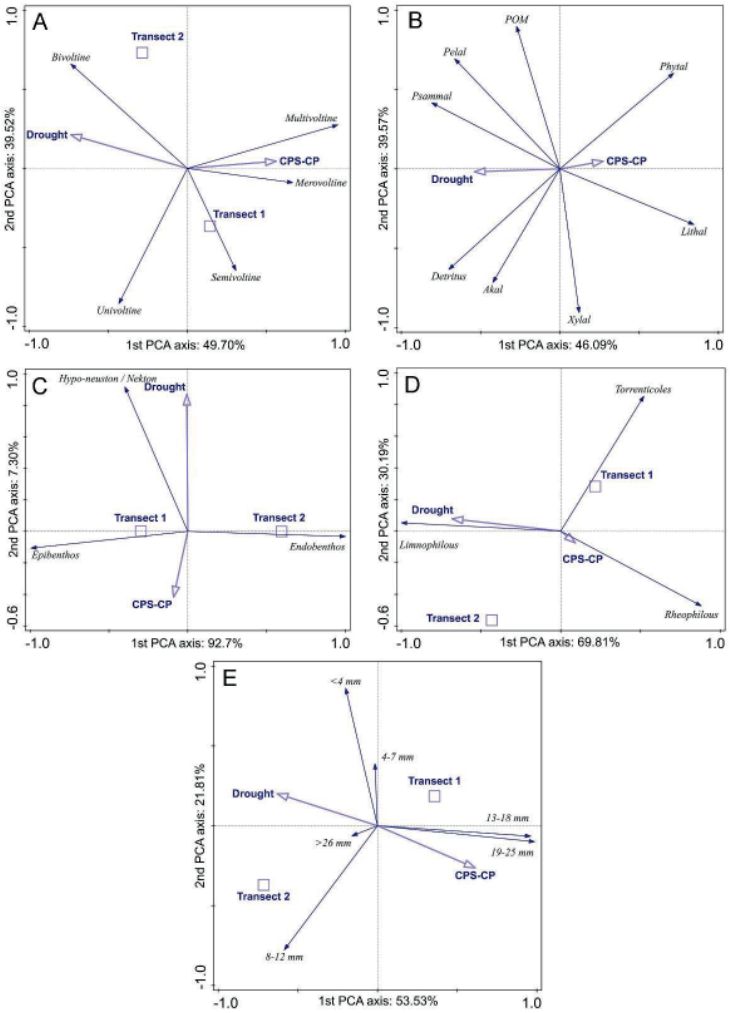

Figure S1. PCA ordination diagrams showing relationships within the composition of particular taxa traits and between these traits and CPS-CP (chlorpyrifos-cypermethrin contamination) and the Drought variable. (**A**) *Reproductive ability* within the arthropod assemblage (explanatory variables account for 20.02% of variation). (**B**) *Micro-habitat preference* within the entire assemblage (explanatory variables account for 18.68% of variation); explanations: *Xylal*—tree trunks, branches, and roots; *Akal*—fine to medium-sized gravel; *POM*—deposits of fine particulate organic matter; *Pelal*—mud and sludge; *Detritus*—deposits of coarse particulate organic matter; *Psammal*—sand; *Lithal*—coarse gravel, cobbles, and blocks; *Phytal*—algae, mosses, and plants. (**C**) *Meso-habitat preference* within the entire assemblage (explanatory variables account for 24.23% of variation). (**D**) *Macro-habitat preference* within the entire assemblage (explanatory variables account for 58.80% of variation). (**E**) *Body length* within the entire assemblage (explanatory variables account for 43.56% of the variation). The permutation test performed on the first ordination axes within RDA was non-significant ($p > 0.05$), as was the power of the explanatory variables to predict particular compositions.

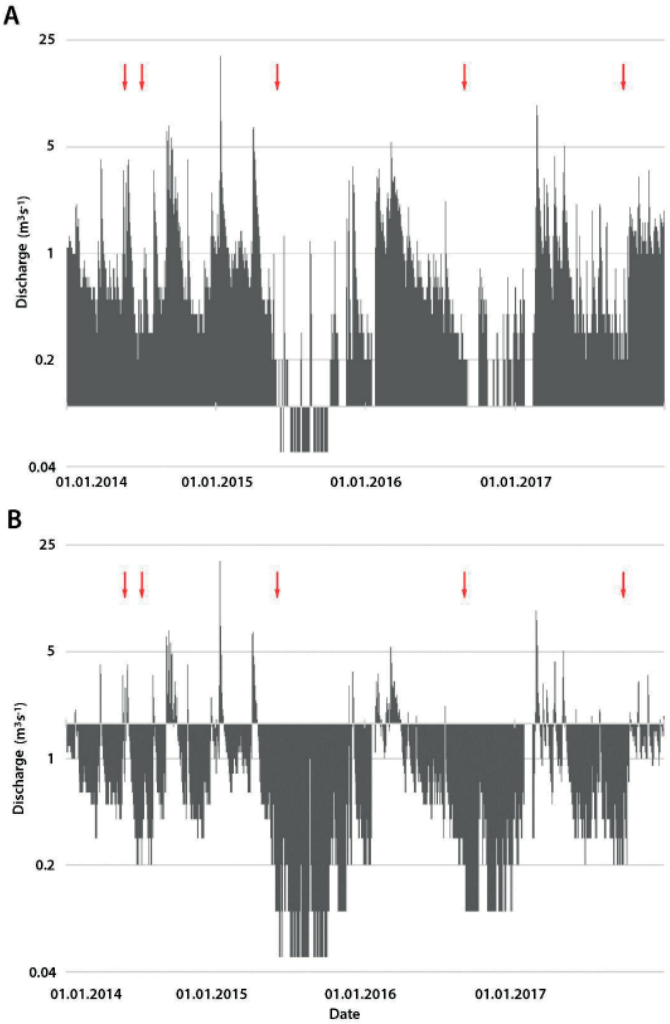

Figure S2. The hydrologic situation during the sampling campaign 2014–2017 (horizontal axes) in the Doubrava river (Spačice, Czech Republic; coordinates of the monitored profile: N 49.8174567, E 15.5949936E). The Doubrava river is a main second-order watercourse draining the area of the Doubravka brook. Very low discharge conditions between 2015 and 2017 demonstrate severe drought conditions in this catchment area. The vertical axes represent discharge ($m^3\ s^{-1}$) in a logarithmic scale (base set on 5). Red arrows point at the sampling date in the Doubravka brook (from left to right: 4th IV 2014—only sediments, 20th IV 2014—both sediment and invertebrates, 17th IV 2015—both sediment and invertebrates, 26th VII 2016 – sediment and invertebrates, and 19th IX – sediment and invertebrates, respectively). (**A**) Peaks are overturned around the baseline horizontal axis which represents dry conditions—Q_{355} ($m^3\ s^{-1}$)—discharge which is exceeded 355 times per year on average. (**B**) Peaks are overturned around the baseline horizontal axis representing a long-term mean discharge.

Figure S3. Photographic documentation of different hydrologic situations through the year 2016 at the Site C (control = unaffected, Transect 1). Dates are written in the lower right corner of each picture.

Figure S4. Photographic documentation of different hydrologic situations through the year 2016 at the Site 1 (affected, Transect 1). Dates are written in the lower right corner of each picture.

Figure S5. Photographic documentation of different hydrologic situations through the year 2016 at the Site 2 (affected, Transect 2). Dates are written in the lower right corner of each picture.

Table S6. Detected taxa and their semi-quantitative abundances detected in samples from Sites C (control = unaffected, Transect 1), 1 (affected, Transect 1), and 2 (affected, Transect 2) during the sampling period 2014–2017. Explanations for semi-quantitative counts: 'x' represents the range of 1–9 individuals, 'xx' represents the range of 10–99 individuals, 'xxx' represents the range of 100–999 individuals, and 'xxxx' represents the range of 1000–9999 individuals; empty cells represent no occurrence.

		2014			2015			2016			2017		
Deeper taxonomic level	**Identified taxon**	**C**	**1**	**2**	**C**	**1**	**2**	**C**	**1**	**2**	**C**	**1**	**2**
Arthropoda													
Insecta													
Diptera													
Chironomidae	Chironomidae gen. sp.	xxx	xxx	xxxx	xx						xxx	xx	xxx
Chironominae													
Chironomini	*Chironomus* sp.										xx		
	Microtendipes chloris gr.	x					xx				xx		
	Cryptochironomus sp.												xx
	Chironomus riparius gr.			xx									
	Paratendipes albimanus							xx	xx				
	Polypedilum convictum						xx						
	Polypedilum pedestre gr.							xx	xx				
	Dicrotendipes notatus									x			
Tanytarsini	*Micropsectra apposita* gr.							xxxx	xxx	xxxx			
	Micropsectra sp.	xx		xxx	xx	x	xx				xxx	xx	
Orthocladiinae	*Rheocricotopus effusus*				xx								

	Paratrissocladius excerptus				xx	xx							
	Orthocladius sp.	xx			xx	xx							
	Brillia bifida	xx	xx			xxx	xxx				xx	xx	
	Epoicocladius ephemerae				x								
	Corynoneura sp.			xx	x		x			xx	xxx		
	Tvetenia verralli	xx		xxx	xx	xxx							
	Paratrichocladius rufiventris			xxx			xxx						
Tanypodinae	*Procladius* (*Holotanypus*) sp.							xx	xxx	xxxx	xxx	x	xx
	Monopelopia tenuicalcar							xx	x				
	Ablabesmyia sp.										xx		
	Macropelopia nebulosa							xxx	xxx	xxxx			
	Tanypodinae gen. sp. (*Krenopelopia* sp. ?)	x											
	Thienemannimyia sp.			xx	xx	xx	xx			xxx	xxx	xx	xxx
Diamesinae	*Diamesa insignipes*				xx	xx	xxx						
	Diamesa tonsa				xx								
Prodiamesinae	*Prodiamesa olivacea*							xxx	xx	xx			
Tipulidae	*Tipula* sp.												xx
	Tipula lateralis				x	x	x						
	Tipula maxima	x									x	x	
Limoniidae	*Paradelphomyia* sp.												x
	Hexatoma sp.							xx	x				

Pediciidae	*Dicranota* sp.	xx											
Simuliidae	*Prosimulium hirtipes*			x									
	Simulium vernum		xx	x									
	Simulium angustipes				x	xx							
Ceratopogonidae	Ceratopogonidae gen. sp.	xx		xx		xxx				xx		xx	
	Atrichopogon sp.										x		
Empididae	*Chelifera* sp.			x			xx		x				
	Clinocera/Wiedemannia sp.			x	x	x							
Culicidae	*Anopheles* sp.						x						
Stratiomyidae	*Nemotelus* sp.											x	
Tabanidae	*Chrysops caecutiens*						x			x			
Psychodidae	*Pericoma* sp.						x						
Odonata													
Cordulegastridae	*Cordulegaster boltonii*						x	x		x			
Coleoptera													
Dytiscidae													
Colymbetinae	*Platambus maculatus* ad.						x	x	xx	xx			
	Platambus maculatus lv.						xx	xx		xxx	xx	xx	
	Agabus didymus ad.								xxx	x			
	Agabus didymus lv.									x			

	Ilybius fuliginosus ad.						xx			
Elmidae	*Elmis latreillei* ad.			x						
	Elmis sp. lv.	xx	xx	x	xx	x		xx	x	
	Riolus sp. lv.				x					
	Oulimnius sp. lv.							xxx	x	xxx
	Esolus sp. lv.		xx							
	Limnius volckmari lv.									x
Hydraenidae	*Hydraena gracilis* ad.		x					x	x	x
	Hydraena excisa ad.		x	x						
Scirtidae	*Elodes minuta* lv.							xxx		xx
Heteroptera										
Notonectidae	*Notonecta* sp.				x	xx				
	Notonecta maculata								x	
Corixidae	*Micronecta* sp.							xx		
Megaloptera										
Sialidae	*Sialis fuliginosa*				xx	x		xx		
Plecoptera										
Perlodidae	*Isoperla* sp.							xx		
Leuctridae	*Leuctra* sp.	x								

Taeniopterygidae	*Brachyptera risi*				xx	x							
Nemouridae	*Nemoura* sp.	x											
Trichoptera													
Limnephilidae	*Halesus digitatus*	xx	xx		xx	x							
	Potamophylax luctuosus	xx											
	Potamophylax latipennis				x			xx					
Beraeidae	*Beraeodes minutus*										xx		
Rhyacophilidae	*Rhyacophila nubile*	x	x		xx	x	x						
	Rhyacophila fasciata				xx	xx							
	Rhyacophila sp.			x									
Polycentropodidae	*Plectrocnemia conspersa*										x		
	Polycentropus flavomaculatus	x					x						
Leptoceridae	*Mystacides longicornis*										xx		
	Mystacides azurea							xx		xx			
	Athripsodes sp.											x	
Hydropsychidae	*Hydropsyche* sp.			x		xx					xx		
	Hydropsyche instabilis	xx	x		xx	x							
	Hydropsyche siltalai	x											
	Hydropsyche angustipennis						x						

Sericostomatidae	*Sericostoma* sp.			x	x								
Goeridae	*Silo nigricornis*									xxx	x	xx	
Psychomyiidae	*Lype reducta*						x						
Odontoceridae	*Odontocerum albicorne*			x									
Ephemeroptera													
Ephemerellidae	*Serratella ignita*	xx											
	Ephemerella mucronata	xxx											
Baetidae	*Centroptilum luteolum*						x			xx		x	
	Baetis sp.									xx			
	Baetis vernus				xx	xx							
	Baetis rhodani	xx		xx	xx								
Ephemeridae	*Ephemera danica*	xx	x	xx	x		x			xx			
Leptohlebiidae	*Habrophlebia lauta*	xx											
	Habrophlebia fusca			x						xx	x	xx	
Heptageniidae	*Electrogena* sp.						x						
	Ecdyonurus torrentis			x									
	Ecdyonurus sp.	xxx	x				x			x			
Crustacea	Ostracoda						xxx	xxx	xx	xxx	xx		

Isopoda	*Asellus aquaticus*					xx	xxx	xx	xx	xxx	xxx
Mollusca											
Bivalvia											
Sphaeriidae	*Pisidium personatum*				x				xxx	xx	xxx
	Pisidium casertanum					xx	xx	xx			
Gastropoda											
Pulmonata											
Planorbidae	*Ancylus fluviatilis*	x	xx	x	xx	xx					
	Gyraulus albus								x		xx
	Gyraulus crista							x	x		x
	Planorbarius corneus							x			
Lymnaeidae	*Lymnaea* (*Radix*) *peregra*	x	x			x			xx		
	Lymnaea (*Galba*) *truncatula*								x		
Allogastropoda											
Valvatidae	*Valvata piscinalis*								xx	x	
Annelida											
Hirudinea											

Erpobdellidae	*Erpobdella octoculata*					x			xx	x		x	x
	Erpobdella vilnensis							x					
Glossiphonidae	*Glossiphonia complanata*							x		x			x
Oligochaeta													
Naididae	*Nais elinguis*				xxx	xxx	xxxx						xxx
	Nais communis		xx	xxx				xxx	xxx	xx			
	Slavina appendiculata										xx	xxx	
Tubificidae	Tubificidae gen. sp.								xxx				
	Pristina aequiseta			xxx									
Enchytraeidae	Enchytraeidae gen. sp.			x					xx				
Lumbriculidae	*Lumbriculus variegatus*				x		xx		xx	x	xx	x	xx
	Stylodrilus sp.						xxxx				xx	xx	xx
	Stylodrilus brachystylus				x		xx						
	Stylodrilus heringianus							xx					
Lumbricidae	*Eiseniella tetraedra*	x	x		x		x						
Platyhelminthes													
Planariidae	*Polycelis nigra*								xxx			x	

CHAPTER 3

EFFECTS OF TRACE METALS AND MUNICIPAL WASTEWATER ON THE EPHEMEROPTERA, PLECOPTERA, AND TRICHOPTERA OF A STREAM COMMUNITY

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My share on this work was about 70%

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Effects of Trace Metals and Municipal Wastewater on the Ephemeroptera, Plecoptera, and Trichoptera of a Stream Community

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Simple Summary: Mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera) (EPT) are aquatic insects that are well known to the general public and are commonly used as indicators of environmental quality in water management. Knowledge of how EPT communities react to human-induced gradients in real environments can be important, for example, during the assessment of the implications of newly planned or currently active human disturbances for natural or cultural landscapes. We sampled a stream ecosystem affected by mining and smelting industries and communal wastewaters with pronounced concentrations of cadmium, lead, and zinc, as well as high levels of pesticides, pharmaceuticals, illegal drugs, and sewage-derived organic matter. Changes in other environmental factors such as increases in temperature were also studied at the affected sites. The abundance and species richness of stoneflies fell rapidly at the study sites. The richness of mayfly families also declined, from four to one, even though overall mayfly abundance was not affected. Conversely, the abundances of caddisflies were higher at the affected sites, and their richness did not decrease. This study will provide feedback for ecotoxicologists who perform better controlled and manipulated tests in laboratories, although any such test results are limited by simple artificial environments.

Abstract: Abundances of EPT larvae sampled in a Central European locality affected by mining and smelting, as well as by the continual inflow of treated communal wastewaters (WWs), were recorded. High concentrations of trace metals in water (maximum 1200 $\mu g \cdot L^{-1}$ for zinc) and sediments (maximum 140,000 $mg \cdot kg^{-1}$ in dry weight for lead) were found at the most contaminated sites. The highest loads of pesticides, pharmaceuticals, and illegal drugs were found under the WW effluent. Other associated factors such as the physicochemical parameters of the water and alterations to microhabitats were also evaluated and taken into account. Although EPT richness was lower at affected sites, abundances did not fall. Stoneflies were dominant at unaffected sites, while caddisflies dominated at affected sites. Only baetid mayflies were detected at the sites contaminated by trace metals and WWs; ephemerellid, heptageniid, and leptophlebiid mayflies were absent from these sites. The site contaminated by trace metals was also inhabited by numerous limnephilid caddisflies, in which limb malformations were detected in up to 11.8% of all specimens of a single taxon. Downstream from the entrance of the WWs, the locality was dominated by hydropsychid caddisflies. The increasing prevalence of predator or passive filter-feeding strategies in these EPT communities was significantly related to increasing water conductivity and acute ecosystemic exposure to 'poorly treated' WWs.

Keywords: anthropogenic disturbances; aquatic insect; environmental gradients; heavy metals; industrial pollution; wastewater treatment plant

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1. Introduction

The aquatic insect orders of mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera) (henceforth, EPT) are frequently used indicators of environmental quality, particularly in running waters [1,2]. Their larvae dominate in unpolluted headwaters where proportions of fine sediment deposits are low and are essential for correct nutrient flow cycling in these environments [3–5]. The contribution of EPT taxa adapted to conditions in downstream ecosystems (lower rhithral and potamal zones) is likewise important, although populations of these specialists, namely certain species of mayflies and stoneflies, are prone to be decimated by the cumulative effects of intensive human activities in lowland areas [6–8].

Given their rapid expansion through the natural environment, the ecological impact of human-induced gradients needs to be monitored, and possible threats are objectively predicted. The contamination of stream ecosystems by toxicants is a global issue; nevertheless, engineering work severely modifies the morphology of whole catchment areas and causes substantial changes in hydrological regimes, habitat structure, and water quality [8–10]. Hence, communities in running waters in cultural landscapes are habitually subjected to multiple anthropogenic factors [11]. Therefore, the manipulative testing of stressors within controlled conditions is now seen as increasingly important. Despite this, natural experiments (observational studies) are still needed to reflect the validity of derived results and vice versa [12].

The experiment described in this work explored an EPT community in a temperate zone in the European Central Highlands ecoregion [13], specifically, in a catchment area severely affected by industrial pollution (trace metals) and treated municipal wastewaters (WWs) whose levels are relatively high in terms of current European standards [14,15]. Measured confounding environmental variables (EVs)—concentrations of pesticides, physicochemical parameters of water, choriotopic composition, and current velocity—were included in the analyses of the changes in community composition. The shift in artificially/hierarchically assigned values for feeding strategies in the EPT community along with measured gradients of the EVs was also tested.

The contamination of streams by trace metals (often generalised as 'heavy metals') is a global phenomenon, and many studies tackling the responses of macroinvertebrate communities have identified mining as the cause of this type of pollution [16–19]. Increased concentrations of cadmium, copper, lead, and zinc—the so-called 'bivalent metals' that bind to both sulphur/nitrogen and oxygen groups—are most often reported as the cause of shifts in stream macroinvertebrate compositions in real environments or as the potential origin of impaired bottom-up control in ecosystems [20,21]. It is easy to misinterpret an oversimple comparison of concentrations measured in a real environment with the lethal values estimated for several EPT taxa under controlled conditions since many contradictions between observational studies and laboratory or mesocosm experiments exist in assessments of the toxicity of trace metals for aquatic insects [22].

Effluent from wastewater treatment plants (WWTPs) often represents an 'entrance gate' for many pollutants, including sewage-derived particulate organic matter (SDPOM), nutrients, pesticides, active pharmaceutical compounds (PhACs), synthetic organic compounds, phthalates, and trace metals into streams [14,23–27]. Over the past two decades, there has been a general decrease in organic matter and nutrient content in outputs from modernised WWTPs in the European Union [28]. Nonetheless, the continual release of compounds that are resistant to conventional purification technologies from WWTPs still occurs [29,30]. In addition, uncontrolled outflows of raw sewage from WWTPs and sewer infrastructure, especially during storm events, persist and represent a further challenge to the protection of natural water resources [31].

This article attempts to decipher the relationships between these human-induced gradients and EPT taxa and their feeding strategies which could be beneficial and permeable in such an impaired environment. It provides a list of taxa that can be considered tolerant to particular types of pollution, and the possible susceptibility of missing taxa is compared

and discussed with previous reports. Since EPT taxa usually represent the essential part of macroinvertebrates in streams [3–5] and the shift in feeding strategies may reflect the change in available food resources, the article also provides partial evidence about the human-induced changes in the functioning of lotic ecosystems, namely, in nutrient cycling and energy flow [32].

2. Materials and Methods

2.1. Locality Description

The two surveyed localities—(i) Obecnický brook and (ii) Litavka river—are situated in central Bohemia (Czech Republic, Central Europe; spring to confluence: (i) N 49.7186939, E 13.8732253–N 49.7083778, E 13.9831206 and (ii) N 49.6563228, E 13.8557881–N 49.9602636, E 14.0847414; Figure 1). EPT larvae were sampled at four sites:

(i) Site 1 was a stretch of the Obecnický brook in the Brdy highlands above the Obecnice reservoir (third-order watercourse according to Strahler's system; N 49.7181961, E 13.9110308–N 49.7176411, E 13.9214808; Figures 1 and S1). This site was assumed to be the least affected by human activities, although forestry management has taken place in the area for many years, and there was once here a military firing range. Although increased acidity in this catchment area due to high emissions of sulphur and nitrogen compounds has been reported in the past, this area seems to have partially recovered [33]. The catchment area consists of spruce plantations, forest-free artificial moorlands (the former artillery range), and peat bogs. The brook's water has very low turbidity, and its rusty brown colour is likely to be caused by humic substances. By comparison, a few spring-fed tributaries had transparent water (an abundant population of acid-sensitive gammarids was observed in one unnamed tributary). Mature spruces grow along the brook edges, along with a few young alders. Particulate organic matter consists mainly of spruce needles, cones, and twigs, as well as leaf litter from the alders, birches, and old unharvested beeches. The brook bottom is densely covered in places by mosses (e.g., *Fontinalis antipyretica*) and species of Marchantiophyta. Despite not monitoring fish populations by electrofishing, brown trout (*Salmo trutta*) were observed during the macrozoobenthic sampling (Table S9). Fišer et al. [34] reported the presence of the brook lamprey (*Lampetra planeri*), brook trout (*Salvelinus fontinalis*), and European perch (*Perca fluviatilis*) at this site four years before our study began. Insects were prevalent in the macrozoobenthic samples, although only a few small oligochaetes, crustaceans, and molluscs were found;

(ii) Site 2 consists of a stretch of the Obecnický brook below the Obecnice reservoir (third-order watercourse according to Strahler's system; N 49.7161703, E 13.9312433–N 49.7161931, E 13.9346444; Figures 1 and S1). Discharge here was usually lower than at Site 1 because of continuous water abstraction from the reservoir. Water retention and discharge manipulation also influence the colour and turbidity, which were both different from those observed at Site 1 (from dark brown with greater turbidity to crystal clear). Site 2 is surrounded by forest where little management occurs, and the brook edges are lined by old alders in the lower part, which increases the site's heterogeneity by forming wide pools or dividing the brook into several branches (the gradient was also approximately one quarter less). Proportionally more leaf litter from deciduous trees was observed here than at Site 1. The brook bottom is covered in places by mosses (*F. antipyretica*). Fish stocks consist of brown trout, brook lamprey, and stone loach (*Barbatula barbatula*) (in decreasing order of abundance; Table S9). Insects prevailed in the macrozoobenthic samples, although small oligochaetes, crustaceans (*Gammarus* sp.), and molluscs (*Pisidium* sp. and *Ancylus fluviatilis*) were relatively abundant;

(iii) Site 3 was on a stretch of the Litavka river below its confluence with the Obecnický brook (fourth-order watercourse according to Strahler's system; from N 49.7100606, E 13.9884817–N 49.7112883, E 13.9961850; Figures 1 and S2). This site has been heavily affected by the local industrial activity (mining, smelting and processing of silver,

iron, lead, zinc, and uranium) that has been active for several centuries [15]. An active industrial complex that recycles, above all, lead waste occupies part of the river's alluvial plain. Heaps of waste material (especially sodium slag) are still stored several meters from the riverbank. Nevertheless, all mining activity has ceased. Here, the effects of agricultural disturbance are also likely to occur as arable fields cover approximately half of the deforested catchment area (excluding human settlements). The rest of the catchment is covered by pastures and meadows. The effect of the municipal WWs is presumably low because of a relatively small human population upstream. The riparian vegetation has been substantially reduced upstream along the Litavka river (third-order watercourse according to Strahler's system), which has been channelled in places between artificial banks. In addition, several shallow reservoirs (up to 7 ha), including some small sludge deposits, are situated upstream. We sampled the upper channelled parts with old reinforced banks and little riparian vegetation, as well as a lower unchannelled, naturally heterogenic stretch (morphologically similar to those described for Site 2) with banks lined with old alders and willows (Figure 1). The fish stocks here consist of common minnow (*Phoxinus phoxinus*), brown trout, and European perch (in descending order of abundance; Table S9). Insects prevailed in the macrozoobenthic samples, and small oligochaetes were relatively abundant, but crustaceans and molluscs were almost totally absent from the macrozoobenthic samples;

(iv) Site 4 was a stretch of the Litavka river downstream from its confluence with the Příbramský brook (fourth-order watercourse according to Strahler's system; from N 49.7113508, E 14.0093011–N 49.7198211, E 14.0133511; Figures 1 and S2). This site was expected to be the most affected by anthropogenic activities because of the combination of industrial pollution from mining and smelting and contamination by treated municipal WWs from the town of Příbram. Treated WWs are continually pumped into the Příbramský brook approximately 900 m upstream from its confluence with the Litavka river. According to Grabicova et al. [14], Site 2 has the greatest concentrations of psychoactive PhACs of all the inspected localities, probably because of the low dilution possibilities (i.e., a small watercourse receiving relatively large amounts of municipal WWs). As at Site 3, we sampled an upper channelled stretch with artificial banks and little riparian vegetation, as well as a lower unchanneled section. Nonetheless, this morphologically heterogenic stretch only had a narrow and irregular fringe of riparian trees (alders and willows), possibly because of the dynamic migration of the river's course across its alluvial plain [15]. The fish stock consists of common minnow, common roach (*Rutilus rutilus*), chub (*Squalius cephalus*), gudgeon (*Gobio gobio*), brown trout, European perch, common bream (*Abramis brama*), common rudd (*Scardinius erythrophthalmus*), stone loach, and rainbow trout (*Oncorhynchus mykiss*) (in descending order of abundance; Table S9). The occurrence of limnophilic fish was due to the presence of aquaculture ponds upstream. There was no clear dominance by insects in the macrozoobenthic samples since small oligochaetes, leeches (e.g., *Erpobdella octoculata*, *Helobdella stagnalis*), and the water louse (*Asellus aquaticus*) were very abundant. Molluscs were detected in low numbers.

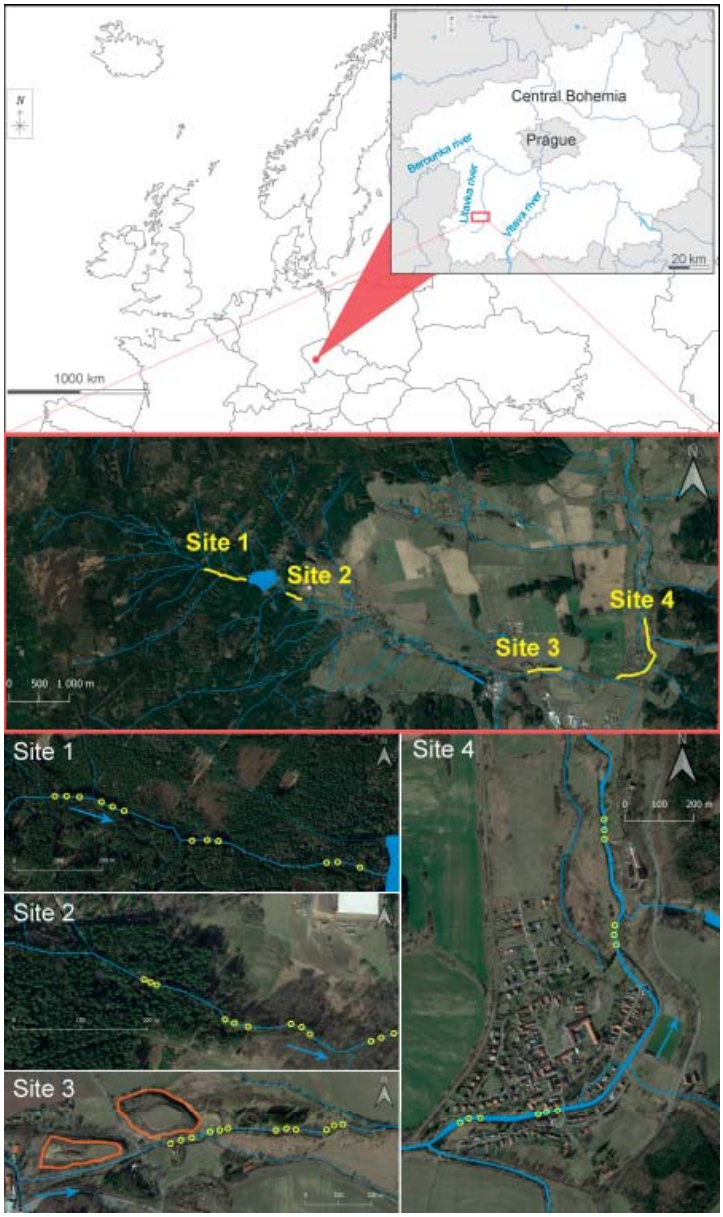

Figure 1. Map of the study area. The yellow circles indicate the sampling points. A set of three sampling points represents a whole plot. The contoured orange-coloured areas in the close-up view of Site 3 (lower left-hand corner) are slag heaps and waste material generated by the smelting industry. The river's flow direction is indicated by blue arrows.

2.2. Sampling and Analysis of Samples

The whole sampling campaign was conducted in 2020, specifically on 11 May, 24 June, 11 August, and 24 September. The macrozoobenthos were sampled using a Surber sampler (30 × 30-cm frame, 500-µm mesh) at four sampling sites and three points (triplicates) at each site (see part 2.1. 'Locality description' and Figure 1). Each triplicate consisted of three subplots within a whole plot or site. The location of the whole plot was chosen at random during each sampling campaign. A diversification of samples within each whole plot was made by sampling riffles (shallow and with high water velocity), inflows into pools (deep and with high water velocity) and the backs of pools (deep and with low water velocity). Samples were processed using a round steel sieve (40 cm diameter, 500 µm mesh) and preserved in 70% technical ethanol. Organisms were sorted in the laboratory from the material taken from the stream bottom. Mayfly, stonefly, and caddisfly larvae were determined to the deepest possible taxonomic level using a binocular microscope and stereomicroscope (Olympus SZX16) and determination keys; their frequencies (abundances) in the samples were recorded. The methodology used to analyse the water samples for pesticides, PACs and drugs, and the water and sediment samples for trace metals is described in detail in the Supplementary Materials (Section Materials and Methods).

Environmental data were taken for both the whole and subplot levels. The environmental variables (EVs) differing only at the whole-plot level (hereafter referred to as 'whole-plot EVs') consisted of (i) physicochemical parameters—conductivity, pH, oxygen concentrations, and water temperature—measured using a HACH® multi-meter, (ii) pesticide and PhAC concentrations in water (measured from samples taken on 11 August 2020), (iii) cadmium, lead, and zinc concentrations measured in sediments and water (measured from samples taken on 24 September 2020), and (iv) daily volumes of sewage (untreated or 'poorly treated' municipal WWs) recorded up to 15 days prior to each sampling day at the Příbram WWTP (data provided by 1. SčV JSC, Příbram, Czech Republic). Detailed monthly data of water and sediment analyses from Sites 1, 2, and 3 (2009–2020) and data of actual water discharge from the same sites were provided by the Czech Hydrometeorological Institute (Prague, Czech Republic) and Povodí Vltavy, State Enterprise (Prague, Czech Republic). In addition, the water temperature was continuously monitored during the sampling campaign by four data loggers (TFA) placed approximately in the middle of each site.

The EVs also differed at the subplot level (hereafter referred to as 'subplot EVs') in terms of their (i) choriotopic compositions, which were empirically estimated for each Surber sampling spot; (ii) current velocities [$m \cdot s^{-1}$] (minimum, maximum and average), measured continuously for 0.5 min above the Surber sampling spot with a flowmeter (MiniController MC20 with the Flowprobe for MiniWater20, Schiltknecht Messtechnik AG, Gossau, Switzerland) placed close to the bottom, in the water column and close to the water surface; (iii) widths and depths at each subplot; and (iv) classification as riffles, pools, inflows into pools, or backs of pools.

2.3. Statistical Analyses

Data were analysed using Canoco 5 version 5.12 (written by Cajo J.F. ter Braak and Petr Šmilauer; Wageningen, the Netherlands, and P. Šmilauer, Czech Republic) [35] and R-studio version 4.1.1 (written by RStudio team; Boston, MA, United States) [36] software. The analysis of shifts in feeding strategies was based on the preferences of individual species according to Graf et al. [37], Buffagni et al. [38], and Graf et al. [39]. The analyses with species community composition as response variables were conducted using unimodal Canonical correspondence analysis (CCA), while analyses with feeding strategies as response variables were performed using linear Redundancy analysis (RDA). Species abundances were log + 1 transformed before the CCA. Detrending was not used because no dependency between positions of samples on the first or second ordination axes was observed. Feeding strategies were averaged by species composition-weighted standardisa-

tion (hereafter referred to as 'averaged feeding strategies'). Only centring by species was applied using RDA.

The significances of axes constrained by (i) site identity, (ii) the identity of sampling times, (iii) whole-plot EVs, and (iv) subplot EVs (see part 2.2. 'Sampling and sample analysis') were tested using Monte-Carlo permutation tests (999 permutations used). Permutations were carried out according to the experiment's hierarchical design: (i) when the whole-plot EVs or the identity of sites were used as explanatory variables, both whole-plots and subplots in the four blocks defined by the four sampling times used as covariates were permuted using cyclic shifts; (ii) when the subplot EVs were used as explanatory variables, only the subplots in the four blocks defined by sampling times were permuted using cyclic shifts; and (iii) when the identity of sampling times was used as an explanatory variable, both whole plots and subplots in the four blocks defined by the four sites were permuted using cyclic shifts. A false discovery rate was used for adjusting *p* values. Forward selection (FS) was used for selecting significant whole- and subplot EVs. 'Hybrid analysis' was chosen when tests on visualised constrained axes were not significant ($p > 0.05$). The significance of differences in choriotopic compositions between sites was tested in a similar way; individual values were transformed by the angular (inverse sine) function and transformed by using them as a response (and even explanatory variables).

Generalised linear models (GLMs) were employed to model trends in shifts of multiple averaged feeding strategies along environmental gradients or along the resulting ordination axes. The significance of differences between sites and sampling times in EPT abundances and richness was tested using GLMs with negative binomial and Poisson distributions, respectively (the quasi-likelihood estimation method was applied in the case of the Poisson distributions). The significance of improvements using Generalised linear mixed-effects models (GLMMs) rather than GLMs was tested by likelihood ratio tests. A post hoc test with Tukey's method for *p* value adjustment was applied.

3. Results

3.1. Environmental Conditions

The differences between sites are summarised in Table 1. Several environmental gradients, including increasing temperature, pH, and conductivity and decreasing oxygen concentrations, changed along with the longitudinal downstream profile (from Site 1 to Site 4). For more information about the basic physicochemical parameters at other sites in the longitudinal profile, see Supplementary Materials (Section Materials and Methods; Table S1). The greatest total concentration of cadmium, zinc, and lead in the waters was detected at Site 3, followed by Site 4, while the highest loads of pesticides and PhACs were found at Site 4 below the discharge of effluent from the WWTP (Table 1). Treated WWs constituted 1/13–1/3 of all discharges at Site 4 (Table S2). The periodical releasing of untreated or 'poorly treated' WWs containing organic material and coarse communal waste (mainly hygienic products) also occurred. The monthly released volumes of this sewage ranged from 299 to 130,016 m^3 in 2020 (Table 2); however, considerable volumes also leaked from sewers upstream from the WWTP but were not included in the analyses. For more information about contamination at other sites along the longitudinal profile, see Supplementary Materials (Section Materials and Methods; Tables S3–S6).

The average current velocities at individual sites were as follows: Site 4 >Site 3 > Site 1 > Site 2 (in descending order from the fastest to the slowest; Table 3). Differences in the choriotopic compositions at sites tested using a RDA were marginal (test on first axis: pseudo-$F = 1.2$, $p = 0.085$; test on all axes: pseudo-$F = 2.8$, $p = 0.003$; Figure S4); only Site 4 and Site 2 were significantly different from the others when tested with the RDA (explained variation = 7.5%, pseudo-$F = 3.8$, p (adj.) = 0.025 and explained variation = 6.0%, pseudo-$F = 2.9$, p (adj.) = 0.049, respectively). Samples from Site 1 compared with those from Site 2 had a much greater proportion of smaller mineral particles (size < 6.3 cm—'microlithal', 'akal', and 'psammal') and 'mosses' (Marchantiophyta only at the Site 1), and a lower proportion of light deposits ('coarse particulate organic matter'—CPOM and

'fine particulate organic matter'—FPOM), debris and xylal (Table 4). Samples from Site 3 resembled most those from Site 1, with similar proportions of smaller mineral particles, deposits, debris, and xylal (Table 4). Finally, Site 4 differed most from the others because of the highest proportions of deposits and debris and the occurrence of filamentous algae and anthropogenic trash (especially wet wipes; Table 4).

Table 1. Resulted mean physicochemical parameters measured at the given sites and concentrations of metals, pesticides, and active pharmaceutical compounds (PhACs) in water samples taken at these sites.

	Site 1	**Site 2**	**Site 3**	**Site 4**
	n = 4 Mean ± SEM	**n = 4 Mean ± SEM**	**n = 4 Mean ± SEM**	**n = 4 Mean ± SEM**
Temperature (°C)	12.05 ± 0.34	13.33 ± 0.61	17.05 ± 1.11	16.80 ± 0.68
pH	7.09 ± 0.24	7.35 ± 0.17	7.45 ± 0.17	7.45 ± 0.09
Oxygen concentration (mg·L^{-1})	9.90 ± 0.10	9.02 ± 0.18	8.91 ± 0.16	7.61 ± 0.27
Conductivity (µS·cm^{-1})	71.40 ± 5.80	84.35 ± 5.98	390.60 ± 39.18	571.00 ± 56.74
Concentrations in Water Samples				
Cadmium (µg·L^{-1})	0.26	0.23	7.90	3.80
Lead (µg·L^{-1})	5.80	9.80	64.00	53.00
Zinc (µg·L^{-1})	15.00	13.00	1200.00	530.00
Sum pesticides (ng·L^{-1})	132.00	70.32	462.90	877.87 ± 125.71 (n = 3)
Sum PhACs (ng·L^{-1})	15.17	13.40	874.58	4636.73 ± 254.79 (n = 3)

Table 2. Monthly volumes of untreated or 'poorly treated' wastewater released from the wastewater treatment plant into Site 4 in 2019 and 2020.

	Volume [m^3]	
	2019	**2020**
January	164,799	1522
February	80,750	55,224
March	58,862	35,861
April	183	3239
May	26,930	24,749
June	34,483	130,016
July	13,340	11,305
August	26,333	22,382
September	817	12,064
October	4967	67,559
November	3270	5941
December	71	299
Total	**414,805**	**370,161**

Table 3. Resulted mean current velocities measured for the macrozoobenthic samples from each site.

	Site 1	**Site 2**	**Site 3**	**Site 4**
Velocity (m·s^{-1})	**n = 12 Mean ± SEM**	**n = 12 Mean ± SEM**	**n = 12 Mean ± SEM**	**n = 9 Mean ± SEM**
Average	0.19 ± 0.04	0.14 ± 0.03	0.27 ± 0.04	0.29 ± 0.05
Maximum	0.44 ± 0.09	0.32 ± 0.06	0.70 ± 0.14	0.62 ± 0.10
Minimum	0.04 ± 0.04	0.02 ± 0.03	0.04 ± 0.04	0.06 ± 0.05

Table 4. Resulted mean choriotopic percentual compositions empirically estimated for each macrozoobenthic sample at all given sites.

	Site 1	Site 2	Site 3	Site 4
Choriotopic Parameter	**n = 12 Mean ± SEM (%)**	**n = 12 Mean ± SEM (%)**	**n = 12 Mean ± SEM (%)**	**n = 12 Mean ± SEM (%)**
Megalithal Large cobbles, boulders and blocks, bedrock; >40 cm	5.75 ± 2.63	18.29 ± 7.87	6.08 ± 2.16	18.50 ± 7.30
Macrolithal Coarse blocks, cobbles, gravel and sand; 20–40 cm	29.92 ± 6.88	17.38 ± 5.28	19.10 ± 4.62	12.60 ± 2.95
Mesolithal Fist to hand-sized cobbles; 6.3–20.0 cm	16.58 ± 3.75	22.29 ± 3.37	28.50 ± 2.41	19.70 ± 3.48
Microlithal Coarse gravel; 2.0–6.3 cm	11.08 ± 1.89	8.67 ± 2.23	18.30 ± 3.29	11.20 ± 0.99
Akal Fine to medium-sized gravel; 0.2–2.0 cm	15.50 ± 3.32	5.21 ± 0.93	11.60 ± 1.97	11.90 ± 2.06
Psammal Sand; <0.2 cm	3.88 ± 1.12	1.21 ± 0.58	3.00 ± 1.04	4.50 ± 2.00
Xylal Deadwood, cones	4.79 ± 1.33	10.38 ± 2.71	2.04 ± 0.82	1.63 ± 0.80
Coarse particulate organic matter CPOM; coarse deposits, e.g., leaves	1.83 ± 0.82	2.04 ± 0.81	0.88 ± 0.26	3.42 ± 1.38
Fine particulate organic matter FPOM; fine deposits	0.75 ± 0.41	3.96 ± 1.54	1.25 ± 0.62	4.25 ± 1.53
Debris Hard and coarse matter—organic or inorganic	2.67 ± 0.77	6.63 ± 2.04	3.13 ± 0.57	4.63 ± 1.10
Mosses *Fontinalis antipyretica* and Marchantiophyta at the Site 1	6.25 ± 3.27	2.71 ± 1.34	1.58 ± 1.21	2.13 ± 0.83
Filamentous algae	0	0	0	2.33 ± 1.37
Roots of riparian trees *Alnus* sp., *Salix* sp. or conifers	0.58 ± 0.42	1.25 ± 0.86	3.33 ± 1.70	0
Submerged riparian vegetation	0.42 ± 0.40	0	1.25 ± 0.63	0.83 ± 0.54
'Wet wipes' Anthropogenic trash	0	0	0	1.38 ± 0.61
'Metal sheets' Anthropogenic trash	0	0	0	1.17 ± 1.12

3.2. EPT Abundance and Richness

In total, 87 EPT taxa were detected in the samples: 11 mayflies, 29 stoneflies, and 47 caddisflies (Table S7). EPT abundances were not substantially different between sites (Figure 2A; Table 5) or sampling times (Table 5). Nevertheless, total EPT richness estimated for individual sites did show a gradual decreasing and significant trend from Site 1 to Site 4 (Figure 2B; Table 5). Sampling time had a nonsignificant effect on EPT richness (Table 5).

The greatest differences between sites in terms of abundance or richness were observed in stoneflies followed by mayflies (Table 5), both of which were impaired at disturbed Sites 3 and 4 (Figure 2C–E). Conversely, caddisflies were more abundant at disturbed Sites 3 and 4 (Figure 2C), although caddisfly richness and family richness did not significantly differ between sites (Figure 2D,E; Table 5). Significant differences between the

four sampling times in abundance or richness were detected in mayflies and stoneflies but not in caddisflies (Table 5). The abundance of mayfly nymphs was lowest in August and highest in May, whilst the opposite was found in stoneflies: the highest stonefly abundance was detected in August and the lowest in May.

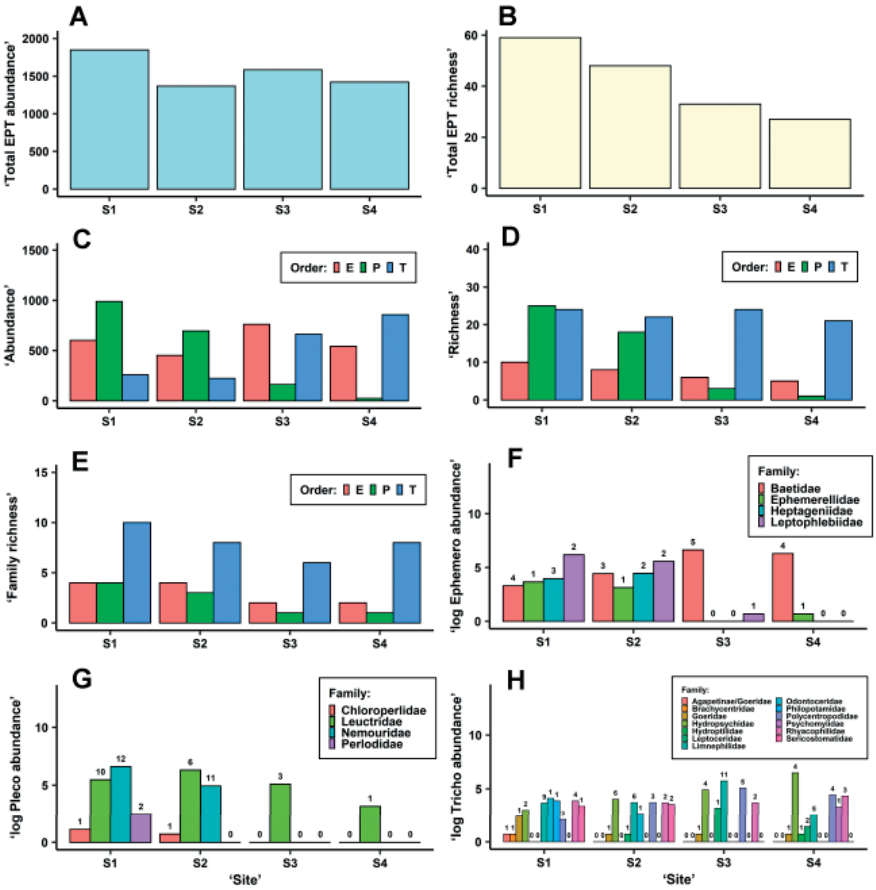

Figure 2. Bar graphs visualising (**A**,**C**) 'abundance' (number of individuals), (**B**,**D**) 'richness' (number of taxa), (**E**) 'family richness' (number of families), and (**F**–**H**) abundances and richness of individual detected families of Ephemeroptera, Plecoptera, and Trichoptera, respectively, summarised for each 'site' from the samples taken at all sampling times. Values in the bar graphs (**F**–**H**) are transformed by a natural logarithm, and the 'richness' is displayed above each column. E—Ephemeroptera, P—Plecoptera, T—Trichoptera.

The stonefly and caddisfly orders significantly differed in abundance between sites (Table 5). Stonefly abundances rapidly declined at disturbed Sites 3 and 4 compared with Sites 1 and 2, whilst caddisfly abundances were lower at Sites 1 and 2 (Figure 2C). Mayfly abundance did not significantly differ between sites (Figure 2C, Table 5) but marginally differed between sampling times (Table 5). No significant interaction was detected between the four-level factor site and sampling time.

Table 5. Results of testing the significance of differences within factors site and sampling time (both four-level factors) in given response variables using Generalised linear models (GLMs) or Generalised linear mixed-effects models (GLMMs). Significant ($p < 0.05$) results are in bold text. No significant interaction between site and sampling time was detected for any response variable ($p > 0.05$). E—Ephemeroptera, P—Plecoptera, and T—Trichoptera.

	Factors	
Response Variable	**Site**	**Sampling Time**
EPT abundance	$\chi^2_3 = 1.19$, $p = 0.76$	$\chi^2_3 = 3.96$, $p = 0.27$
EPT richness	$\mathbf{F_{3,44} = 2.82, p = 0.049}$	$F_{3,41} = 1.14$, $p = 0.34$
E abundance	$\chi^2_3 = 1.22$, $p = 0.75$	$\chi^2_3 = 7.56$, $p = 0.055$
E richness	$F_{3,44} = 0.14$, $p = 0.93$	$\mathbf{F_{3,44} = 3.19, p = 0.03}$
P abundance	$\mathbf{\chi^2_3 = 56.33, p < 0.001}$	$\mathbf{\chi^2_3 = 9.70, p = 0.02}$
P richness	$\mathbf{\chi^2_3 = 124.03, p < 0.001}$	$\mathbf{\chi^2_3 = 8.14, p = 0.04}$
T abundance	$\mathbf{\chi^2_3 = 12.59, p = 0.006}$	$\chi^2_3 = 6.11$, $p = 0.11$
T richness	$F_{3,44} = 1.03$, $p = 0.39$	$F_{3,44} = 0.43$, $p = 0.73$
E family richness	$\mathbf{F_{3,41} = 6.83, p < 0.001}$	$F_{3,41} = 2.48$, $p = 0.074$
P family richness	$\mathbf{F_{3,44} = 23.70, p < 0.001}$	$F_{3,41} = 1.02$, $p = 0.39$
T family richness	$F_{3,44} = 0.47$, $p = 0.70$	$F_{3,44} = 0.23$, $p = 0.88$

The total abundances and species richness shown in Figure 2F,G indicate that practically only members of the Baetidae family (namely *Baetis fuscatus*, *B. rhodani*, *B.* aff. *scambus*, and *B. vernus*) and the Leuctridae family (stoneflies) were detected at disturbed Sites 3 and 4. At these sites, *Habrophlebia lauta* (Leptophlebidae) and *Seratella ignita* (Ephemerellidae) all but completely disappeared, while *Paraleptophlebia submarginata* (Leptophlebiidae), *Ecdyonurus torrentis*, and members of the genus *Rhithrogena* (both Heptageniidae) were totally absent, although these latter taxa were detected upstream at the interconnected Sites 1 and 3 (Figure 2F, Table S7). Of the stoneflies, only *Leuctra albida*, *L. fusca*, and *L. geniculata* were detected at Site 3 (*L. geniculata* was only detected at this site, Table S7) and only *L. fusca* in low abundances and body sizes was detected at Site 4 (Figure 2G, Table S7). *Siphonoperla torrentium* (Chloroperlidae), several species belonging to the genera *Protonemura*, *Amphinemura*, and *Nemoura* (Nemouridae), all very abundant at Sites 1 and 2, along with *Diura bicaudata* and *Perlodes* aff. *microcephalus* (Perlodidae) were completely absent in the samples taken from disturbed Sites 3 and 4 (Figure 2G, Table S7). Nevertheless, compared with Site 1, the richness of Leuctridae decreased also at Site 2 below the water reservoir (Figure 2G), and, for instance, *Leuctra nigra*, frequent in samples from Site 1, was missing from samples from Site 2 (Table S7).

Of the caddisflies, Site 1 had a greater richness of trichopterans than Site 2; the abundant family Philopotamidae, for example, was no more detected at Sites 2, 3, and 4; finally, members of the Goeridae were detected only sporadically at particular sites (Figure 2H). On the other hand, the richness of Hydropsychidae was lowest at Site 1. *Odontocerum albicorne* (Odontoceridae) and *Sericostoma flavicorne/personatum* (Sericostomatidae), very abundant at both Sites 1 and 2, were completely missing from the samples from Sites 3 and 4 (Figure 2H, Table S7). One of the dominant families at Site 3 was Limnephilidae, represented above all by species from the genera *Potamophylax*, *Halesus*, and *Chaetopteryx* (in descending order of total abundance; Figure 2H, Table S7). One of the dominant families at Site 4 below the WWTP was Hydropsychidae, represented by *Hydropsyche siltalai*, *H. angustipennis*, and *H. incognita/pellucidula* (in descending order of total abundance; Figure 2H, Table S7); the population of limnephilids at Site 4 was substantially lower (Figure 2H). At both disturbed Sites 3 and 4, the family Polycentropodidae was much more abundant than at Sites 1 and 2 (Figure 2H). A substantially greater proportion in the total abundances of *Cyrnus trimaculatus* than *Polycentropus flavomaculatus* was observed at Site 4 than at Site 3 (2.54 and 0.03, respectively; Table S7). The Leptoceridae represented by *Athripsodes bilineatus* was most abundant at Site 3, while the Psychomyidae, represented only by *Psychomyia pusilla*, were detected only at Site 4 (Figure 2H; Table S7). The total abundances of the family Rhyacophil-

idae were almost identical at all sites (Figure 2H). Even though the clear morphological determination of larvae from the group *Rhyacophila* sensu stricto is not always possible [40], *Rhyacophila* cf. *aurata* was dominant in samples from Sites 3 and 4 (especially Site 3), whilst *Rhyacophila nubila* gr. was commonest in samples from Sites 1 and 2.

3.3. Shift in EPT Community Composition along Environmental Gradients

The differences in species compositions between sites were significant (test on first axis: pseudo-F = 1.9, p = 0.001; test on all axes: pseudo-F = 4.0, p = 0.001; Figure S5), as were the differences in composition of species between sampling times (but only the composition of specimens > 0.5 mm in size; test on first axis: pseudo-F = 0.7, p = 0.024; test on all axes: pseudo-F = 1.8, p = 0.001; Figure S6). The whole- and subplot EVs significantly correlated with the shift in species composition (p < 0.05); those selected by FS are shown in Figures 3 and 4, respectively.

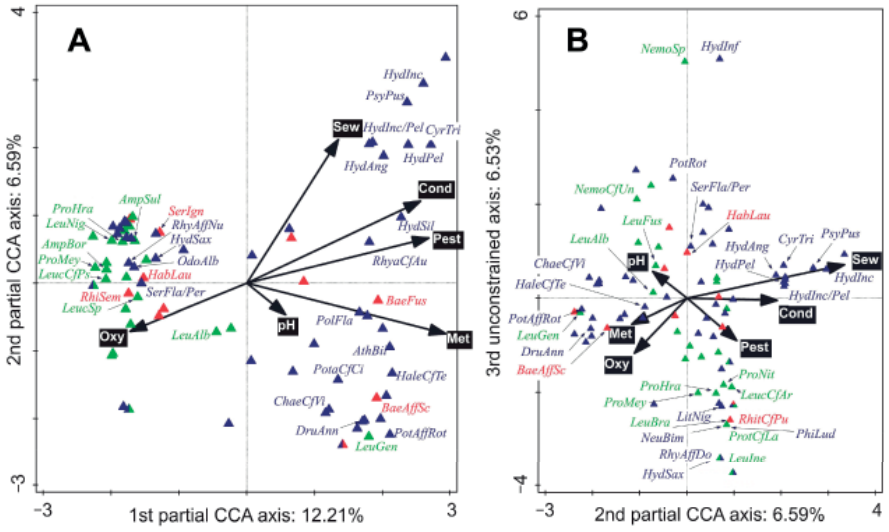

Figure 3. Species + whole-plot environmental variables (EVs) biplots: the first 33 best fitting species are labelled (Ephemeroptera, Plecoptera and Trichoptera labelled in red, green, and blue, respectively). A partial canonical correspondence analysis (CCA) was used: (**A**) first and second ordination axes; (**B**) second and third ordination axes. Ordination axes were constrained by the EVs selected by forward selection, which accounted for 30.48% of the explained variation. Test on first axis: pseudo-F = 0.5, p = 0.001; test on all axes: pseudo-F = 2.3, p = 0.001. Conditional effects (from the greatest to the lowest explained variation): 'Log-transformed sum of cadmium, lead and zinc (Met)': explained variation = 11.7%, pseudo-F = 5.7, p (adj.) = 0.003; 'Conductivity (Cond)': explained variation = 6.1%, pseudo-F = 3.1, p (adj.) = 0.020; 'Oxygen concentration (Oxy)': explained variation = 4.0%, pseudo-F = 2.1, p (adj.) = 0.012; 'Log-transformed sum pesticides (Pest)': explained variation = 3.0%, pseudo-F = 1.6, p (adj.) = 0.045; 'pH' explained variation = 2.9%, pseudo-F = 1.6, p (adj.) = 0.047; 'Total volume of released sewage for three days prior to sampling time (Sew)': explained variation = 2.9%, pseudo-F = 1.6, p (adj.) = 0.036. Abbreviations: *AmpBor—Amphinemura borealis*, *AmpSul—A. sulcicollis*, *AthBil—Athripsodes bilineatus*, *BaeAffSc—Baetis* aff. *scambus*, *BaeFus—B. fuscatus*, *ChaeCfVi—Chaetopteryx* cf. *villosa*, *CyrTri—Cyrnus trimaculatus*, *DruAnn—Drusus annulatus*, *HabLau—Habrophlebia lauta*, *HaleCfTe—Halesus* cf. *tesselatus*, *HydInf—Hydatophylax infumatus*, *HydAng—Hydropsyche angustipennis*, *HydInc—H. incognita*, *HydInc/Pel—H. incognita/pellucidula*, *HydPel—H. pellucidula*, *HydSax—H. saxonica*, *HydSil—H. siltalai*, *LeuAlb—Leuctra albida*, *LeucCfAr—L.* cf. *armata*, *LeuBra—L. braueri*,

LeucCfPs—*L.* cf. *pseudocingulata*, *LeucSp*—*Leuctra* sp., *LeuFus*—*L. fusca*, *LeuGen*—*L. geniculata*, *LeuIne*—*L. inermis*, *LeuNig*—*L. nigra*, *NemoCfUn*—*Nemoura* cf. *uncinata*, *NemoSp*—*Nemoura* sp., *NeuBim*—*Neureclipsis bimaculata*, *OdoAlb*—*Odontocerum albicorne*, *PhiLud*—*Philopotamus ludificatus*, *PolFla*—*Polycentropus flavomaculatus*, *PotaCfCi*—*Potamophylax* cf. *cingulatus*, *PotAffRot*—*P.* aff. *rotundipennis*, *PotRot*—*P. rotundipennis*, *ProHra*—*Protonemura hrabei*, *ProMey*—*P. meyeri/nitida*, *ProNit*—*P. nitida*, *ProtCfLa*—*P.* cf. *lateralis*, *PsyPus*—*Psychomyia pusilla*, *RhiSem*—*Rhithrogena semicolorata*, *RhitCfPu*—*R.* cf. *puytoraci*, *RhyaCfAu*—*Rhyacophila* cf. *aurata*, *RhyAffDo*—*R.* aff. *dorsalis*, *RhyAffNu*—*R.* aff. *nubila*, *SerFla/Per*—*Sericostoma flavicorne/personatum*, *SerIgn*—*Serratella ignita*.

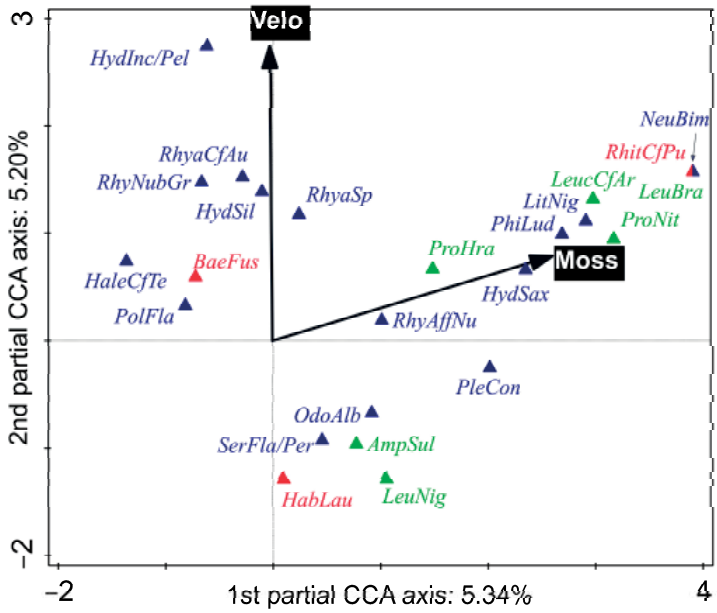

Figure 4. Species + subplot environmental variables (EVs) biplot: the first 24 best-fitting species are shown (Ephemeroptera, Plecoptera and Trichoptera labelled in red, green and blue, respectively). A partial Canonical correspondence analysis (CCA) was used. Ordination axes were constrained by the EVs selected by forward selection, which accounted for 10.54% of the explained variation. Test on first axis: pseudo-F = 0.1, p = 0.024; test on all axes: pseudo-F = 1.4, p = 0.001. Conditional effects (from the greatest to the lowest explained variation): 'Arcsine-transformed proportion of mosses in choriotope (Mosses)': explained variation = 5.3%, pseudo-F = 2.4, p (adj.) = 0.015 and 'Mean current velocity (Velo)': explained variation = 5.2%, pseudo-F = 2.5, p (adj.) = 0.023. Abbreviations: *AmpSul*—*Amphinemura sulcicollis*, *BaeFus*—*B. fuscatus*, *HabLau*—*Habrophlebia lauta*, *HaleCfTe*—*Halesus* cf. *tesselatus*, *HydInc/Pel*—*Hydropsyche incognita/pellucidula*, *HydSax*—*H. saxonica*, *HydSil*—*H. siltalai*, *LeuBra*—*L. braueri*, *LeucCfAr*—*L.* cf. *armata*, *LeuNig*—*L. nigra*, *LitNig*—*Lithax niger*, *NeuBim*—*Neureclipsis bimaculata*, *OdoAlb*—*Odontocerum albicorne*, *PhiLud*—*Philopotamus ludificatus*, *PleCon*—*Plectrocnemia conspersa*, *PolFla*—*Polycentropus flavomaculatus*, *ProHra*—*Protonemura hrabei*, *RhitCfPu*—*Rhithrogena* cf. *puytoraci*, *RhyaCfAu*—*Rhyacophila* cf. *aurata*, *RhyAffNu*—*R.* aff. *nubila*, *RhyaSp*—*Rhyacophila* sp. (early instar larvae), *RhyNubGr*—*R. nubila* gr., *SerFla/Per*—*Sericostoma flavicorne/personatum*.

3.4. Shift in Averaged Feeding Strategies along Environmental Gradients

The differences in compositions of the averaged feeding strategies between sites were significant in general; however, only the composition at Site 4 was significantly different from all other sites ($p < 0.05$; Figure 5A). There was also a significant shift in the whole-plot EVs ($p < 0.05$). Significant relationships with 'conductivity' (32.5% of explained variability), 'total volume of released sewage for three days prior to sampling time' (5.9% of explained variability), 'oxygen concentration' (2.8% of explained variability), and 'total concentration of PhACs' (2.3% of explained variability) were detected ($p < 0.05$). 'Passive filter feeder' and 'predator' feeding strategies were dominant in the WW-polluted environment; the 'grazer and scraper' feeding strategy seemed to be limited by the most polluted WW; and the 'gatherer/collector' and 'shredders' feeding strategies were generally suppressed in the most polluted environment (Figures 5B and S7B,C). The shift in the subplot EVs was not significant (test on first axis: pseudo-$F = 0.6$, $p = 0.379$; test on all axes: pseudo-$F = 1.9$, $p = 0.168$).

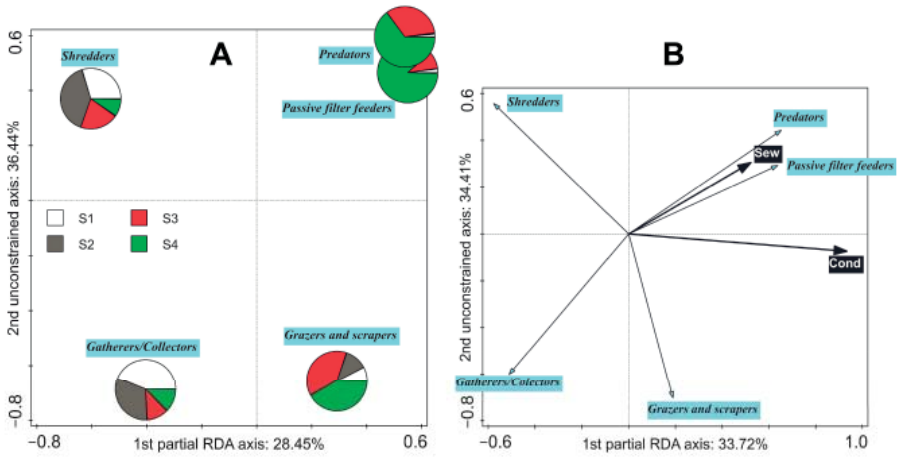

Figure 5. (**A**) Averaged feeding strategies pie chart and (**B**) Averaged feeding strategies + whole-plot environmental variables (EVs) biplot. Partial Redundancy analyses (RDA) were used. First ordination axes were constrained: (**A**) by the factor 'Site' that accounted for 30.98% of the explained variation and (**B**) by the EVs selected by forward selection, which accounted for 38.91% of the explained variation. (**A**) Test on first axis: pseudo-$F = 5.4$, $p = 0.003$; test on all axes: pseudo-$F = 6.1$, $p = 0.026$; (**B**) test on first axis: pseudo-$F = 2.5$, $p = 0.006$; test on all axes: pseudo-$F = 5.7$, $p = 0.009$. Conditional effects (from the greatest to the lowest explained variation): (**A**) 'Site 4 (S4)' explained variation = 22.0%, pseudo-$F = 12.1$, p (adj.) = 0.027; 'Site 3 (S3)' explained variation = 8.6%, pseudo-$F = 5.2$, p (adj.) = 0.090; and 'Site 2 (S2)' explained variation = 0.3%, pseudo-$F = 0.2$, p (adj.) = 0.943. (**B**) 'Conductivity (Cond)': explained variation = 32.5%, pseudo-$F = 20.7$, p (adj.) = 0.005 and 'Total volume of released sewage for three days prior to sampling time (Sew)': explained variation = 6.4%, pseudo-$F = 4.4$, p (adj.) = 0.045.

3.5. Malformations and Mortality of Caddisflies

A substantial proportion of final instar caddisfly larvae in *Halesus* cf. *tesselatus*, *Potamophylax* aff. *Rotundipennis*, and *R.* cf. *aurata* sampled at Site 3 had malformed limbs (11.8%, 7.0%, and 7.7%, respectively). Peculiarly fused terminal parts of walking legs (between tibiae and tarsal claws) or overall shortened walking legs were observed in the two given limnephilid taxa; deformed and shortened anal claws were observed in *R.* cf. *aurata* (Figure 6). Malformed specimens of *H.* cf. *tesselatus* were also observed at Site 4 (28.6%), although at the latter site, the total number of sampled specimens was substantially lower than at Site 3 (7 vs. 76 specimens at Site 3). There were no malformed specimens in the

abundant *R.* cf. *aurata* in Site 4; however, there was one malformed specimen out of a total of 20 specimens of *R. nubila* gr. at Site 4.

Figure 6. Malformed limbs of *Halesus* cf. *tesselatus* (two pictures on the left) and *Rhyacophila* cf. *aurata* (right side) sampled at Sites 3 and 4.

We also observed a drift of dead limnephilid pupae at Site 3 in September during the macrozoobenthic sampling. We found dead and decaying larvae and pupae of *Drusus annulatus*, *H.* cf. *tesselatus*, and Limnephilidae gen. sp. inc. in the given samples (14 dead limnephilid specimens and 7 living limnephilid specimens in total). Several dead and decaying pupae of *Hydropsyche* sp. were also found in the samples from Site 2 taken in June.

4. Discussion

4.1. Variability in the EPT Community along an Environmental Gradient

The EPT community can significantly reflect the quality of the environment (Figures 3–5). Even though our study only consisted of a small-scale natural experiment (i.e., we only sampled two mutually interlinked watercourses), the results match, in general, those of a study of whole macroinvertebrate communities carried out in 23 Swiss streams [41]. As in our work, in the Swiss study, about 30% of community variability was explained by water quality; conductivity (which correlates to total dissolved solids) together with oxygen concentrations explained 10.1% in our study, while concentrations of pesticides explained 3.0% of community variability in both studies (Figure 3).

The changes in EPT abundance and richness related to pollution by trace metals observed in our study generally resemble reports from other sites, the greatest similarity being in the susceptibility of mayflies—and especially heptageniids—to this type of pollution (Figure 2). Sites 3 and 4 were both warmer—maximums up to 23 °C—than unpolluted Sites 1 and 2 (Table 1; Figure S3), a relevant finding given that the temperature gradient, which is often related to the toxicity of chemical compounds and the solubility of oxygen in water, is considered a good predictor of change in the composition of EPT communities [42]. The increasing temperature gradient observed when moving downstream is undoubtedly natural; however, the difference in the temperature regime between the contaminated and uncontaminated sites is also likely to be the product of the canalisation, the removal of the riparian vegetation, and the presence of shallow reservoirs upstream on the Litavka

river (see part 2.1 'Locality description' [43]). That many species of mayflies and stoneflies would avoid high temperatures, mine effluent and certain trace metals was predicted beforehand [17–19,44–46]. Our results show that baetids were the most resistant (or resilient) to the trace metal contamination, and within this group, the dominant taxa were the *Baetis fuscatus* group (i.e., *B. fuscatus* and *B.* aff. *scambus*, whose separation was not possible since the taxon identified and presented here as *B.* aff. *scambus* shared features of both species). The total abundance of *B. fuscatus* gr. compensated for the decrease in abundances of other mayfly taxa so that this small-sized baetid could act as a proxy for other mayflies in human-disturbed sites. The other most commonly detected baetids, *B. rhodani* and *B. vernus*, did not show any substantial changes in abundance between sites. Nevertheless, trace metal pollution can have an effect later on in the life cycle of baetids and other aquatic insects by rapidly causing increased mortality in emerging imagines (or subimagines) [47,48].

Stoneflies are generally the aquatic insects most sensitive to conditions modified by humans [49,50]. There was significantly decreased richness in stoneflies—even at Site 2 compared with Site 1—and their abundance only significantly fell at Sites 3 and 4 (Figure 2; Table 5). We interpret their decrease in richness at Site 2, situated below the reservoir, as the result of the discontinuity in the river network, i.e., a change in the deposition regime (a lower proportion of smaller mineral particles and a greater proportion of light deposits; Table 4), and the separation of Site 2 from the network of tributaries in the crenel zone situated upstream from the reservoir (Figure 1). However, there are several indications that stoneflies are less sensitive to metals than mayflies. Many stoneflies (as well as caddisflies) are more tolerant to increased acidity than many mayflies [51]. Lower pH leads to natural access to metals (e.g., aluminium) originally bound to substrates [52,53]. Observational work studying the effects of contamination from mining has reported relatively high resistance in some stoneflies, including *Amphinemura sulcicollis*, *Leuctra fusca*, and *L. inermis*, taxa that are similar to those found in our study [16,19]. Little evidence exists regarding the levels of trace metals concentrations that are lethal for stoneflies [54], although certain North American stoneflies have been found to withstand (in significantly reduced numbers) concentrations of 11, 120, and 1100 $\mu g \cdot L^{-1}$ of cadmium, copper, and zinc, respectively, for 10 days in microcosm conditions. By comparison, numbers of heptageniids and *Baetis tricaudatus* fell significantly in only half of these concentrations [46].

Therefore, the significant reduction in stonefly abundance and richness at both contaminated sites cannot be conclusively attributed to pollution by trace metals. The absence there of most of the stoneflies detected upstream could also be due to water warming since most stoneflies prefer lower temperatures than those recorded at Sites 3 and 4 [39,42]. However, the interaction of multiple effects, including habitat degradation and several types of pollution, are assumed to take place.

According to our data, the less sensitive stoneflies were able to withstand the heavy industrial trace metal pollution but were then eliminated by the municipal WW, despite the advanced technology used in the WW treatment [14]. A synergic effect of multiple stressors is likely to occur. We can demonstrate that specimens of all sizes of *Leuctra fusca* gr. (species identified as *L. albida* and *L. fusca* in this study [55]) and *L. geniculata* occurred in the water contaminated by trace metals at Site 3 (Table S7) but not in the water contaminated by municipal WWs. Only *L. fusca* gr. (the only species identified as *L. fusca*) of small sizes were detected at Site 4, contaminated by both trace metals and municipal WWs. It is important to note that the organic pollution produced by the WWs sometimes reaches high levels. According to unpublished data provided by the Czech Hydrometeorological Institute and Povodí Vltavy, State Enterprise, the average estimated five-day biochemical oxygen demand (BOD_5) was equal to 2.39 $mg \cdot L^{-1}$ at Site 4; however, the estimated maximum was 17.00 $mg \cdot L^{-1}$ just four months before the beginning of our sampling campaign. In the Příbramský brook (Auxiliary Site 4 downstream from the effluent from the WWTP, see Supplementary Materials), the estimated BOD_5 at the same time was

24.00 mg·L^{-1} (the average and maximum of the estimated BOD_5 were 4.76 and 60.00 mg·L^{-1} in 2013–2020, respectively).

Caddisflies were the least harmed insects in the EPT community. Their richness was not significantly different between sites, and their abundance was significantly higher at Site 4 than at Sites 1 and 2 (Figure 2; Table 5). Many caddisflies are acid-tolerant or acid environment specialists (e.g., *Chaetopterygopsis maclachlani* and *Rhyacophila polonica* detected during our study [51,56]), and some are reported to be tolerant to trace metal pollution (e.g., *Hydropsyche* sp., leptocerids, *Rhyacophila* sp. [17,18,46]). Ubiquitous caddisfly taxa can occupy warmer sites polluted by organic materials with low oxygen concentrations (e.g., potamal hydropsychids and *Psychomyia pusilla* [56,57]). Nevertheless, caddisflies are generally very sensitive to pesticides and avoid sedimentation and droughts, as does the EPT group in general [11,58,59].

The vast majority of EPT taxa situated on the right-hand side of the CCA biplot (Figure 3A) were caddisflies. The relative abundances of these caddisflies were positively correlated to the human-induced gradients. The caddisfly communities at Sites 1 and 2 were quite similar; however, net-spinning philopotamids were lacking from Site 2 and were replaced by net-spinning hydropsychids and polycentropodids (Figure 2H). Changing conditions along the longitudinal profile (e.g., food source and current velocity) probably played an important role in shaping the net-spinning caddisfly community. Philopotamids prefer diatoms and fine detritus and have been reported to be quite sensitive to organic pollution [60,61]). According to our data, philopotamids were found at the subplots with the greatest maximum water velocity (0.73–0.97 m·s^{-1}). Slower water speeds caused by water abstraction [60] combined with the natural longitudinal stream gradient [37,60] favoured hydropsychids and polycentropodids. Unlike philopotamids, hydropsychids and polycentropodids are either facultatively or obligatory carnivorous [37,62].

The altered conditions at Sites 3 and 4 probably prevent the occurrence of the eruciform caddisflies *O. albicorne* and *S. flavicorne/personatum*, which were dominant in the samples from Sites 1 and 2. The warmer temperatures (and poorer oxygen conditions) are expected to have a negative effect. Haidekker and Hering [42] have reported that *O. albicorne* tolerates well mean summer temperatures up to 16 °C. However, this figure was exceeded at Site 4 by 1.24 °C (Figure S2), and so, based on individual measurements (Table 1), we assume that a similar temperature regime exists at Site 3. Furthermore, both these taxa are partially endobenthic and are more exposed to the toxic compounds that accumulate in sedimented particles [63]; the long larval development time in *S. flavicorne/personatum* [64] will also prolong the exposure of its larvae to toxicants. On the other hand, high abundances of large eruciform caddisflies—limnephilids of the genus *Halesus* and *Potamophylax*—were found at Site 3. These taxa are probably tolerant to trace metal pollution. Nonetheless, we found malformed walking legs in up to 11.8% of individuals that, in light of previous studies, could be a sign of trace metal contamination [65] or other persistent and hazardous compounds generated by the industrial complex upstream from Site 3 [66].

The lower abundances and richness of limnephilids observed at Site 4 downstream from the WWTP effluent could be a consequence of the interaction between trace metals and WW pollution (or the WW pollution alone). Hydropsychids, polycentropodids, rhyacophilids, and psychomyids (*P. pusilla*) were most abundant downstream from the WW effluent. The taxa are shown in the upper right-hand corner of Figure 3A were positively related to the 'total volume of released sewage three days prior to the sampling' (hereafter referred to as '3-day sewage volumes'), which probably contained a high proportion of SDPOM—a mixture of organic detritus and microorganisms such as bacteria and algae that is a high-quality food resource downstream from WW effluents [23]. On the other hand, the released sewage could trigger a drift of benthic prey organisms with greater oxygen demands (e.g., mayfly nymphs), and SDPOM could be used by periphyton and macrophytes (aquatic and riparian), which can serve as microhabitats or food. For example, the carnivorous *C. trimaculatus* with a more pronounced microhabitat preference for macrophytes [37] was more common than *P. flavomaculatus* at Site 4 than Site 3. The most

abundant hydropsychids can use multiple food resources and, aside from predating on small benthic organisms and consuming detritus, they also graze on algae [37,67]. Besides crustaceans, insects (including EPT), and terrestrial prey, detritus also constitute a substantial portion of gut content in polycentropodids [68]. It cannot be rejected that hydropsychids and polycentropodids could have a detrimental impact on the populations of other EPT taxa. Nevertheless, their net spinning increases heterogeneity of current velocities and nets represent valuable microhabitats, e.g., positive relationships to ephemerellid mayfly and chironomids were reported [69,70]. We associate the high abundance of early instars of *H. siltalai* in September at Sites 3 and 4 with the presence of aquatic mosses (Figure 4).

The proportion of mosses in the choriotope (explained community variation = 5.3%) and mean current velocity (explained variation community variation = 5.2%) had the highest parsimonious and significant power to explain EPT community composition at the subplot level (Figure 4). These two variables were positively correlated because mosses were detected mainly in shallow, fast-flowing habitats. Mosses (as well as roots) provide better vertical zonation possibilities for aquatic organisms, as well as food and shelter [71]. The information provided by Buffagni et al. [38], Graf et al. [37], and Graf et al. [39] does not correspond particularly well to our results: of the species best corresponding to the presence of aquatic mosses only *Hydropsyche saxonica, Plectrocnemia conspersa*, and *Protonemura hrabei* were reported to have a certain preference for macrophytes (mosses included). Nevertheless, Krno [72] suggests that the goerid *Lithax niger* is a bryophyte dweller and reports that *Rhyacophila obliterata* can only live on emergent mosses, whilst *R. nubila*, *R. polonica* can also live on submerged mosses. Similar findings have been reported by Glime [71]. Conversely, Graf et al. [37] consider all goerids and rhyacophilids to inhabit micro-/mesolithal and macro-/megalithal and suggest that they are 'specialised for lithal'.

A high mean velocity best corresponded to the highest relative abundance of *H. pellucidula/incognita* (the larvae of these two species are often not separable [40]): fitted optimum over 0.60 $m \cdot s^{-1}$; *R.* cf. *aurata* and *R. nubila* gr.: fitted optimum around 0.50 $m \cdot s^{-1}$; and *H. siltalai*: fitted optimum 0.47 $m \cdot s^{-1}$ (Figure 4). The mayfly *B. fuscatus* had a fitted optimum around 0.35 $m \cdot s^{-1}$ (Figure 4). By contrast, according to our data, *O. albicorne* had a fitted optimal mean current velocity around 0.14 $m \cdot s^{-1}$, *S. flavicorne/personatum* 0.10 $m \cdot s^{-1}$, and the mayfly *H. lauta* less than 0.10 $m \cdot s^{-1}$.

4.2. Shift in Averaged Feeding Strategies in the EPT Community along Environmental Gradients

The analysis of shifts in ecological traits and, above all, their relationship to environmental characteristics has recently become a key topic in community ecology [73]. Substantial changes in the composition of feeding strategies (functional feeding groups) due to disturbances are assumed to occur in the macrozoobenthos [74,75]. Significant differences in the composition of average EPT feeding strategies were detected between localities (Figure 5A). Conductivity and 3-day sewage volumes were found to be the most parsimonious significant predictors in our dataset (Figure 5B). Both these EVs were positively related to the input of various materials and were positively related to both passive filter feeder and predator feeding strategies (represented almost only by caddisflies; Figure S7B). It suggests in higher susceptibility of EPT shredders to the pollution or unavailability of the CPOM (main food resource utilised by shredders) edible for these organisms (e.g., crystalline deposits containing trace metals can precipitate on the external surface of alder leaves because of fungal grown [21]). Even though shredder abundance will be compensated by numerous *Asellus aquaticus* and gatherers/collectors by chironomids and oligochaetes (see part 2.1. 'Locality description'), the abundance of winged adults of the aquatic insect directly utilising CPOM may be substantially lowered and could cause an alteration in nutrient and energy flows through the environment.

Despite that, 3-day sewage volumes positively correlated to the responses of passive filter feeders, predators and shredders and negatively to the responses of gatherers/collectors (Figure S8A), the greatest 15-day sewage volumes (Figure S8B) positively correlated to the responses of gatherers/collectors and grazers and scrapers. Nevertheless, this trend

was not strongly associated with the '15-day sewage volumes' because the dominance of gatherers/collectors and grazers and scrapers was caused by a high abundance of nymphs of the mayfly *B. fuscatus* gr., which probably colonised this site after drifting from an upstream stretch after a high discharge event (up to 8.5 times the average discharge). This hydrological situation occurred at the same time as the release of a high volume of sewage. The sewage release ended four days before the sampling time. The greatest similarity in the overall EPT community of samples to the samples from Site 3 is shown in the overlap of the ellipses in Figure S4. This similarity was subsequentially lost when the heavy rains stopped: the greatest dissimilarity between samples from individual sites was observed in August, followed by September. Nonzero values of high 3-day sewage volumes were linked to EPT compositions sampled in both these months, as well as in May. The significant linkage between 3-day sewage volumes and the composition of feeding strategies, as a result of FS in RDA (Figure 5B), supports the presumption that the population of campodeiform net-spinning caddisflies is directly or indirectly dependent on the supply of 'poorly treated' WWs.

5. Conclusions

This study highlights the impacts on the EPT community of trace metal pollution originating from mining and smelting and effluent from a municipal WWTP, which releases continuously treated WW and, periodical, 'poorly' treated WW. According to our data and previously published studies, trace metal pollution can have quite different consequences for the EPT community than, for example, pesticides and several caddisflies, especially limnephilids, were found to be very abundant at the affected site. Additionally, malformations in caddisfly larvae and mortality of caddisfly pupae were observed. Even though the affected sites were warmer, the absence of ephemerellid, heptageniid, and leptophlebiid mayflies is attributable to trace metal pollution, given the results from previous studies. The factors responsible for the decline in the stonefly community are less clear, and, except for higher water temperature, other kinds of pollutants and habitat degradation can be assumed to have a negative impact. Nevertheless, *L. fusca* gr. and *L. geniculata* can be considered tolerant to trace metal pollution but sensitive to WW pollution or its combination with trace metals. The municipal WW combined with trace metal contamination led to the greatest changes in the EPT community, including changes in the composition of feeding strategies. Caddisfly passive filter feeders and predators—mainly hydropsychids, polycentropodids, and rhyacophilids—dominated the most polluted environment. EPT shredders and collectors/gatherers dominated in unaffected sites. Our results demonstrate how the EPT community reacts to human-induced gradients in natural environments, which is important knowledge for assessing the implication of planned or current human disturbances in natural or cultural landscapes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology11050648/s1, Materials and Methods; Results, Figure S1: photographic documentation of Site 1 and Site 2, Figure S2: photographic documentation of Site 3, Site 4 and Auxiliary Site 4, Figure S3: summer temperature regime monitored by data loggers (TFA), Table S1: physicochemical parameters of water at longitudinal profile, Table S2: parameters of discharge at monitored sites, Table S3: contamination of water and sediments by trace metals at longitudinal profile, Table S4: contamination of water by trace metals at Auxiliary Site 3 and Site 4 during 2018–2020, Table S5: pesticides and pharmaceutical active compounds in water samples taken at the longitudinal profile, Table S6: list of pesticide and pharmaceutical active compounds analysed in water samples with particular limits of quantification (LOQs), Figure S4: classified sample diagram resulted from choriotopic compositions using the RDA (sampling sites), Table S7: list of EPT taxa detected at given sites and times, Figure S5: classified CCA sample diagram resulted from species compositions and CCA species pie chart (sampling sites), Figure S6: CCA Species pie chart (sampling times), Figure S7: plotted significant averaged feeding strategies response curves, Figure S8: plotted significant averaged feeding strategies response curves against '3-d Sew' and '15-d Sew', Table S8: the statistics of relationship strengths for plots in Figures S7 and S8, Table S9: abundance and biomass

of fishes detected at the particulate sites during 20 May and 8 October in 2020, Figure S9: plotted significant relationship between EPT family richness and Metal index. References [14,76–78] are cited in the supplementary materials.

Author Contributions: Conceptualization, M.L. and M.B.; methodology, M.L.; formal analysis, M.L.; investigation, M.L., J.Č., P.N., F.L. and M.B.; writing—original draft preparation, M.L.; writing—review and editing, M.B. and P.N.; supervision, M.B. All authors have read and agreed to the published version of the manuscript.

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Figure S1. Photographic documentation of Site 1 (upper part) and Site 2 (down part). Pictures were taken in March 2022. The picture in the upper left corner shows the confluence of the Obecnický brook and a spring-fed nameless tributary. There were subnormal discharges at both sites.

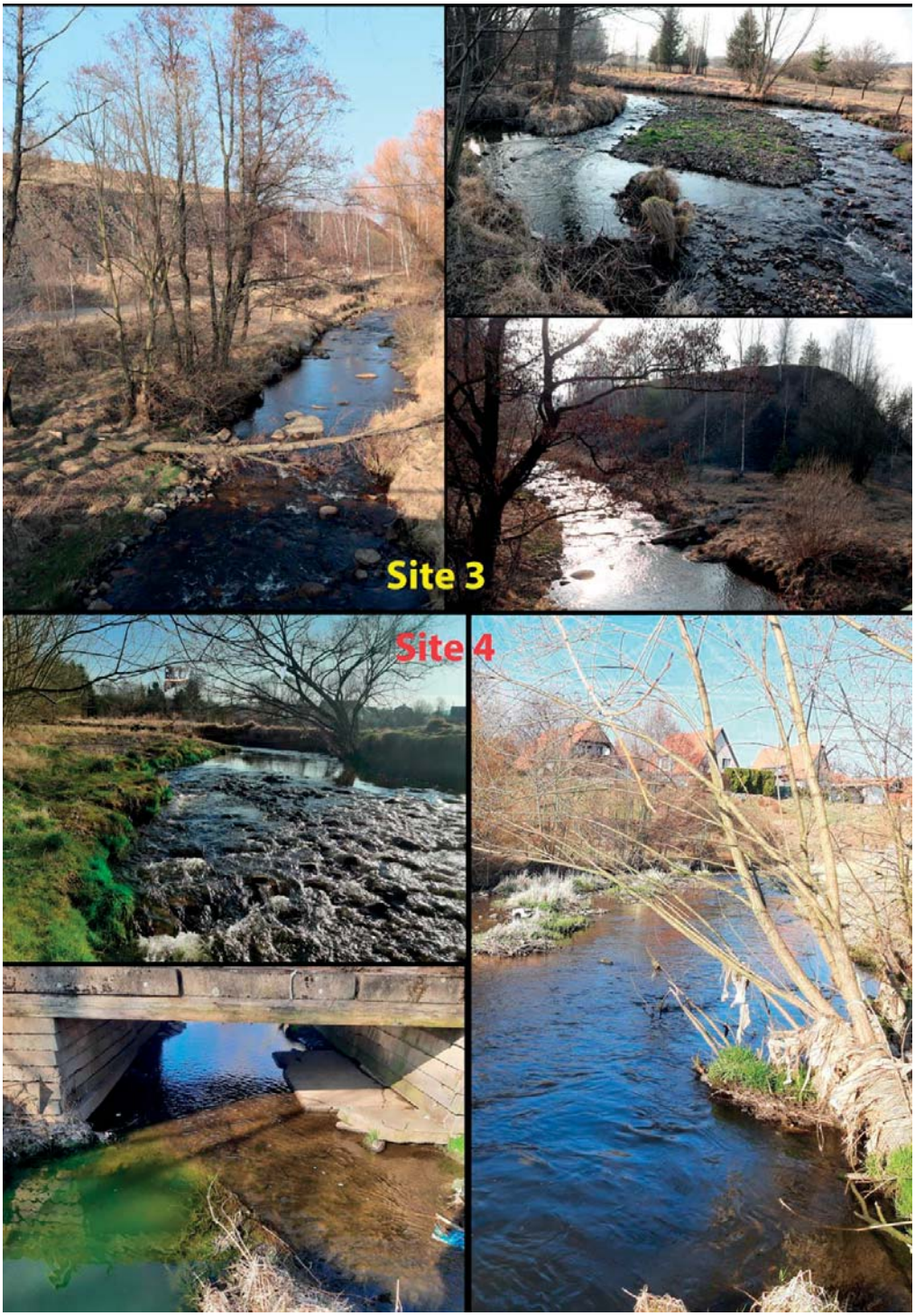

Figure S2. Photographic documentation of Site 3 (upper part) and Site 4 (down part). Pictures were taken in March 2022. The non-channelised part of the Site 3 is shown in the picture in upper right corner. Heaps of waste materials (mainly sodium slag) are visible in the pictures of the channelised part of Site 3. A periodically active effluent of 'poorly treated communal wastewaters' flowing into Auxiliary site 4 is

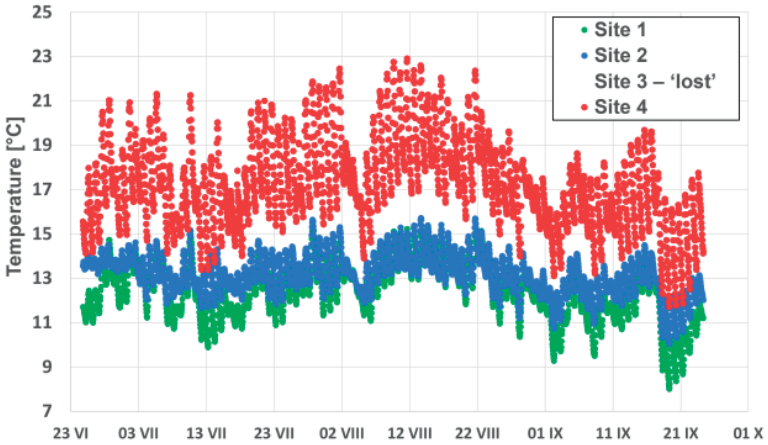

Figure S3. Temperature monitored by dataloggers (TFA) during the time interval between 24 June to 24 September 2020 at given sites. The datalogger placed in the Site 3 was lost.

Table S1. Physicochemical parameters of water measured at particular sites. Comparison between Sites 1–4 and Auxiliary sites 1–4 is not fully relevant because the times of measuring parameters at auxiliary sites do not fully correspond to the times of measuring parameters at Sites 1–4. Aux. site = Auxiliary site.

	Site 1	Site 2	Aux. site 1	Aux. site 2	Site 3	Aux. site 3	Aux. site 4	Site 4
	n = 4	n = 4	n = 3	n = 3	n = 4	n = 3	n = 3	n = 4
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Temperature (°C)	12.05 ± 0.34	13.33 ± 0.61	17.85 ± 2.73	15.24 ± 1.91	17.05 ± 1.11	15.76 ± 1.33	19.59 ± 1.37	16.80 ± 0.68
pH	7.09 ± 0.24	7.35 ± 0.17	8.22 ± 0.05	7.74 ± 0.02	7.45 ± 0.17	8.81 ± 0.69	7.44 ± 0.15	7.45 ± 0.09
Oxygen concentration (mg·L^{-1})	9.90 ± 0.10	9.02 ± 0.18	9.97 ± 0.40	9.11 ± 0.35	8.91 ± 0.16	9.62 ± 0.67	5.36 ± 0.78	7.61 ± 0.27
Conductivity (µS·cm^{-1})	71.40 ± 5.80	84.35 ± 5.98	392.33 ± 61.52	362.00 ± 48.42	390.60 ± 39.18	609.32 ± 76.97	838.67 ± 9.66	571.00 ± 56.74

Table S2. Parameters of discharge at particular sites continually measured by Povodí Vltavy, State Enterprise between February and September of 2020.

	Site 1	Site 2	Site 3	Site 4*
Average discharge ± SD (m^3·s^{-1})	0.11 ± 0.14	0.08 ± 0.12	Not available	0.88 ± 0.87
The lowest observed discharge (m^3·s^{-1})	0.017	0.015	Not available	0.187
The highest observed discharge (m^3·s^{-1})	1.328	1.340	Not available	7.480

*** The hydrological station measuring discharge is situated approximately 5.4 km downstream the Site 4. Therefore, the given parameters can be slightly higher**

Table S3. Concentrations of metals and organic carbon in sediment samples and concentrations of metals in water samples. Samples were taken during 24 September 2020 in the particular sites. The sites are ordered from the most upstream site to the most downstream site. Aux. site = Auxiliary site.

	Site 1	Site 2	Aux. site 1	Aux. site 2	Site 3	Aux. site 3	Aux. site 4	Site 4
Concentrations in sediment samples (mg·kg^{-1} DW)								
Cadmium	1.70	2.60	76.00	240.00	30.00	71.00	24.00	51.00
Lead	170	87	9100	140000	3700	5900	1100	2200
Zinc	180	170	11000	3400	3800	7400	3600	5200
Total organic carbon	27000	25000	39000	62000	19000	74000	69000	78000
Concentrations in water samples (µg·L^{-1})								
Cadmium	0.26	0.23	3.50	3.10	7.90	7.20	1.30	3.80
Cadmium dissolved	< 0.05	< 0.05	2.40	0.69	6.50	4.30	0.11	0.50
Lead	5.80	9.80	22.00	690.00	64.00	100.00	38.00	53.00
Lead dissolved	< 0.50	3.20	3.50	8.60	14.00	7.90	1.00	1.70
Zinc	15	13	1000	120	1200	910	210	530
Zinc dissolved	< 5	<5	820	31	1000	640	48	180

Table S4. Concentrations of cadmium (Cd), lead (Pb), and zinc (Zn) in water samples estimated in water samples from Auxiliary site 4 (Aux. site 4) and Site 4. Data provided by the Czech Hydrometeorological Institute (Prague, Czech Republic) and PovodíVltavy, State Enterprise (Prague, Czech Republic).

		2018			2019			2020		
		Cd (µg·L^{-1})	Pb (µg·L^{-1})	Zn (µg·L^{-1})	Cd (µg·L^{-1})	Pb (µg·L^{-1})	Zn (µg·L^{-1})	Cd (µg·L^{-1})	Pb (µg·L^{-1})	Zn (µg·L^{-1})
Aux. site 4	Mean ± SEM	0.53 ± 0.09	22.43 ± 4.06	143.58 ± 13.21	0.87 ± 0.22	34.57 ± 7.83	146.75 ± 20.45	0.67 ± 0.10	24.82 ± 4.33	148.42 ± 19.37
	Min	0.13	3.60	53.00	0.17	6.10	41.00	0.23	7.30	81.00
	Max	1.10	54.00	220.00	3.20	92.00	260.00	1.30	53.00	340.00
Site 4	Mean ± SEM	3.20 ± 0.42	26.19 ± 5.18	430.00 ± 21.21	3.89 ± 0.46	90.61 ± 62.01	610.00 ± 67.27	3.40 ± 0.21	52.67 ± 10.75	484.17 ± 22.53
	Min	1.80	7.30	320.00	1.80	8.30	290.00	2.40	14.00	390.00
	Max	7.60	71.00	580.00	8.30	800.00	1200.00	4.80	130.00	640.00

Table S5. Pesticides and pharmaceutical active compounds (PhACs) in water samples taken in August 2020 in the particular sites. Only the compounds below the limits of quantifications are involved. The sites are ordered from the most upstream site to the most downstream site. Max = the concentration of the compound with the highest concentration. Aux. site = Auxiliary site.

	Site 1	Site 2	Aux. site 1	Aux. site 2	Site 3	Aux. site 3	Aux. site 4	Site 4
	n = 1	n = 1	n = 1	n = 1	n = 1	n = 1	n = 1	n = 4 Mean ± SEM
Sum pesticides (ng·L^{-1})	132.00	70.32	695.40	1096.10	462.90	1304.00	1692.62	877.87 ± 125.71
N pesticides	8	11	13	22	21	21	37	29.25 ± 0.83
Max pesticides (ng·L^{-1})	48	13	230	320	130	440	650	207.50 ± 37.00
Sum PhACs (ng·L^{-1})	15.17	13.40	1781.47	230.07	874.58	471.76	10,036.78	4636.73 ± 254.79
N PhACs	6	4	31	18	28	20	48	45.25 ± 1.38
Max PhACs (ng·L^{-1})	7.4	8.7	610.0	59.0	310.0	130.0	3100.0	1275.00 ± 47.87

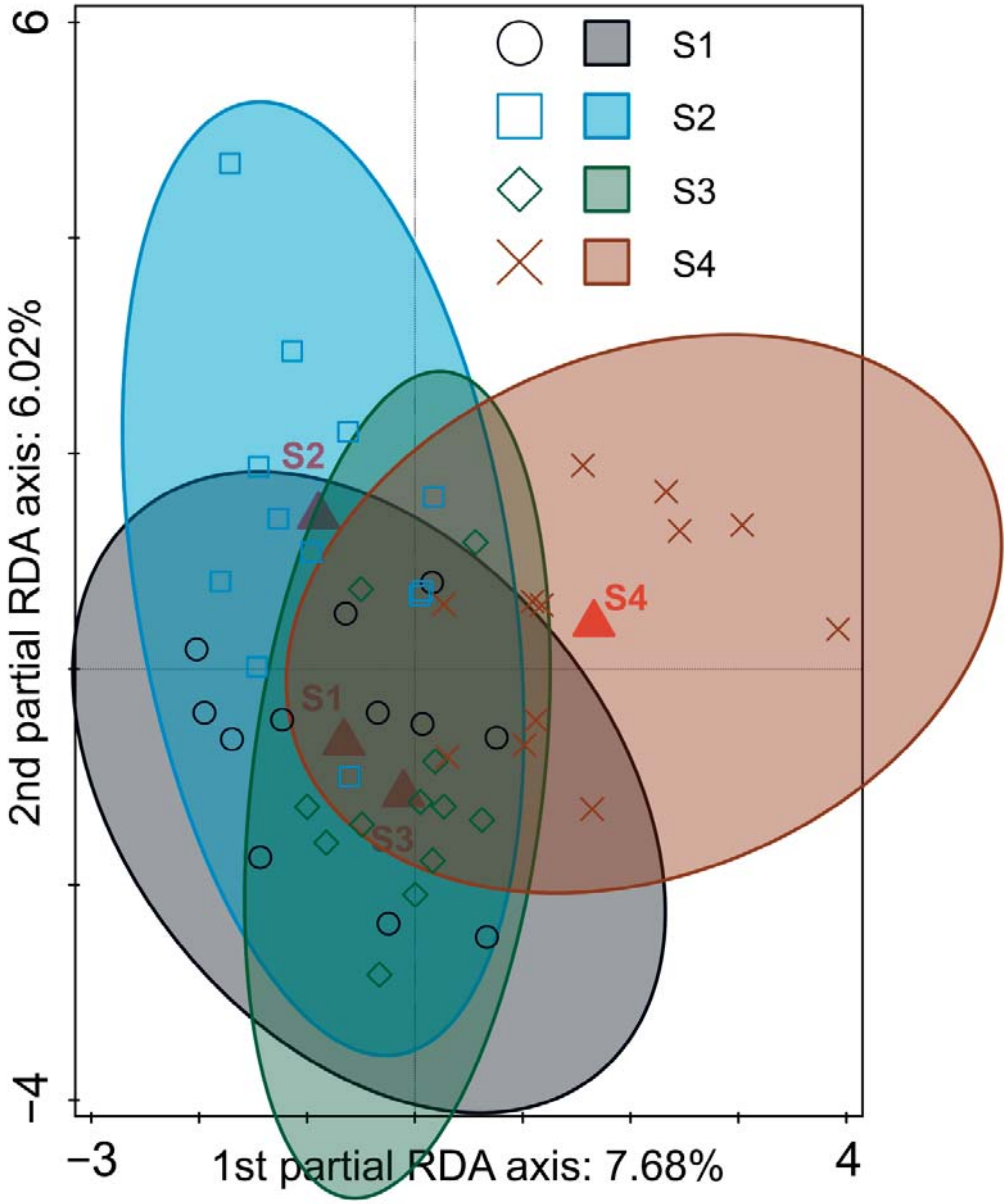

Figure S4. Classified sample diagram (caseR scores) resulted from choriotopic compositions. Partial Redundancy analysis (RDA) was used. Ordination axes were constrained by the factor 'Site' which accounted for 16.80% of the explained variation. Test on first axis: pseudo-*F* = 1.2, *p* = 0.085, test on all axis: pseudo-*F* = 2.8, *p* = 0.003. Conditional effects (from the greatest to the lowest size): 'Site 4 (S4)': explained variation = 7.5%, pseudo-*F* = 3.8, *p* (adj.) = 0.025; 'Site 2 (S2)': explained variation = 6.0%, pseudo-*F* = 2.9, *p* (adj.) = 0.049; and 'Site 1 (S1)': explained variation = 3.4%, pseudo-*F* = 1.7, *p* (adj.) = 0.216. S3 = Site 3.

Table S7. List of EPT taxa detected at the given sites and times and their semi-quantitative abundances. V = 11 May, VI = 24 June, VIII = 11 August, and IX = 24 September. Explanations for semi-quantitative counts: '**X**' represents the range of 1–9 individuals, '**XX**' represents the range of 10–99 individuals, '**XXX**' represents the range of 100–999 individuals; empty cells represent no occurrence.

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
EPHEMEROPTERA	XXX	XX	XX	XXX	XXX	XXX	XX	XX	XXX	XXX	XX	XXX	X	XXX	XXX	XX
Baetidae	X	X	XX	X	XX	XX	X	X	XXX	XXX	XX	XXX	X	XXX	XXX	XX
Baetis fuscatus (Linnaeus, 1761)		X	X			X			XXX	XX	XX	XX	X	XXX	XX	X
Baetis rhodani (Pictet, 1845)	X		X	X	XX	XX	X	X	X	XX		XX		XX	XX	X
Baetis* aff. *scambus (*fuscatus* gr.) (Eaton, 187)			X						XX	XX	XX	XX	X	X		
Baetis vernus (Curtis, 1834)	X		X	X		XX		X		X				XX	XX	X
Centroptilum luteolum (Müller, 1776)											X					
Ephemerellidae		XX	XX			XX									X	
Serratella ignita (Poda, 1761)		XX	XX			XX									X	
Heptageniidae	XX	X		XX	XX	X		XX								
Ecdyonurus torrentis (Kimmins, 1942)	X			X	X	X		XX								
Rhithrogena* cf. *puytoraci (Sowa & Degrange, 1987)		X														
Rhithrogena semicolorata (Curtis, 1834)	X	X		XX	XX			XX								
Leptophlebiidae	XXX	XX	XX	XXX	XX	XXX	XX	XX	X							
Habrophlebia lauta (Eaton, 1884)	XXX	XX	XX	XXX	XX	XXX	XX	XX	X							
Paraleptophlebia submarginata (Stephens, 1835)	X				X	X		X								
PLECOPTERA	XXX	XXX	XXX	XXX	XX	XXX	XXX	XXX	X	XX	XXX	XX		X	XX	
Chloroperlidae				X	X											
Siphonoperla torrentium (Pictet, 1841)				X	X											
Leuctridae	XX	XX	XX	XXX	XX	XXX	XXX	XX	X	XX	XXX	XX		X	XX	

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
Leuctra albida (Kempny, 1899)	XX	X	X	XX	XX	XX	XX	X	X	XX	XX	X				
Leuctra* cf. *armata (Kempny, 1899)	X	XX														
Leuctra* cf. *autumnalis (Aubert, 1948)		X						X								
Leuctra braueri (Kempny, 1898)		X														
Leuctra fusca (Linnaeus, 1758)		X	X	XX	X	XXX	XXX	X	X	X	XX	XX		X	XX	
Leuctra geniculata (Stephens, 1836)									X	X	XX					
Leuctra inermis (Kempny, 1899)			X													
Leuctra* cf. *leptogaster (Aubert, 1949)		XX	X	X	X			XX								
Leuctra major (Brinck, 1949)				X												
Leuctra nigra (Olivier, 1811)	XX	X	X	X												
Leuctra* cf. *pseudocingulata (Mendl, 1968)		X	X	XX				X								
Leuctra* aff. *pseudosignifera (Aubert, 1954)								X								
***Leuctra* sp.** (Stephens, 1836) indet.	X	X	XX	XX		X		XX		X						
Nemouridae	XXX	XXX	XXX	XXX	XX	XX	XX	XX								
Amphinemura borealis (Morton, 1894)	X		X	XXX	X											
Amphinemura standfussi (Ris, 1902)			X		X											
Amphinemura sulcicollis (Stephens, 1836)	XX	XX		X	X	X										
Amphinemura sulcicollis/triangularis (Stephens, 1836/Ris, 1902)	X		X		X											
***Amphinemura* sp.** (Ris, 1902) indet.	X	X			X			X								
Nemoura avicularis (Morton, 1894)				X				X								

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
Nemoura* aff. *cambrica (Stephens, 1836) – early instar nymphs								X								
Nemoura cinerea (Retzius, 1783)					X			X								
Nemoura* cf. *uncinata (Despax, 1934) – early instar nymphs			XX	XX			XX	XX								
***Nemoura* sp.** (Latreille, 1796) indet. – early instar nymphs			X				X									
Protonemura* cf. *aestiva (Kis, 1965)		X														
Protonemura hrabei (Raušer, 1956)		XX	XXX	XX		XX										
Protonemura intricata (Ris, 192)	XX	XX			X											
Protonemura* cf. *lateralis (Pictet, 1836)	XX	XX	X													
Protonemura meyeri/nitida (Pictet, 1841/ Pictet, 1836)			XX	XX				X								
Protonemura nitida (Pictet, 1836)		XX		X												
Perlodidae		X		XX												
Perlodes* aff. *microcephalus (Pictet, 1833) – early instar nymphs				X												
Diura bicaudata (Linnaeus, 1758). – early instar nymphs		X		X												
TRICHOPTERA	XX	XXX	XX	XX	XX	XXX	XX	XX	XXX	XXX	XXX	XXX	XX	XX	XXX	XXX
Goeridae		X	X	X			X				X					X
Goera pilosa (Fabricius, 1775)																X
Lithax niger (Hagen, 1859)		X	X	X												
Goeridae gen. sp. inc. (*Lithax niger*, Hagen, 1859 / *Silo nigricornis*, Pictet, 1834) – early instar larvae							X				X					
Hydropsychidae	X	X	X	X	X	XX	X	X	X	X	X	XXX	X	XX	XXX	XXX

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
Hydropsyche angustipennis (Curtis, 1834)						X				X		X	X	X	XX	XX
Hydropsyche fulvipes (Curtis, 1834)						X										
Hydropsyche* aff. *guttata (Pictet, 1834) – early instar larvae											X	X				
Hydropsyche incognita (Pitsch, 1993)															XX	XX
Hydropsyche* aff. *incognita/pellucidula (Pitsch, 1993/Curtis, 1834) – early instar larvae								X							X	X
Hydropsyche instabilis (Curtis, 1834)					X	X										
Hydropsyche pellucidula (Curtis, 1834)											X	X			XX	XX
Hydropsyche saxonica (McLachlan, 1884)	X	X	X	X	X	XX		X								
Hydropsyche saxonica/fulvipes (McLachlan, 1884/Curtis, 1834) – early instar larvae		X	X			X										
Hydropsyche siltalai (Döhler, 1964)						X			X	X	X	XXX	X	X	XX	XXX
***Hydropsyche* sp.** indet. (Pictet, 1834) – early instar larvae							X	X				X			X	
Hydroptilidae													X			
***Hydroptila* spp**. (Dalman, 1819)													X			
Leptoceridae								X	XX	X		X			X	X
Athripsodes bilineatus (Linnaeus, 1758)								X	XX	X		X				X
Mystacides azurea (Linnaeus, 1761)												X			X	
Limnephilidae	XX	XX	X		XX	X	XX	X	XX	XXX	XX	X	X	X	X	

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
Annitella obscurata (McLachlan, 1876)	X				X											
Annitella thuringica (Ulmer, 199)	X				X				X							
Drusus annulatus (Stephens, 1837)									X		XX	X				
Chaetopterygopsis maclachlani (Stein, 1874)	X	X														
Chaetopteryx major (McLachlan, 1876)		X								X					X	
Chaetopteryx villosa (Fabricius, 1798) – "dark-coloured"					X		X									
Chaetopteryx* cf. *villosa (Fabricius, 1798) – "light-coloured"					X		X		XX	XX	XX	X				
Halesus* cf. *digitatus (Schrank, 1781)											X					
Halesus* cf. *tesselatus (Rambur, 1842)									XX	XX	XX	X	X	X	X	
Halesus radiatus (Curtis, 1834)		X								X	X					
Hydatophylax infumatus (McLachlan, 1865) – early instar larvae			X													
Potamophylax* cf. *cingulatus (Stephens, 1837)					X				X	X	X					
Potamophylax* cf. *latipennis (Curtis, 1834)	X							X	X		X					
Potamophylax* cf. *luctuosus (Piller & Mitterpacher, 1783)		X									X					
Potamophylax rotundipennis (Brauer, 1857)		X	X			X				X			X			
Potamophylax* aff. *rotundipennis (Brauer, 1857)									X	XXX	X					
Limnephilidae gen sp. inc. (*Chaetopteryx villosa/Anitella obscurata*) – early instar larvae													X			

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
Limnephilidae gen sp. inc. (*Micropterna* sp./*Potamophylax rotundipennis*) – early instar larvae													X			
Limnephilidae gen. sp. – early instar larvae	X	X										X				
Odontoceridae	X	XX	X	XX	X	X		X								
Odontocerum albicorne (Scopoli, 1763)	X	XX	X	XX	X	X		X								
Philopotamidae		XX	XX													
Philopotamus ludificatus (McLachlan, 1878)		XX	XX													
Philopotamus ludificatus/variegatus (McLachlan, 1878/Scopoli, 1763) – early instar larvae		X														
Polycentropodidae	X	X			X	XX	X	X	XX	XX	XX	XX	X	X	XX	XX
Cyrnus trimaculatus (Curtis, 1834)										X				X		XX
Neureclipsis bimaculata (Linnaeus, 1758)		X														
Plectrocnemia conspersa (Curtis, 1834)	X	X							X							
Plectrocnemia* cf. *geniculata (McLachlan, 1871)	X															
***Plectrocnemia* sp.** (Stephens, 1836) – early instar larvae					X				X		X	X			X	X
Polycentropus* cf. *excisus (Klapálek, 1894)					X	X			X	X	X	X				
Polycentropus flavomaculatus (Pictet, 1834)						XX	X	X	XX	XX	X	XX	X		XX	XX

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
Polycentropodidae gen. sp. inc. (*Neureclipsis bimaculata*, Linnaeus, 1758) – early instar larvae												X			X	
Polycentropodidae gen. sp. inc. (*Neureclipsis bimaculata*, Linnaeus, 1758 / *Plectrocnemia* sp., Stephens, 1836)						X										
Polycentropodidae gen. sp. inc. (*Plectrocnemia* sp., Stephens, 1836) – early instar larvae										X	X	X				
Polycentropodidae gen. sp. inc. (*Polycentropus* sp., McLachlan, 1878) – early instar larvae					X	X										
Psychomyiidae															XX	XX
Psychomyia pusilla (Fabricius, 1781)															XX	XX
Rhyacophilidae	X	XX	XX	X	X	XX	X	X	X	X	XX	XX		X	XX	XX
Rhyacophila* cf. *aurata (Brauer, 1857)			X	X					X	X	XX	X			XX	XX
Rhyacophila* aff. *dorsalis (*nubila* gr.) (Curtis, 1834)			X													
Rhyacophila* cf. *fasciata (Hagen, 1859)															X	
Rhyacophila* aff. *nubila (Zetterstedt, 184)		X	X	X	X	X		X								
***Rhyacophila nubila* gr.** (Zetterstedt, 184)		X	X			X		X	X			X			XX	X
Rhyacophila* cf. *obliterata (McLachlan, 1863)						X										
Rhyacophila* cf. *polonica/praemorsa (McLachlan, 1879/ McLachlan, 1879)		X														
***Rhyacophila* sensu stricto** (Pictet, 1834) – early instar larvae		X	X		X	XX	X		X		X			X	X	

	Site 1				Site 2				Site 3				Site 4			
Taxon	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**	**V**	**VI**	**VIII**	**IX**
***Rhyacophila* sp.** inc. (Pictet, 1834) – early instar larvae	X	X	X	X	X	X	X		X			X		X	X	X
***Rhyacophila* sp.** inc. (Pictet, 1834) – pupae		X		X								X		X		X
Sericostomatidae	X	XX	X	X	X	XX	X	X								
Sericostoma flavicorne/personatum (Schneider, 1845/Kirby & Spence, 1826)	X	XX	X	X	X	XX	X	X								
Sericostomatidae gen. sp. inc. (*Notidobia ciliaris* ?) (Linnaeus, 1761) – early instar larvae								X								
Trichoptera fam. gen. sp. indet. (Agapetinae/Goeridae ?) – early instar larvae		X														
Trichoptera fam. gen. sp. indet. (Brachycentridae?) – early instar larvae	X															

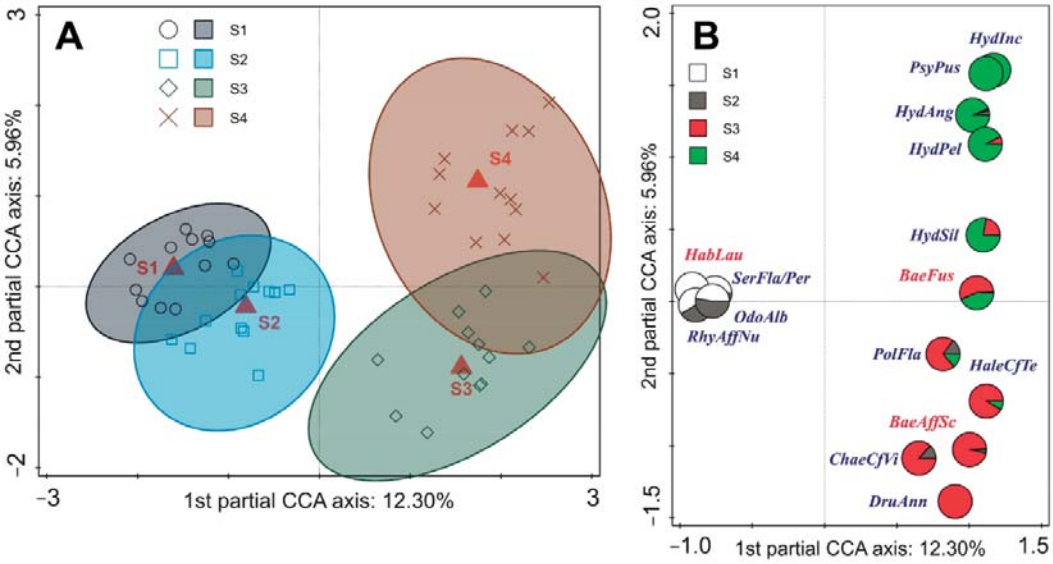

Figure S5. (A) Classified sample diagram (caseR scores) resulted from species compositions and (**B**) Species pie chart: the first 15 best fitting species are labelled (Ephemeroptera, Plecoptera, and Trichoptera labaled in red, green, and blue, respectively). Partial Canonical correspondence analysis (CCA) was used. Ordination axes were constrained by the factor 'Site' which accounted for 22.71% of the explained variation. Test on first axis: pseudo-F = 1.9, p = 0.001; test on all axes: pseudo-F = 4.0, p = 0.001. Conditional effects (from the greatest to the lowest explained variation): 'Site 1 (S1)': explained variation = 8.8%, pseudo-F = 4.1, p (adj.) = 0.002; 'Site 2 (S2)': explained variation = 8.0%, pseudo-F = 4.0, p (adj.) = 0.002; and 'Site 3 (S3)': explained variation = 5.9%, pseudo-F = 3.1, p (adj.) = 0.002. Abbreviations: *BaeAffSc* = *Baetis* aff. *scambus*, *BaeFus* = *B. fuscatus*, *ChaeCfVi* = *Chaetopteryx cf. villosa*, *DruAnn* = *Drusus annulatus*, *HabLau* = *Habrophlebia lauta*, *HaleCfTe* = *Halesus* cf. *tesselatus*, *HydAng* = *Hydropsyche angustipennis*, *HydInc* = *H. incognita*, *HydPel* = *H. pellucidula*, *HydSil* = *H. siltalai*, *OdoAlb* = *Odontocerum albicorne*, *PsyPus* = *Psychomyia pusilla*, *RhyAffNu* = *Rhyacophila* aff. *nubila*, *SerFla/Per* = *Sericostoma flavicorne/personatum*, S4 = Site 4.

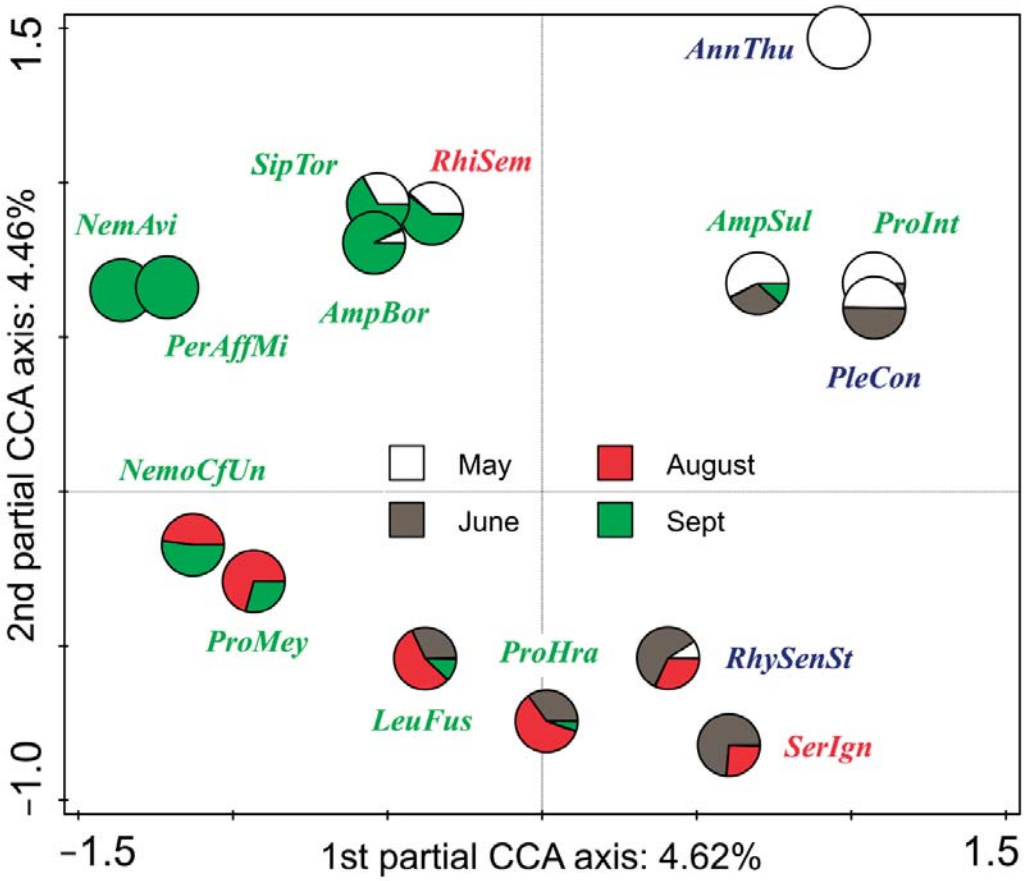

Figure S6. Species pie chart: the first 15 best fitting species are labelled (Ephemeroptera, Plecoptera, and Trichoptera labaled in red, green, and blue, respectively). Partial Canonical correspondence analysis (CCA) was used. Ordination axes were constrained by the factor 'Sampling time' which accounted for 11.62% of the explained variation. Test on first axis: pseudo-*F* = 0.7, *p* = 0.024; test on all axes: pseudo-*F* = 1.8, *p* = 0.001. Conditional effects (from the greatest to the lowest explained variation): 'September': explained variation = 4.1%, pseudo-*F* = 1.8, *p* (adj.) = 0.017; 'May': explained variation = 4.1%, pseudo-*F* = 1.9, *p* (adj.) = 0.017, and 'June': explained variation = 3.4%, pseudo-*F* = 1.6, *p* (adj.) = 0.02. Abbreviations: *AnnThu* = *Annitella thuringica*, *AmpBor* = *Amphinemura borealis*, *AmpSul* = *A. sulcicollis*, *LeuFus* = *Leuctra fusca*, *NemAvi* = *Nemoura avicularis*, *NemoCfUn* = *N.* cf. *uncinata*, *PerAffMic* = *Perlodes* aff. *microcephalus*, *PleCon* = *Plectrocnemia conspersa*, *ProHra* = *Protonemura hrabei*, *ProInt* = *P. intricata*, *ProMey* = *P. meyeri/nitida*, *RhiSem* = *Rhithrogena semicolorata*, *RhySenSt* = *Rhyacophila* sensu stricto (early instar larvae), *SerIgn* = *Serratella ignita*.

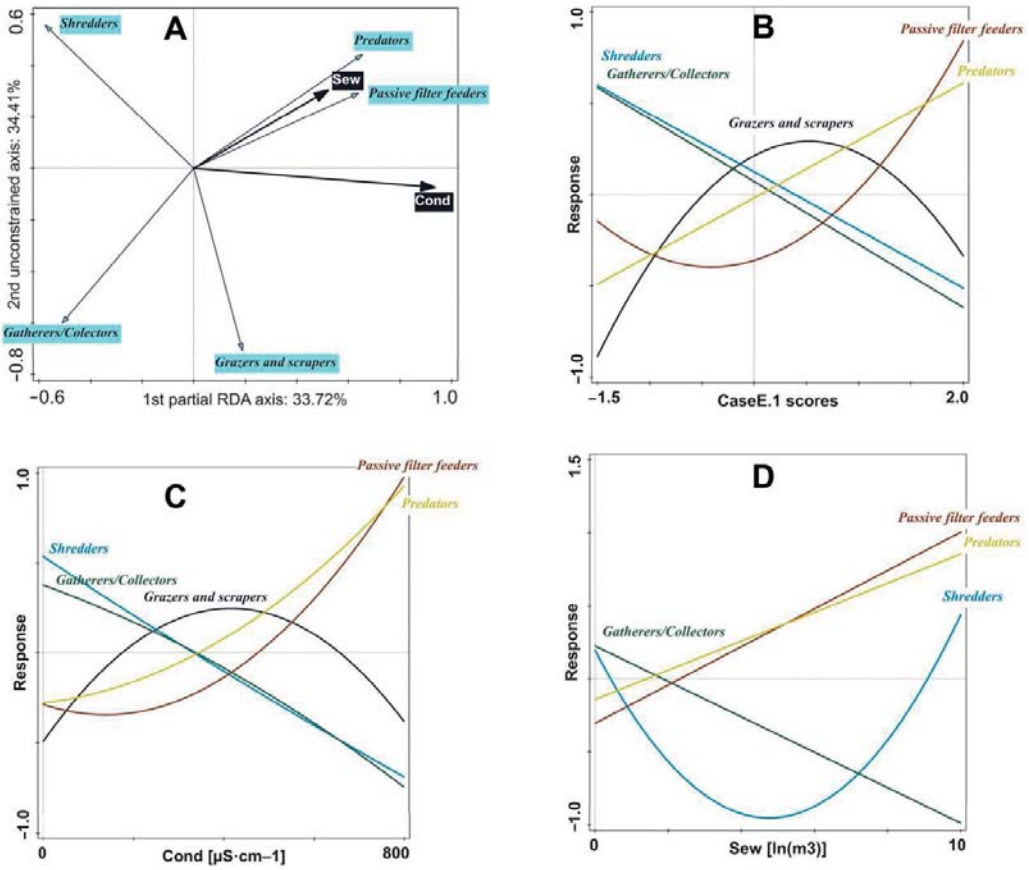

Figure S7. (**A**) Averaged feeding strategies + whole-plot environmental variables (EVs) biplot. (**B**) Plotted significant averaged feeding strategies response curves against 'CaseE.1 scores': fitted case scores of the '1st partial RDA axis' from (**A**). (**C**) Plotted significant averaged feeding strategies response curves against 'Conductivity (Cond)'. (**D**) Plotted significant averaged feeding strategies response curves against log-transformed 'Total volume of released sewage for three days prior to particular sampling time (Sew)'. (**A** and **B**) Partial Redundancy analysis (RDA) was used. The first ordination axis was constrained by the particular EVs selected by forward selection. 'Cond': explained variation = 32.5%, pseudo-F = 20.7, p (adj.) = 0.005 and 'Sew': explained variation = 6.4%, pseudo-F = 4.4, p (adj.) = 0.045. (**B**, **C** and **D**) Generalized linear models (GLMs) with gaussian distribution (with the *identity* link function) were used. Quadratic fit was chosen for the response where the fit was significantly improved compared to the linear one. See Table S8 for the statistics of relationship strengths.

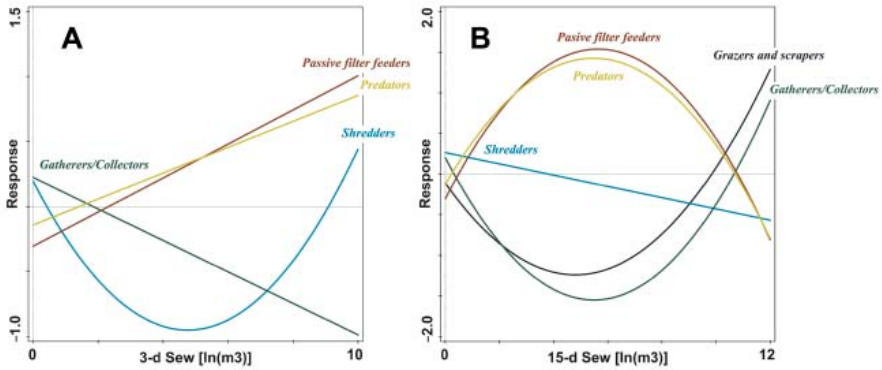

Figure S8. (**A**) Plotted significant averaged feeding strategies response curves against log-transformed 'Total volume of released sewage for three days prior to particular sampling time (3-d Sew)' (**B**) Plotted significant averaged feeding stretegies response curves against log-transformed 'Total volume of released sewage for 15 days prior to particular sampling time (15-d Sew)'. (**A** and **B**) Generalised linear models (GLMs) with gaussian distribution (with the *identity* link function) were used. Quadratic fit was chosen for the response where the fit was thus improved. See Table S8 for the statistics of relationship strengths.

Table S8. The statistics of relationship strengths for plots in Figures S7 and S8. Abbreviations: Gat = Gatheres/Collectors, Gra = Grazers and scrapers, Pff = Passive filter feeders, Pre = Predators, Shr = Shredders.

	Feeding strategies				
	Gat	**Gra**	**Pff**	**Pre**	**Shr**
	$R^2 = 30.3\%$	$R^2 = 22.4\%$	$R^2 = 44.4\%$	$R^2 = 41.9\%$	$R^2 = 23.7\%$
Fig. S7B	$F_{1,46} = 20.0$	$F_{2,45} = 6.4$	$F_{2,45} = 17.9$	$F_{1,46} = 33.2$	$F_{2,45} = 14.3$
	$p < 0.001$	$p = 0.003$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	$R^2 = 25.8\%$	$R^2 = 17.1\%$	$R^2 = 39.8\%$	$R^2 = 46.8\%$	$R^2 = 29.4\%$
Fig. S7C	$F_{1,46} = 16.0$	$F_{2,45} = 4.6$	$F_{2,45} = 14.9$	$F_{1,46} = 40.4$	$F_{2,45} = 19.1$
	$p < 0.001$	$p = 0.015$	$p < 0.001$	$p < 0.001$	$p < 0.001$
	$R^2 = 34.3\%$		$R^2 = 46.5\%$	$R^2 = 38.2\%$	$R^2 = 14.0\%$
Figs. S7D and S8A	$F_{1,46} = 24.0$	$F_{1,46} = 1.37$	$F_{1,46} = 40.0$	$F_{1,46} = 28.5$	$F_{2,45} = 3.7$
	$p < 0.001$	$p = 0.248$	$p < 0.001$	$p < 0.001$	$p < 0.034$
	$R^2 = 38.5\%$	$R^2 = 30.6\%$	$R^2 = 54.9\%$	$R^2 = 49.1\%$	$R^2 = 18.3\%$
Fig. S8B	$F_{2,45} = 14.1$	$F_{2,45} = 9.9$	$F_{2,45} = 27.4$	$F_{2,45} = 21.7$	$F_{1,46} = 10.3$
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.002$

CHAPTER 4

SIGNAL CRAYFISH AS A THREAT FOR EUROPEAN ECTOSYMBIONTS – OVERLOOKED BIODIVERSITY LOSSES

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My share on this work was about 35%

Signal crayfish as a threat for European ectosymbionts – overlooked biodiversity losses

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ABSTRACT

The decline of native crayfish biodiversity caused by introductions of invasive crayfish has been well documented; however, the fate of their associated biota remains overlooked. Native European branchiobdellids (Annelida: Clitellata) – crayfish ectosymbionts – were monitored in the Czech Republic at three localities with past or present sympatric occurrence of the native noble crayfish *Astacus astacus* (AA) and invasive signal crayfish *Pacifastacus leniusculus* (PL). No branchiobdellids were detected in two localities with abundant populations of PL but with absences of AA, although they were recently recorded there. Two species of branchiobdellids (*Branchiobdella parasita* and *B. pentadonta*) were detected on both crayfish species in the locality where AA still coexists with PL. However, PL harboured a significantly lower abundance of both branchiobdellids than AA (80.7% and 90.8% decrease, respectively) as well as a significantly lower number of their attached cocoons (95.7% and 95.0% decrease, respectively). Nevertheless, the abundance of branchiobdellids was positively correlated with the carapace length in both crayfish species. Other potential predictors related to crayfish biological parameters (e.g. sex or missing appendages) were not significantly related to the abundances of both branchiobdellids or their cocoons. We also performed laboratory experiments with both branchiobdellids distributed on size-matched individuals of AA and PL to verify the results of field observations. The probability for branchiobdellid survival was significantly higher on AA in both branchiodellids for all the time of the experiments. The absence or decreased abundances of the native branchiobdellids in the invasive crayfish suggest their limited capability to deal with the replacement of host species.

Keywords: *Astacus astacus*, *Branchiobdella*, ectosymbionts, host switch, *Pacifastacus leniusculus*

1. Introduction

Anthropogenic translocation of species with subsequent biological invasions is considered among the major threats causing biodiversity alterations and generating ecological and socioeconomic impacts worldwide (Rodríguez et al., 2005; Dudgeon et al., 2006; Gallardo et al., 2016; Diagne et al., 2020). Several crayfish species have been introduced worldwide into the aquatic environment through their utilisation in fishery and aquaculture (Hobbs III et al., 1989; Twardochleb et al., 2013; Henttonen and Huner, 2017). Highly invasive populations of non-native crayfish established in Europe contribute to the continuing drop in native European crayfish populations (de Almeida, 2013; Kouba et al., 2014). While most of the attention was focused on investigating a direct or indirect impact from invasive American crayfish on native European crayfish species (Holdich and Domaniewski, 1995; Holdich et al., 1995) or other biotas (Guan and Wiles, 1997; Ruokonen et al., 2014), less attention has been paid to the fate of native crayfish symbionts including Branchobdellidans (Annelida: Clitellata) – ectosymbionts of northern hemisphere freshwater decapods (Gelder, 1999). European species are represented by a single genus, *Branchiobdella*, whilst North American species belong to ten genera, e.g. *Xironogiton*, *Cambarincola*, *Ankyrodrilus*, and *Pterodrilus* (Brinkhurst and Gelder, 1991; Williams et al., 2013). Their presence can favour crayfish fitness, increase growth rate and reduce mortality since they clean the crayfish body surface and gills from growths of fouling epibionts (Brown et al., 2002; Brown et al., 2012; Farrell et al., 2014).

Although several cases have been reported of the presence of North American branchiobdellidans on non-native crayfish species outside their native range (Gelder et al., 1994; Gelder, 1999; Gelder et al., 2012; James et al., 2015), reports about native European branchiobdellids on non-native crayfish species remain scarce (Gelder, 1999; Ďuriš et al., 2006; Bláha et al., 2018). A necessary prerequisite is a sympatric occurrence of infested native and non-native crayfish species. Non-native hosts could serve native branchiobdellidan symbionts, presuming their host opportunity; however, no comparison of branchiobdellid infestations between alien and indigenous crayfish species have been published. A few studies from North America were focused on the impact of crayfish invasion on indigenous branchiobdellids (Bailey, 2017; Creed et al., 2021). Creed et al. (2021) reported a decline of *Cambarincola ingens*, the native ectosymbiont of the crayfish *Cambarus chasmodactylus*, after the introduction of the crayfish *Faxonius cristavarius* and linked it to the dilution effect (originally proposed to describe the negative effect of increased host diversity on parasite abundance). In addition, branchiobdellidans actively discriminate between sympatric hosts (Brown and Creed, 2004). Many factors may drive the decision whether to switch to another host or stay on the original host. Most of these factors could be linked to the potential host's behaviour and general activity (Brown and Creed, 2004; Farrell et al., 2014; Skelton et al., 2015). The potential switching ability is ultimately demonstrated when the native host is extinct.

Therefore, we studied branchiobdellid assemblages in the invasive *Pacifastacus leniusculus* (PL) populations that previously co-occurred or still co-occur with the populations of native *Astacus astacus* (AA). Although the negative impact of alien crayfish species on native branchiobdellid assemblages was presumed, we hypothesised that either there is (i) no effect of alien crayfish species on native branchiobdellid assemblages, or (ii) alien crayfish have an effect on branchiobdellid assemblage, and thus native branchiobdellid abundances are more likely altered in sites with a sympatric occurrence of PL and AA and sites inhabited only by PL.

2. Materials & methods

2.1. Field study

2.1.1. Sampling sites

The occurrence of branchiobdellids on native AA and invasive PL individuals from sympatric populations was investigated at three different Czech localities in 2021. The first locality is a pond situated on the Šebkovický brook in Čáslavice, Vysočina (49.1478761N, 15.7545950E), previously investigated in 2008 (Bláha et al., 2018) and reinvestigated for this study in May and October 2021 (Figure 1). The second locality is Žďárka brook near Chlumek, Vysočina (49°22'10.5"N, 15°51'12.1"E), previously investigated in 2013 (Jurek, 2014), and reinvestigated for this study in October 2021 (Figure 1). The third locality is Křesánovský brook, situated in South Bohemia near the Šumava National Park (N 49.0631294, E 13.7549014), which was monitored in May 2021 for this study (Figure 1).

Šebkovický brook and the Nový pond were originally settled by AA. After the poisoning of the pond with insecticides inappropriately applied on the nearby field, the AA population was presumed extinct. This stimulated the introduction of PL juveniles to the pond in 1980, imported from Sweden. The Nový pond was one of a few places where this species was first introduced in the country. However, the AA population also recovered from the upper section of the brook, and a sympatric population of these crayfish species was established. The AA population gradually declined, but it still remains present in a low abundance in the pond (J. Gleta, personal communication, 2022). PL strongly dominates and is spreading in the catchment. In our study, three areas were investigated: (i) a 30 m stretch of the brook upstream of the pond (capture by hand-net); (ii) the pond – stony dam (14 traps baited with fish meat); and (iii) a 30 m stretch of the brook downstream of the pond (hand-net capture).

Žďárka brook was originally settled by AA; nevertheless, PL was introduced into one of the connected ponds most likely after 1980. In 2013, a dominance of PL and most probably the last few individuals of AA in bad condition were recorded by Jurek (2014). For this study, 100 m of the brook was investigated for actual crayfish and branchiobdellid presence using hand-net capture.

Similarly, the Křesánovský brook was originally settled by AA. However, a small pond on the brook most probably served as a source of PL more than 10 years ago, deduced from the abundance of PL. For the present study, the Křesánovský brook was divided into two main areas according to the presence of PL: (i) the upper stretch (upstream, approx. 150 m long stretch) – the zone inhabited by both crayfish species (mixed zone/invasion front), and (ii) the lower stretch (approx. 450 m long) – the zone where the PL population is settled (core zone), with a rare occurrence of AA. The lower stretch of Křesánovský brook was regularly monitored (hand-net capture) each month from April to October between 2018 and 2021. All caught PL individuals were measured, weighed and removed from the brook (Table 1). In total, 13,359 individuals of PL were captured and removed.

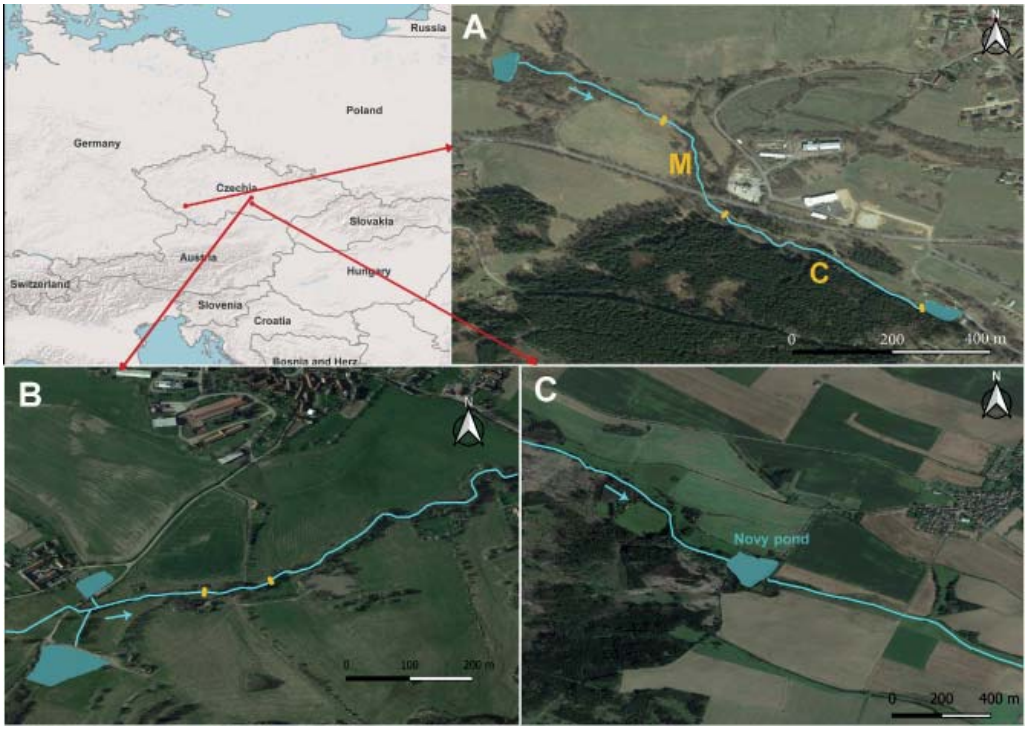

Figure 1. *Map of sampling sites, the Křesánovský brook (A) with the core (C) and mixed zone (M), (B) the Žďárka brook and (C) the Šebkovický brook upstream and downstream of the Nový pond. Blue arrows indicate the direction of water flow.*

2.1.2. Data collecting and manipulation with animals

As AA is a critically endangered species, a permit was needed to handle it at the Křesánovský brook (Czech National Council Act No. 114/92 on the Protection of *Nature* and the Landscape, permit No. KUJCK 150766/2017 OZZL). *Branchiobdella parasita* and *B. pentadonta* and their egg cocoons (hereinafter referred as 'cocoons') were determined and quantified directly after each crayfish was caught. Due to the relatively distinctive morphological differences between adult specimens of these species, they were identified by the naked eye. Cocoons of both species were similarly distinguished according to their different size. Nevertheless, some branchiobdellids were collected from both crayfish species for subsequent morphological and molecular confirmation of species identity. Basic morphometric measurements were taken for each crayfish individual (carapace length – CL and weight – W). Crayfish were sexed and screened for missing or regenerating claws. After investigation, specimens of AA were released at the place where they were initially caught. The invasive PL was taken out from the locality and used for experimental or educational purposes at the Faculty of Fisheries and Protection of Waters in Vodňany (FFPW).

2.2. Morphological and molecular confirmation of branchiobdellid identity

Ethanol-preserved specimens were taken to the laboratory at FFPW and were morphologically determined according to the taxonomic key by Nesemann and Neubert (1999). The head was dissected for microscopic determination of jaw morphology. The rest of the body was used for DNA extraction using the E.Z.N.A.® Tissue DNA Mini Kits (Peqlab, Erlangen, Germany). A part of the mitochondrial gene Cytochrome c oxidase subunit I was amplified using universal PCR primers LCO1490 and HCO2198 (Folmer et al., 1994). PCR products were purified with NucleoSpin® (Macherey-Nagel, Düren, Germany) and sequenced on an ABI automatic capillary sequencer (series 373) (Macrogen, Seoul, South Korea) using amplification primers. Nucleotide BLAST web service (Sayers et al., 2019) and Neighbor-Joining methods implemented in GENEIOUS (Kearse et al., 2012) were used to match sequences to particular branchiobdellid species.

2.3. Laboratory experiment

2.3.1. Animals

Individuals of PL were caught by hand nets in the Křesánovský brook, and AA individuals were captured in crayfish traps in the forest pond Zlatý in Písecké Hory (49.2234911N, 14.2771547E). Crayfish were transferred to the experimental facility of the FFPW. They were measured for basic morphometry (CL and W) and treated in carbonated water to remove potential branchiobdellids. After 2 minutes of exposure, crayfish were visually checked for branchiobdellid presence and kept individually in the recirculation aquaculture system.

Branchiobdellids were collected from AA individuals caught in localities with high fouling environments. Individuals of *B. parasita* were obtained in the outflow channel from a carp culture pond (49.0223733N, 14.1479125E) in the South Bohemia region in July 2021. *B. pentadonta* individuals were collected from a pond in the village Svatoslav, South Moravia (49.3051444N, 16.3078714E) in August 2021. In both cases, branchiobdellids were gently wiped from collected crayfish hosts and transferred in plastic boxes to laboratory incubators in the experimental facility of FFPW. Branchiobdellids were kept there under constant conditions (temperature 20 °C, 12/12 light and dark regime) until their experimental use.

2.3.2. Experimental design

Ten individuals of both crayfish species were individually placed in 60 L tanks and kept in standardised conditions (temperature 20 °C, 12/12 light and dark regime) and fed once per two days with carrot and chironomid larvae. Positions of individual crayfish species in the tanks were randomised in space to ensure the independence of observations. After one week of acclimatisation, a small petri dish containing ten specimens of *B. parasita* was added to every tank containing crayfish. The number of branchiobdellids on crayfish was documented after 2, 98, 314, 410, 506, and 602 hours. The eventual moulting of crayfish was recorded. After this experiment, the remaining branchiobdellids were removed from the crayfish individuals. The crayfish individuals were reused for a second infestation experiment, where 20 *B. pentadonta* individuals were added into every crayfish tank, and their number on crayfish was recorded after 2, 170, 338, and 506 hours.

2.4. Statistical analysis

All statistical tests were performed in R software (CRAN, version 4.1.1) using basic default libraries, *effects*, *lme4* and *pwr* libraries. All plots were made in R, using the *ggplot2* library.

2.4.1. Field study

Abundances of branchiobdellids

Several explanatory variables (related to caught crayfish individuals), including categorical variables: (i) 'crayfish species' (AA and PL), (ii) 'sex' (male and female), and (iii) 'conditions' (presence of eggs, one claw missing, one claw regenerated, one claw regenerated and second claw missing, and reference – no eggs and no claw missing or regenerated), as well as numerical variables: (iv) 'CL' [mm] and (v) 'W' [g], were tested for their significance as predictors of shifts in individual abundances of the branchiobdellid species (*B. parasita* and *B. pentadonta*) and their laid cocoons. Generalised linear models (GLMs) and generalised linear mixed-effects models (GLMMs) with an assumed Poisson distribution (the log link function applied) were handled for these purposes. In cases of models with overdispersion, the quasi-likelihood estimation method was applied in GLMs. Similarly, the overdispersion was fitted as a random variability at the level of individual observations in GLMMs. Differences between the particular crayfish species in response variables and mutual first-order interactions between 'crayfish species' and the other predictors were of the main interest in this study tested by partial ^{2} tests or *F*-tests.

Relationships within branchiobdellid communities

Potential differences in relationships within branchiobdellid communities sampled on different crayfish species (AA and PL) were estimated using six second-order partial correlations applied to detrend relationships given by assumed inter-community constraints. Partial correlations were calculated for each crayfish species as well as the whole dataset of both species together (AA + PL) using four sets of response variables: (i) '*B. parasita* abundance', (ii) '*B. parasita* cocoon abundance', (iii) '*B. pentadonta* abundance', and (iv) '*B. pentadonta* cocoon abundance'. All variables were log+1 transformed. Since none of the given response variables fulfilled a requirement of data normality ($p<0.05$; tested by Shapiro-Wilk test of normality), non-parametric methods for partial correlation calculation, Spearman and Kendall, tests were applied. The approximal test power (1–β) was calculated for each relationship using Pearson's r to decide on tested hypotheses. For visualisation of individual relationships, partial regression plots (added-variable plots) were computed, where sample-specific residuals from two linear regressions ($\varepsilon_1 = Y_1 - \beta_0 - \beta_1 X_1 - \beta_2 X_2$ and $\varepsilon_2 = Y_2 - \beta_0 - \beta_1 X_1 - \beta_2 X_2$) were plotted against each other.

2.4.2. Laboratory experiment

Effects of 'crayfish species', 'sex', 'CL', 'W', 'moulting' (not moulted/moulted), and 'time' [h] on the probability of *B. pentadonta* to survive (observed number related to the initial number) was tested using GLMM with assumed binomial distribution with the logistic link function. Since observations were made on the same individuals over time (repeated-measurements design), 'identity of individuals' was used as a random-effect factor. Data from the experiment with *B. parasita* were analysed in the same way; however, only the 'crayfish species', 'time',

'moulting' and 'identity of individuals' were noted. The partial effects of particular variables were tested using the χ^2 test.

Effects of 'crayfish species' and 'branchiobdellid species' (*B. parasita* and *B. pentadonta*) on the probability of initial success of infestation were analysed using GLM with assumed binomial distribution with the logistic link function. The success was calculated as number of branchiobdellids of each species observed on individual crayfish related to the total number of branchiobdellids (sum of branchiodellids 'on' and 'off' each crayfish) after 2 hours from the start of the experiments. Since massive overdispersion was detected (Dispersion parameter ≤ 5.28) a quasi-likelihood estimation method was applied.

3. Results

3.1. Field study

No crayfish were caught in the Čáslavický pond and only PL individuals with no branchiobdellids attached were recorded in Šebkovický brook as well as in Ždárka brook. A similar situation was observed in Křesánovský brook in the core zone site, where all caught PL had no branchiobdellids attached. In the mixed zone, 36 individuals of AA and 44 individuals of PL were caught and surveyed for branchiobdellid presence. Two species, *B. parasita* and *B. pentadonta* were identified on both crayfish species. Significantly higher abundances of both branchiobdellid species were found on AA than on PL (Tables 1 and 2).

Table 1. *The number of crayfish caught and their biological parameters; mean, standard deviation and min.-max. of branchiobdellids, and their cocoons from crayfish individuals between sites of investigated localities.*

Locality	Stretch	Species	No. of inds.	CL (mm) ± SD	W (g) ± SD	Sex ratio	*B. parasita*		*B. pentadonta*	
						M:F	Mean counts of inds. ± SD	Mean counts of cocoons ± SD	Mean counts of inds. ± SD	Mean counts of cocoons ± SD
The Šebkovický brook	Upstream	PL	6	36.2±6.9	19±15.2	5:1	–	–	–	–
	Pond	–	–	–	–	–	–	–	–	
	Downstream	PL	56	38.7± 7.3	23±13.7	34:22	–	–	–	–
The Žďárka brook	–	PL	72	–	–	–	–	–	–	–
The Křesánovský brook	Mixed zone	AA	36	29.3±8.6	8.3±8.4	22:14	2.0±3.1	17.3±17.9	5.9±12.5	21.2±38.2
		PL	44	30.6±6.2	9.9±6.5	19:25	0.4±0.6	3.3±6.5	0.7±2.8	3.6±11.3
	Core zone	PL	494	21.1±10.1	5.3 ±9.3	261:233	-	-	-	-

3.2. Molecular and morphological determination of branchiobdellids

The occurrence of two European native branchiobdellid species, *B. parasita* and *B. pentadonta*, was confirmed on both crayfish species from the mixed zone of the Křesánovský brook. Morphology of jaws clearly showed the affiliation to particular species (Figure 2), also corroborated by molecular identification. We identified one haplotype of *B. parasita* GenBank accession number OM250467, which BLAST analysis assigned 92.38 % similar to FJ655048 *B. parasita* sequence (Füreder et al., 2002), while four haplotypes of *B. pentadonta* (OM250468–471) were identified. BLAST analysis assigned these four haplotypes as highly similar to *B. italica* (FJ655027, FJ655064; Füreder et al., 2002) and *B. pentadonta* sequences (FJ655023; Füreder et al., 2002; 98.78–99.29 and 98.5–99.11%, respectively); however, Neighbour-Joining clustering analysis assigned our haplotypes to the cluster of *B. pentadonta* sequences (Figure 2).

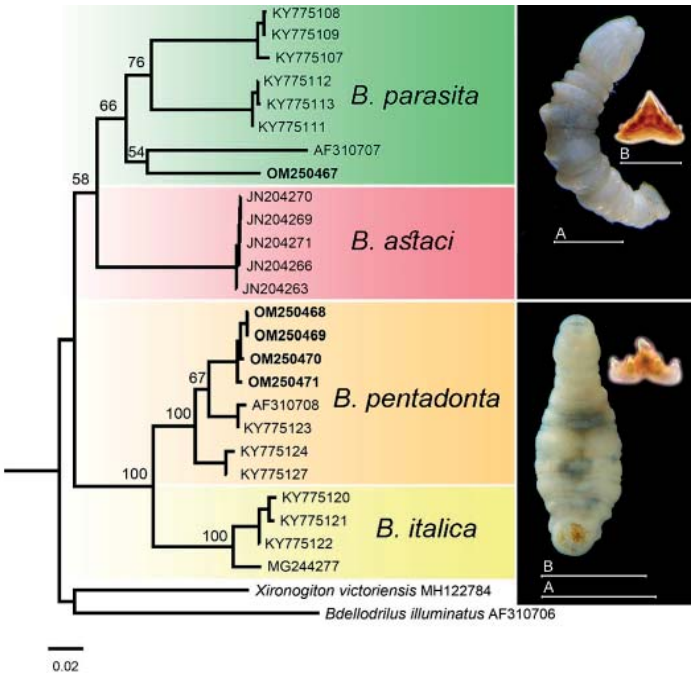

Figure 2. *Phylogram based on COI sequences depicting relationship and species assignment of our sequences (written in bold) to particular Branchiobdella species displayed at the right side of the phylogram Branchiobdella parasita (upper) and B. pentadonta (lower) body shape and claw morphology. Scale A and B represent 1 mm and 50 m and refer to the body and claw size, respectively.*

In both crayfish species, *B. parasita* specimens were mostly attached on the backs of the carapace or the sides of the first abdominal segment. This species was only rarely found on the dorsal part of the rostrum of AA (Figure S1). *B. parasita* was observed on crayfish individuals with CL>26.1 and >16.1 mm on PL and AA, respectively. Their cocoons were laid mainly on the backs of both crayfish species' carapaces, between pleopods, and between the bases of walking legs. On the other hand, individuals of *B. pentadonta* and their cocoons were primarily found on the ventral side of chelipeds of both crayfish species, and occasionally on the dorsal side of chelipeds. Individuals of *B. pentadonta* were observed on AA with CL>23.0 mm. *B. pentadonta* was identified on PL with CL>29.0 mm; however, higher infestation by this species was observed only on the biggest PL individual (CL>48.2 mm).

3.3. Abundance of branchiobdellids

There were significant differences between crayfish species in abundances of both branchiobdellid species and their laid cocoons and a significant positive correlation between CL and abundances of both branchiobdellid species and their cocoons in both crayfish species ($p<0.05$; Table 2). Abundances of both branchiobdellids and their cocoons found on PL were significantly lower compared to AA ($p<0.05$) – from 80.7% decrease in *B. parasita* up to 95.7% decrease in *B. parasita* cocoons, according to the GLMM regression coefficients (Table 2). There was no significant interaction between crayfish species and CL in all tested abundances ($p>0.05$; Table 2). Of the remaining predictors – sex and conditions – were not significant ($p>0.05$; Table 2), except for testing the abundance of *B. parasita*. The categorical variable conditions (missing claws, etc.) had a significant effect ($p<0.05$; Table 2); however, this result was only approximated because the particular model showed lack of convergence (Table 2). The tested interaction between crayfish species and conditions was not significant ($p>0.05$), and this result is approximate as well (Table 2). Significant relationships fitted by GLMs are visualised in Figure 3.

Table 2. *Results of χ^2 tests performed within Generalised linear mixed-effects models (GLMMs) and fitted regression coefficients for significant variables ($p<0.05$). Table 2 continues on the next page.*

	Crayfish species (AA and PL)				Carapace length – CL (mm)			
Response variable	**χ^2**	**df**	***p*-value**	**Fitted differences (range of SE)**	**χ^2**	**df**	***p*-value**	**Fitted estimates (range of SE)**
Branchiobdella parasita abundance	24.23	1	<0.001	80.7% (73.0–86.2%) lower in PL	18.96	1	<0.001	8.6% (6.7–10.6%) increase per each mm
Branchiobdella pentadonta abundance	15.77	1	<0.001	90.8% (83.9–94.7%) lower in PL	38.72	1	<0.001	27.9% (23.1–32.8%) increase per each mm
Branchiobdella parasita cocoon abundance	24.21	1	<0.001	95.7% (91.2–97.9%) lower in PL	12.56	1	<0.001	16.4% (11.4–21.6%) increase per each mm
Branchiobdella pentadonta cocoon abundance	5.57	1	0.02	95.0% (82.0–98.6%) lower in PL	19.22	1	<0.001	54.3% (40.8–69.0%) increase per each mm

	Sex			Conditions				Interaction between crayfish species and carapace length			Interaction between crayfish species and conditions		
Response variable	**χ^2**	**df**	***p*-value**	**χ^2**	**df**	***p*-value**	**Fitted difference**	**χ^2**	**df**	***p*-value**	**χ^2**	**df**	***p*-value**
Branchiobdella parasita abundance	1.89	1	0.17	13.56	4	0.01	Lack of convergence	0.14	1	0.71	1.57	3	0.67
Branchiobdella pentadonta abundance	2.07	1	0.15	2.64	4	0.62	---	0.10	1	0.75	---	–	---
Branchiobdella parasita cocoon abundance	---	---	---	6.41	4	0.17	---	0.56	1	0.46	---	–	---
Branchiobdella pentadonta cocoon abundance	0.36	1	0.55	1.87	4	0.76	---	0.65	1	0.42	---	–	---

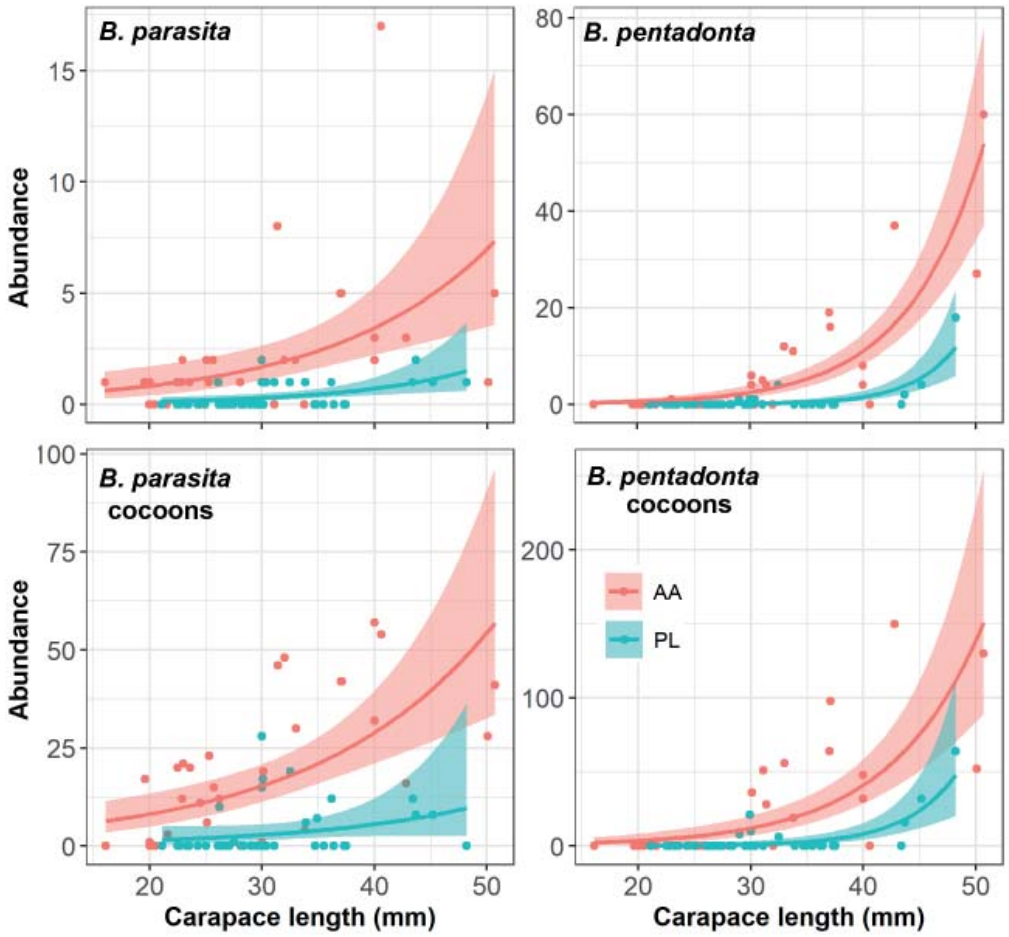

Figure 3. *Significant effects of Crayfish species (AA = Astacus astacus, PL = Pacifastacus leniusculus) and Carapace length (mm) on abundances of two branchiodellid species and their cocoons. Generalised linear models (GLMs) with assumed Poisson distribution with the log link function were employed (regarding the value of dispersion parameters, the quasi-likelihood estimation method was applied). Light red and blue areas represent particular 95% confidence regions.*

3.4. Relationships within branchiobdellid communities

Fitted relationships between abundances of both branchiobdellid species and their cocoons revealed hardly any stronger competition in reproduction between *B. parasita* and *B. pentadonta*, neither on AA nor on PL. The abundances of both branchiobdellid species were positively correlated ($p<0.05$) to the abundances of their cocoons ($\rho>0.70$ in *B. parasita* and $\rho>0.73$ in *B. pentadonta*; Figure 4). However, there could be potential differences between crayfish species in some relationships supported by the statistical results. Since the abundances of both branchiobdellid species and their cocoons were significantly higher in AA compared to PL, it cannot be rejected that larger-sized *B. parasita* in higher abundances may slightly oust *B. pentadonta* (or vice-versa), regarding their negative correlation ($p>0.05$) and weak test power

(β = 0.41) on AA. Contradictorily, their relationship on PL was positive and significant according to the non-parametric methods (p<0.05). The remaining correlations are not supported by stronger test power either in one species (β>0.41); thus, it is no more interpreted.

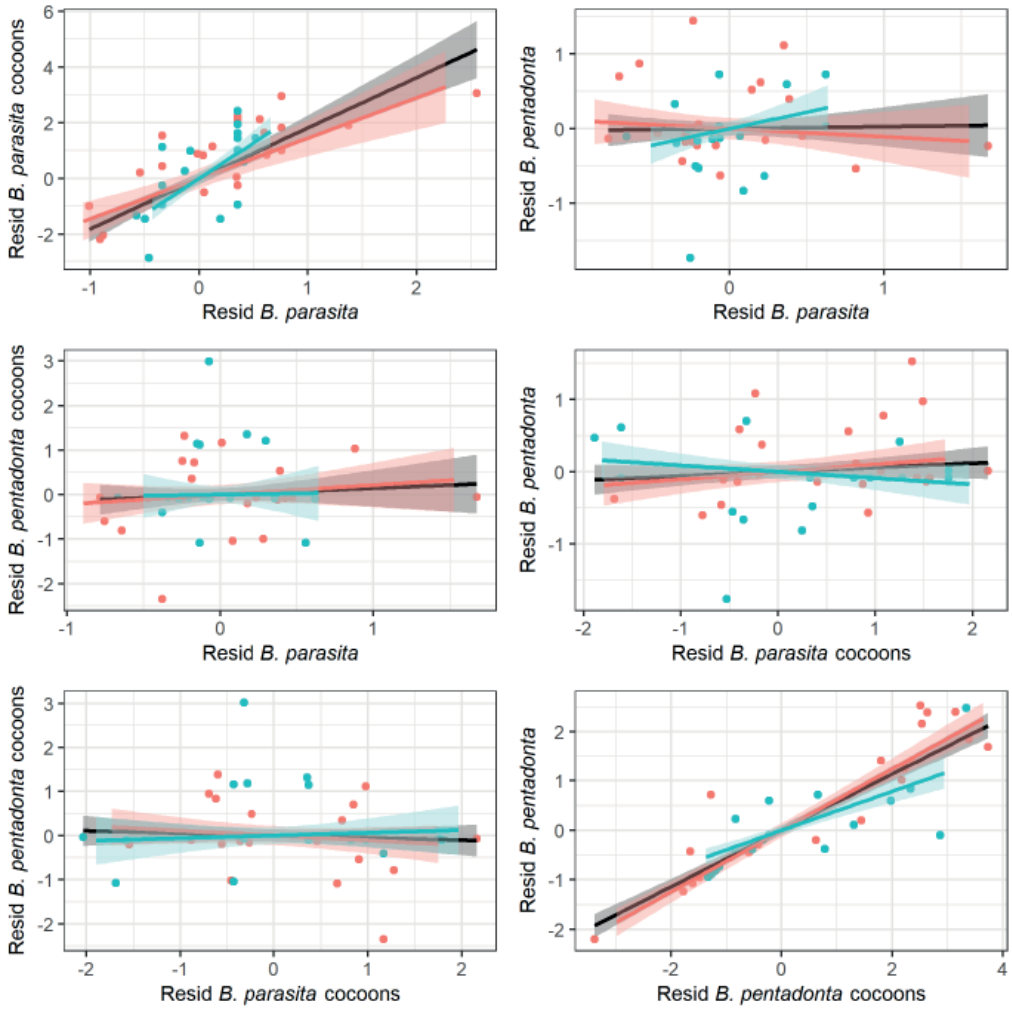

Figure 4. *Partial regression plots (added-variable plots) constructed based on log+1 transformed abundances of two branchiodellid species and their cocoons sampled on native noble crayfish Astacus astacus (AA; red lines and points ; n=36) and on invasive signal crayfish Pacifastacus leniusculus (PL; blue lines and points; n=44). Black lines represent common relationships (AA+PL). Shaded areas represent 95% confidence region. Abbreviation: Resid = residuals.*

3.5. Laboratory experiment

The numbers of *B. pentadonta* and *B. parasita* were significantly affected by crayfish species (χ^2_1=19.96, p<0.001; χ^2_1=9.03, p=0.003, respectively) and time (χ^2_3=184.30, p<0.001; χ^2_5=117.36, p<0.001, respectively), whilst the effect of the interaction between crayfish species and time was significant only for *B. pentadonta* (χ^2_3=12.23, p=0.007; Figure 5). In

addition, we found a significant negative effect of moulting (χ^2_1=13.07, p=0.003) on the number of attached individuals of *B. pentadonta*; the 67.5% (95% confidence intervals: 58.1–74.6%) decrease in the crayfish after moulting was fitted. The effects of sex, carapace length and weight were not significant (however, the sizes of crayfish were similar contradictory to the field study) .

We found no significant differences between crayfish and branchiobdellid species in initial infestation success ($F_{1,32}$=0.09, p=0.764 and $F_{1,32}$=3.11, p=0.088, respectively).

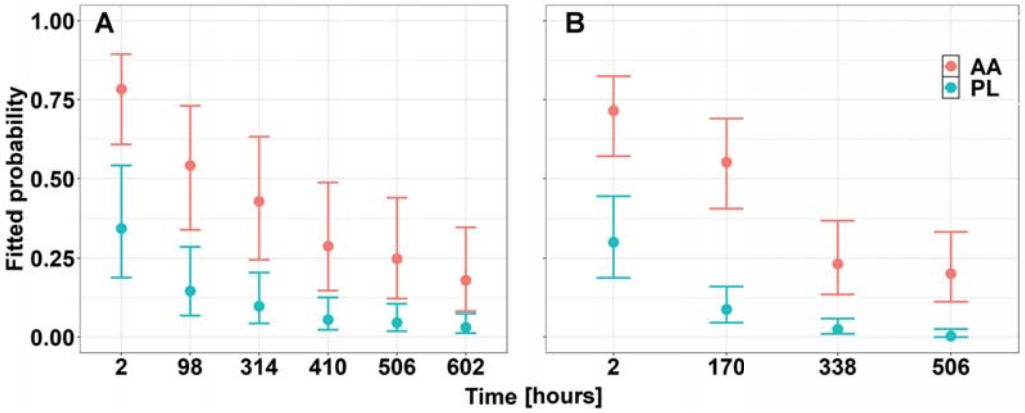

Figure 5. *Relationships between crayfish species – Astacus astacus (AA) and Pacifastacus leniusculus (PL) – over time in fitted proportions related to the initial number of branchiobdellids: (A) Branchiobdella parasita – the initial number was 10, and (B) B. pentadonta – the initial number was 20. The whiskers correspond to the 95% confidence intervals.*

4. Discussion

The native branchiobdellid ectosymbiont assemblage on crayfish was surveyed at three sites with the sympatric occurrence of native *Astacus astacus* and invasive *Pacifastastacus leniusculus.* In contrast to the situation in 2008 (Bláha et al., 2018), Šebkovický brook was inhabited only by a population of PL during our reinvestigation in 2021. Furthermore, no branchiobdellids were found there. We can only presume that the population of AA is gone or more likely, that the current situation of the population is below the limit of observation. This pattern also corresponds with the finding of our study that PL can get rid of native branchiobdellids more effectively than AA. This can result in the disappearance of native branchiobdellids if native crayfish as a source of native branchiobdellids become extinct. A similar situation was observed at the second surveyed locality, the core zone of invasion in the Křesanovský brook. Here, we reported a very dense population of PL (over 10 ind.m^{-2}), but with no branchiobdellids attached. The core zone of this brook has been surveyed for four years, and no attached branchiobdellids have been found there, despite the enormous number of potential PL hosts (>13,300 individuals, unpublished data). The only branchiobdellid evidence was taken on very rarely captured AA, probably migrated from upper stretches. There is undoubtedly some possibility of branchiobdellid transfer from those AA individuals to PL but this did not result in PL infestation.

Our observations are consistent with others (Dražina et al., 2018), who report a clear separation of epifauna (besides branchiobdellids) between native and alien crayfish species in Croatia. Although, only native European species of branchiobdellids (*B. astaci*, *B. italica*, *B. parasita*, and *B. pentadonta*,) were found on native crayfish species (AA and *Austropotamobius*

pallipes), while no branchiobdellids were found on alien crayfish *Procambarus virginalis*. Moreover, only one species native to North America, *Xironogiton victoriensis,* was found on PL caught in Mura river, where no AA has been reported since 2007 (Hudina et al., 2009).

Despite that, the transmission of branchiobdellids between sympatric species is highly variable, most probably driven by cost/benefit relationships and explained in terms of fitness outcomes for individual ectosymbionts (Brown and Creed, 2004; Skelton et al., 2015). It is well known that non-native crayfish species in Europe are considered more active even during the day than their native counterparts (Lozán, 2000; Styrishave et al., 2007; Musil et al., 2010). Also, the grooming behaviour in non-native crayfish species could play a substantial role in the long-term survival of branchiobdellid assemblage (Brown and Creed, 2004). These facts most likely caused an absence of branchiobdellid species in the core zone of Křesánovský brook, while in the mixed zone, where both crayfish species are still in permanent contact, an ectosymbiont assemblage can occur, continually supplemented by new material from native crayfish individuals. Hence the branchiobdellids persist there on PL, although to a significantly lesser extent.

It is not surprising that the abundances of both branchiobdellid species increased with increasing crayfish carapace length. The larger available surface can explain this trend observed in both crayfish species for colonisation by branchiobdellids and for other fouling epibionts serving as their food (Skelton et al., 2014; Skelton et al., 2016). In addition, crayfish juveniles, in general, have a higher moulting frequency which, together with higher regulation of ectosymbionts (Thomas et al., 2016), could result in a lower abundance of branchiobdellids. Nevertheless, some interspecies differences in infestation of both crayfish species by branchiobdellid species were recorded in this study, probably suggesting different biological parameters between AA and PL. No specimens of *B. parasita* were observed on PL individuals <26.1 mm CL, while AA individuals were found infested by *B. parasita* from 12.1 mm CL. In addition, PL individuals hosted a maximum of two individuals of *B. parasita*, contrasting with up to 17 individuals on AA. Altogether, 32 individuals of *B. pentadonta* were found on PL, out of which 18 individuals infested the largest recorded PL individual (CL 48.2 mm). *B. pentadonta* was not recorded on smaller individuals of PL (CL<29.0 mm), while in the case of AA, *B. pentadonta* was found on individuals from 23.0 mm CL. Lowered abundances of *B. parasita* (by 80.7%) and *B. pentadonta* (by 90.8%) on PL suggest intolerance between non-native hosts and native ectosymbionts. Infestation of only larger PL compared to AA individuals and lowered branchiobdellid abundances can also reflect the higher growth rate and thus more frequent moulting in PL (Westman et al., 2002; Buřič et al., 2021), as well as a higher grooming rate in PL observed in the experimental infestation. PL in its native range serves as a host for branchiobdellids of the genus *Cambarincola* (Vogt, 2017), which most probably could rule out its general intolerance to ectosymbionts. A differently structured exoskeleton in PL and native AA must also be considered. The surface of the carapace, claws, and abdomen of PL is smooth compared to that of AA (with abundant tubercles and a few spines; Souty-Grosset et al., 2006). These body parts are usually inhabited by *B. parasita* and *B. pentadonta* (Nesemann and Neubert, 1999). That also means relatively less space for fouling epibionts that serves as their food (Dobrzycka Krahel et al., 2022), and with accessibility for grooming together, fewer options for branchiobdellids to hide from being eaten by the host.

Together with reduced abundances of both branchiobdellid species on PL, the number of branchiobdellid cocoons on PL was lower by 95.7% in *B. parasita* and 95.0% in *B. pentadonta* compared to AA. However, it suggests some capability of branchiobdellids to reproduce on alien crayfish species. In fact, this ability can be very limited by the factors mentioned above and thus can lead to extirpation of native branchiobdellids, as confirmed at three investigated localities.

Thomas et al. (2016) reported intraguild predation between two North American branchiobdellid species of the genus *Cambarincola*. Contrastingly, we found only a non-significant correlation between abundances of *B. parasita* and *B. pentadonta* on both crayfish species despite *B. pentadonta* being a relatively smaller species than *B. parasita*. Our observation suggests their relationship was rather neutralistic.

We can only speculate about the dilution effect described by Creed et al. (2021) caused by a new intruder in the case of PL, where the higher level of host biodiversity could harm one or both partners by reducing the number of competent hosts available for ectosymbiont dispersal. This could be obvious in the core zone in Křesánovský brook, where no branchiobdellids were detected, and in the contact zone, where both crayfish species hosted both branchiobdellids. Unfortunately, we do not have data about branchiobdellid species numbers from the upstream zone that contained only AA. These data could help us better understand and suggest a dilution effect, which could decrease symbiont populations and, therefore, decrease beneficial services for the host.

Our laboratory and field observations showed PL to be more effective in grooming behaviour to reduce branchiobdellid assemblages. It seems that grooming behaviour is species-specific, and the level of infestation which triggers intensive grooming varies strongly (Farrell et al., 2014). European native crayfish species are generally known to show lower activity (Twardochleb et al., 2013) compared to non-native species, including grooming behaviour, which was found to be more intensive in American crayfish species and could be caused by much higher branchiobdellid diversity in North America and by parasitic behaviour of some species to their host (Gelder et al., 1999). Given the reported transfer of European native branchiobdellids onto alien crayfish species (Gelder et al., 1999; Ďuriš et al., 2006; Bláha et al., 2018) and based on our field study and laboratory experiment, we suggest putting similar observations into the context of long-term monitoring.

A combination of all the factors mentioned above likely plays a role in the lower abundance of branchiobdellids in PL, resulting in the gradual extinction of branchiobdellids in well-settled populations of PL which have replaced indigenous crayfish species. The indigenous biodiversity almost invisibly declines due to the displacement of indigenous AA and their ectosymbionts *B. parasita* and *B. pentadonta*. It is reasonable to believe that similar inconspicuous processes lead to losses of many native species, which remain overlooked. A deep understanding of such invaders' effects is crucial for saving the gene pool of native organisms, even those not important at first sight. This study should contribute to knowledge about the interaction between non-native hosts and native ectosymbionts and highlight possible newly identified threats to the native branchiobdellid species.

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Ethics approval: The authors declare that they have strictly followed all the rules and principles of ethical and professional conducts while completing the research work.

Consent to publication: All the authors have agreed to be listed as per the order mentioned in the manuscript.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Supplementary materials

Figure S1. *Photodocumentation of branchiobdellids and their cocoons on a noble crayfish (Astacus astacus) from the Křesánovský brook.*

CHAPTER 5

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

General discussion

Consequences resulting from increasing expansion of human activities pose a threat to freshwater ecosystems (Dudgeon, 2019). Resilient freshwater macroinvertebrate fauna consists of several basic keystone taxonomic units (Annelida, Arthropoda, Mollusca, Nematoda, and Platyhelminthes) that seem to flexible cope with various types and strengths of human interference. Nevertheless, to enhance and maintain the quality of freshwater environments, which represent a vital resource for humankind, more profound knowledge of ecological and biological relationships, new perspectives and reflection of recent trends may prevent stagnation in this field. Although possibilities for determining causal effects in real environments are limited, natural experiments must not be replaced by easier-to-perform laboratory experiments or computer modelling (see Table 2 in *General introduction*).

As was suggested by Rosenzweig and Abramsky (1993) or Lake (2000), macroinvertebrate productivity and species richness can be negatively correlated to disturbance intensity or frequency (see Figure 2 in General Introduction). On the other hand, a system with few disturbances seems to generate lower species richness than the system with an intermediate level of disturbance ("intermediate disturbance hypothesis"). However, anthropogenic landscape modifications have expanded to such an extent that about three-quarters of Earth's land surface was altered by humans within the last millennium (Winkler et al., 2021). Agricultural land represents the world's largest terrestrial biome (only croplands occupy about 12% of Earth's ice-free land; pastures and forest plantations are omitted) (Foley et al., 2011). Associated risks and negative consequences, such as habitat and biodiversity loss, have arisen for (not only) adjacent freshwater ecosystems, which have been strongly influenced by the terrestrial expansion of human activities. Among freshwater animals, freshwater molluscs, crayfishes, stoneflies, amphibians, and fishes exhibit the greatest level of risk (Master et al., 2000). Even though the conceptual ecosystemic functionality of macroinvertebrates shows substantial signs of robustness, a rapid fall in the population of keystone organisms in freshwater communities with sparse diversity may cause an unpredictable impact on production capacities and socioeconomic relationships (Covich et al., 1999).

The objectives of this thesis were focused on the evaluation of responses of natural stream macroinvertebrate communities to human activities, namely 1) releasing wastewaters from wastewater treatment plants, 2) handling with pesticides and 3) introducing indigenous species. Co-occurring factors were emphasised and quantified when it was possible. In addition, the effect of industrial pollution on the community of aquatic macroinvertebrates (pronounced contamination by trace metals) was assessed. The presented studies were performed in the Czech Republic (Central Europe) at several localities: the Doubravka brook (Vysočina region), the Litavka river and Obecnický brook (Central Bohemian region), the Křesánovský Brook (South Bohemian region). Auxiliary data from two localities, the Šebkovický brook and Žďárka brook (both in the Vysočina region) were used. Results of one laboratory experiment performed to prove findings from the observational study are also reported (**Chapter 4**).

A brief summary of the main results:

Chapter 2: Insecticides and drought as a fatal combination for a stream macroinvertebrate assemblage in a catchment area exploited by large-scale agriculture

- Negative correlations between abundances of most detected aquatic macroinvertebrate taxa and insecticide contamination were observed.
- Hydrological droughts correlated most positively to the abundances of coleopteran adults, heteropterans, ostracods, and water lice (*Asellus aquaticus*) and negatively to mayflies, stoneflies and caddisflies (EPT taxa).
- The dominance of predators and active filter feeders was found in conditions of severe hydrological droughts.

Chapter 3: Effects of trace metals and municipal wastewater on the Ephemeroptera, Plecoptera, and Trichoptera of a stream community

- The contamination by trace metals (cadmium, lead and zinc) of smelting and mining origin was associated with the loss of ephemerellid, heptageniid and leptophlebiid mayflies.
- Substantially reduced richness and abundance of stoneflies in the contaminated sites could also be attributed to human-induced habitat alterations and warmer temperatures.
- Caddisflies generally exhibited the highest tolerance to the contamination, although unexpected malformations were detected in larvae of the genes *Halesus*, *Potamophylax* and *Rhyacophila.*
- The subsequent loads of treated and "poorly treated" municipal wastewaters led to the almost complete absence of stoneflies and dominance of caddisfly predators and passive filter feeders, represented mainly by hydropsychids, polycentropodids and rhyacophilids.

Chapter 4: Signal Crayfish as a Threat for European ectosymbionts – overlooked biodiversity losses

- Native crayfish ectosymbionts, branchiodellids *Branchiobdella parasita* and *B. pentadonta*, showed significantly decreased abundances in invasive signal crayfish (*Pacifastacus leniusculus*) compared to those in native noble crayfish (*Astacus astacus*) caught in the same locality.
- Based on the longer-term observations from two other localities with only previously recorded branchiobdellid occurrence, where populations of noble crayfish recently collapsed, the introduction of the invasive signal crayfish was assumed to be able to eliminate these native crayfish ectosymbionts.
- Under the laboratory conditions, signal crayfish infested by both branchiobdellids showed more intensive grooming compared to infested noble crayfish, which was most likely responsible for significantly decreased branchiobdellid survival in signal crayfish.
- The relationships between two branchiobdellids sharing the same hosts were rather neutralistic, even though there were indices *B. parasita* may slightly oust out *B. pentadonta.*

In Chapter 2, we analysed the response of the stream macroinvertebrate community inhabiting a catchment area that has been exploited by practices of large-scale agriculture (large uniform blocks of fields, extensive surface and subsurface drainage, and utilisation of heavy agricultural machinery and agriculture chemicals) to droughts and the insecticide contamination. The studied locality was contaminated by extremelly high concentrations of insecticides (a mixture of organophosphate chlorpyrifos and pyrethroid cypermethrin), which were illegally spilt by a staff of the local agriculture enterprise into a drainage ditch flowing

into the brook. The contamination caused observed mass mortality in stock of noble crayfish and fish (the brown trout, *Salmo trutta*, and stone loach, *Barbatula barbatula*). Benthic samples were taken for four years from the initial contamination. During the following period, the locality was impacted by severe droughts, which sometimes led to the almost complete absence of surface water in the brook channel. Hence, a precise evaluation of the temporal requirement of macroinvertebrate communities for recovery from insecticide contamination was hardly possible. Recovery times needed for stream macroinvertebrate communities after direct/indirect application of pesticides can last 0.2–10 years, depending on compound-specific persistence and resilience capacity of disturbed environment (Milner, 2009). For example, Hutchens et al. (1998), who evaluated dynamics of macroinvertebrate community in the stream experimentally treated by already banned organochloride insecticide methoxychlor, observed possible residual signs of disturbance after 5–10 years from the application (significantly higher within-year variability in biomass and/or abundances of some taxa compared to the control non-treated stream). Nevertheless, macroinvertebrates' total abundance and biomass recovered after 1/3 and 2 years, respectively (Cuffney et al., 1984; Wallace et al., 1986). Even though, the stream was contaminated from its spring unlike in our study, it flowed through a natural-close environment with a lower occurrence of anthropogenic factors. Periodical runoffs of soil and agriculture chemicals (pesticides, fertilisers), severe hydrological droughts and homogenisation of surrounding terrestrial and aquatic habitats (transformation of surrounding initial headwater network into drainage ditches) were the factors that we faced by evaluations.

We tried to test the size of the effects of drought status and initial contamination since their detrimental impact was strongly assumed regarding the resulted community indices (e.g. Shannon diversity index – H', BMWP score, $SPEAR_{pesticides}$, and the relative abundance of EPT taxa). Three sites were sampled within two spatial transects (0 and 6 km) with different contamination and drought status. The distance from potential upstream and downstream refugia is the crucial factor of recovery dynamics after pesticide exposure (Knillmann et al., 2018). Therefore, susceptible species can colonise the most polluted sites faster since they are often close to non-polluted sites, as was in our study. However, numerous potential refugial areas, such as the hyporheic exchange zones and cut-off stream branches, can occur in contaminated stretches with well-conserved fluvial morphology and natural riparian vegetation (Orlinskiy et al., 2015; Woessner, 2017). The mosaic character of natural initial river networks provides numerous niches and resilient macroinvertebrate communities with high β-diversity (Clarke et al., 2008); thus, an adverse effect of disturbances may be substantially mitigated.

While contamination of the environment is regulated by legislation, lower emphasis is taken on the negative impacts of engineering interferences in the landscape, causing habitat homogenisation. Besides the loss of aquatic biota and sociocultural impact on people, these interferences may pose a serious risk of a hardly reversible loss of quality water resources (including groundwaters) and the increasing risk of drought and flood impacts (Bedient et al., 2008; Blann et al., 2009). Nonetheless, even the effectiveness of conventional monitoring of aquatic environment contamination is often limited because short-term peaks of toxicants are often missed, which can make the environmental data more optimistic (Stehle and Schulz, 2015). We used sediment sampling since the contamination of sediments should usually indicate that both environments, the bottom and water column, are at risk.

Droughts can have a detrimental impact on stream macroinvertebrate communities. If the key condition in running waters – water flow – is ceased, the macroinvertebrate community undergoes substantial alterations in taxonomic composition and function structures (feeding strategies were significantly altered in our study). In spite of that, the non-polluted site which was strongly affected by droughts in the same way as the initially polluted sites was still inhabited by the EPT taxa, generally susceptible to droughts and deteriorating oxygen

concentration, in contrast to the initially polluted sites. Although there are not enough proofs from our data, a possible synergistic effect of droughts and environmental contamination on the negative response of stream macroinvertebrates was suggested and corresponded to the studies reported by, e.g. Arenas-Sánchez et al. (2016) or Munz et al. (2017). We are also convinced that the consequence of the long-term landscape agricultural overexploitation substantially worsened the hydrological conditions.

Chapter 3 represents the results of a natural experiment aimed at stream EPT (Ephemeroptera-Plecoptera-Trichoptera) community response to human-induced disturbances and gradients, particularly the response to industrial pollution and the effluent from a municipal wastewater treatment plant. In addition, the role of extreme temperatures and hydrological droughts amplified by presented water abstraction from drinking water reservoirs would have been observed; however, the particular year of the sampling campaign, 2020, was with lower temperatures and relatively rich for precipitation compared to the previous several years with extremely hot summers and low precipitations. The upper parts (reference sites) of the non-contaminated Obecnický brook (above and under the reservoir) was inhabited by numerous and rich stonefly community, which included threatened species on the red list (Bojková and Soldán, 2013); namely *Amphinemura borealis* (Category II – "critically endangered" in CZ; however, given specimens should be reviewed regarding uncertainties in morphological characteristics within the genus *Amphinemura*, as well as low likelihood for its occurrence), *Leuctra major* (Category III – "endangered" in CZ), *Amphinemura standfussi*, *A.* aff. *triangularis*, *Protonemura hrabei*, and *P.* aff. *meyeri* ("vulnerable" in CZ). Except for endangered stoneflies, we detected also threatened caddisfly taxa, namely *Hydatophylax infumatus*, *Hydropsyche fulvipes* and *Plectrocnemia* cf. *geniculata* (Category V – "near threatened" in CZ) (Chvojka et al., 2005). Nevertheless, we were faced with problems with morphological determination in the genus *Leuctra* and *Protonemura*, which was caused by a lack of contemporary determination keys enabling clear morphological separation. Determination of mayflies and caddisflies was easier, even though we had problems with determining limnephilid caddisflies, especially at the Site 3 contaminated by trace metals. Namely, larvae of taxon *Potamophylax* aff. *rotundipennis* showed less clear and peculiar morphological features, despite being in the final larval instar.

Contradictory to the non-polluted sites of the Obecnický brook, the polluted sites were dominated by caddisflies. Even though we did not detect any threatened caddisfly taxa in polluted sites, their richness values were constant between all sites, whilst the richness of mayflies and stoneflies were significantly reduced in general. Based on these results and e.g. study of Conti et al. (2014), we assume caddisflies can cope with human activities much better than mayflies and stoneflies, although the sum of all three orders is frequently used as a biotic index (Carter et al., 2017). Therefore, using the sum of EPT may bias estimated environmental status. Since there are more potamal caddisfly taxa in Europe than the potamal mayflies and stoneflies (184 potamal caddisflies, 118 potamal mayflies and 47 potamal stoneflies summarised according to from Graf et al., 2008; Buffagni et al., 2009 and Graf et al., 2009, respectively), the adaptation for living in the potamal river zone could be one of the general prerequisites for resistance to anthropogenic alterations in EPT. The potamal zones naturally show the warmest temperatures, highest organic loads, and worse oxygen concentrations than the crenal and rhithral zones. Furthermore, the potamal zones have been exposed to the greatest anthropogenic pressures in the last centuries; thus, enhanced resistance to deteriorated conditions had been evolved in particular potamal species. According to Bojková and Soldán (2013), the stoneflies *Leuctra fusca*, *L. geniculata* and *Nemoura cinerea* (all of them show a certain preference for a potamal zone) can cope with human-induced habitat alterations and all types of pollution. This and another report by Pařil et al. (2008) well correspond to our findings. We showed that *L. fusca* gr. and *L. geniculata* can also withstand high pollution by trace metals; however, the environment

polluted by combination of trace metals and municipal treated wastewaters (also frequently supplied by "poorly treated" wastewaters) was virtually without stoneflies. In addition, epibenthic and epiphytic mesohabitat preference and short and desynchronised lifecycle can be also advantageous species traits for living in a human-altered environment. Despite this, we did not find any significant relationships between disturbance gradients and length of lifecycle or meso-habitat preferences within the studies in **Chapters 2** and **3** (the results published only within **Chapter 2**). Based on the particular method of Community-Weighted Mean (CWM) and Redundancy analysis (RDA) (Šmilauer and Lepš, 2014), we also tested shifts along disturbance gradients in macro- and micro-habitat preference traits, body length, time of emergence, and temperature preference that were non-significant. However, instead of rejecting these given possible relationships, we state that if there are such relationships, they are not such strong as it was between environmental gradients and feeding strategies or taxonomic compositions. Application of an alternative statistical method or a correction of the dataset of ecological/biological traits could bring positive outputs (Crabot et al., 2021). Nevertheless, a large-scale experiment or a meta-analysis are needed to prove the evidence provided by the small-scale experiments.

Chapter 4 deals with the effects of signal crayfish introductions (non-native crayfish species in Europe) on the community of native crayfish epibionts – branchiobdellids *B. parasita* and *B. pentadonta*. Based on both natural and laboratory experiments, we found substantially decreased abundances of both branchiobdellids in signal crayfish compared to those in the native noble crayfish. Within the natural experiment, we also detected rapid decreases in the numbers of cocoons of both branchiobdellids, which indicate a reduced ability in these species to reproduce on the non-native crayfish host. We also tried to reveal the relationships between the abundances of both branchiobdellid species and their cocoons on different crayfish hosts. The between-species relationships were rather neutralistic; however, there were statistically supported indices that bigger-sized *B. parasita* in higher abundances could compete out *B. pentadonta* (or vice-versa) in native noble crayfish, whilst their relationship was contradictory positive in non-native signal crayfish. We interpret the trend as a result of the much higher abundances of both branchiobdellids in noble crayfish than in signal crayfish, potentially leading to a competitive behaviour due to a lack of space. It is worth mentioning that some individuals of signal crayfish were able to fully get rid of branchiobdellids just during the first hours from the infestation in laboratory conditions, whilst the same reaction was not observed in the native crayfish species. Those individuals of signal crayfish which do not exhibit such intensive grooming can alternatively make less suboptimal conditions for bigger-sized epibionts. In other words, some individuals of signal crayfish can be strongly intolerant to branchiobdellids, while other individuals can be more tolerant but most likely do not provide favourable conditions for the branchiodellids, such as the availability of preferred food and prey (Dražina et al., 2018) and less frequent moulting (Thomas et al., 2016), compared to the native noble crayfish.

We assume that both branchiobdellid species will disappear with the disappearance of native crayfish hosts. It seems that branchiobdellidans can be narrowly specialised on specific hosts, even though they can temporarily colonise non-native species. Massive large-scale spreading of branchiobdellidans out of their formal origin has not been documented (although it can easily escape our attention; Vedia et al., 2015), while the invasive spreading of some of their hosts represents a global phenomenon (e.g. Lodge et al., 2012). Therefore, it seems branchiobdellidans may be strongly dependent on co-occurring environment-specific factors in general. The communities of other epibionts, such as Ciliophora, Rotifera, Copepoda, or Ostracoda, can also vary highly between crayfish species (Dražina et al., 2018). Nonetheless, information about epizoic algal and fungal communities inhabiting crayfish is

scarce (Nekuie Fard et al., 2011; Falasco et al., 2018). Thus, these factors and the following environmental conditions can play a crucial role in the distribution and possible adaptability of branchiobdellidans. It cannot be excluded that new communities and ecological strengths have been forming in crayfish introduced out of their origin as they interact with local organisms, while the "hitchhiker" organisms transferred with them may not be always adaptable to new conditions, although they are specialised to living with their host in their native distribution range. Our study represents an enhanced example of the potential negative effect of non-native species on the native ones and intervenes in a little researched but inspirational ecological relationship in crayfish ectosymbionts.

Conclusions

Aquatic ecosystems are exposed to multiple stressors induced by human activities. This thesis aims to give an overview of some known or less known anthropogenic processes that have the potential to affect aquatic macroinvertebrates. Although their communities stay at the fringes of mainstream societal and economic concerns, their destabilisation indicates worsening status in the aquatic environments. Damage to water resources by various kinds of pollution along with a disrupted hydrological regime had also a strong destabilizing potential for humankind. In the case of damage to nature due to the novel technologies and energetic demands of the population, the conservation of pristineness of the water environment is not virtually taken into account – the instant benefit over the loss tends to be indisputable. However, the negative consequences of those impacts may over a short or longer time outweigh these economic benefits. In ideal, it ought to be strongly considered in advance and reflected by appropriate countermeasures. For example, the increasing severity of droughts and high temperatures in the past decades should be locally reflected by increased limits for pollutant release, since aquatic ecosystems show higher vulnerability during a depressed water level while there can also be an increased risk for fluvial groundwaters contamination. Much attention is paid to pollution; nevertheless, the side effects of engineering interventions on water regimes in fluvial landscapes – especially artificial initial riverine networking – should be also precisely studied and eventually mitigated. The biological invasions mediated by human transfers represent another dimension of human disturbance, although natural redistribution of macroinvertebrate species during altered climate conditions, e.g. via airflow or aquatic birds must be considered too. This thesis highlights the importance of assessing human impacts on aquatic ecosystems in natural conditions. Combining with the results of laboratory studies, the experiments carried out in the natural environment provide a realistic evaluation of aquatic macroinvertebrate community responses to human impacts and non-native species introductions.

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English summary

Macroinvertebrates represent an essential part of aquatic environments contributing to various ecosystem services. Disregarding the importance of their habitats and stable and functionally diverse communities can cause hardly reversible losses. This thesis aimed to reveal relationships between responses of aquatic macroinvertebrate communities and the consequences of human activities. The general negative relationship between the gradient of agricultural insecticide contamination and abundances of higher taxonomic units of the stream macroinvertebrates was observed (**Chapter 2**), even though shown resilience or resistance of the black fly *Simulium vernum* gr. and the planariid *Polycelis nigra* to the contamination was likely possible. The gradient of droughts correlated the most positively to the abundances of coleopteran adults, heteropterans, ostracods, and water lice and negatively to mayflies (Ephemeroptera), stoneflies (Plecoptera) and caddisflies (Trichoptera) – hereinafter referred as "EPT" taxa. Disturbing synergic effects of droughts and agricultural runoff were assumed based on the comparisons between communities sampled in the control site and initially contaminated downstream sites. The practices in modern agricultural systems can be considered one of the greatest threats to aquatic macroinvertebrate fauna due to (i) their increasing relative contribution to the altering of the total land surface, (ii) high direct and/or indirect involvement in the loss and degradation of unique aquatic habitats, (iii) potential negative influence to climate and hydrological conditions, and (iv) large-scale application of products highly toxic to aquatic organisms.

Results summarised in **Chapter 3** show decreasing richness of EPT taxa (however, not their abundances) along with the involvement of an increasing number of anthropogenic factors. Nevertheless, only the mayflies and especially stoneflies showed significant negative responses, whilst caddisflies generally exhibited resistance to the presumably strongest anthropogenic factors; namely to contamination by trace metals (cadmium, lead and zinc) and to subsequent loads of treated and "poorly treated" municipal wastewaters containing pesticides, pharmaceutical active compounds, sewage-derived organic matter, and undoubtedly other not identified kinds of pollutants. Despite the higher caddisfly abundance, there were detected signs of worsened health status in hydropsychids, limnephilids and rhyacophilids, particularly detected malformations and the presence of dead pupae in both types of contaminated environments. However, the environment polluted by wastewaters exhibited a significant relationship to a highly increased relative contribution of passive filter feeders and predators within the EPT community; it likely reflected consumption of drifting sewage-derived organic matter by omnivorous caddisflies, possible higher availability of drifting prey organisms (e.g. chironomids and water lice) and steady water flow. Since wastewater treatment technologies have been enhanced in many countries and there is a lack of published evidence about their complex effect on aquatic ecosystems, future studies are required.

Chapter 4 reveals the negative effect of non-native signal crayfish (*Pacifastacus leniusculus*) on the native branchiodellid community. According to our results, the total replacement of the native noble crayfish (*Astacus astacus*) by signal crayfish can lead to the disappearance of two species *Branchiobdella parasita* and *B. pentadonta*. Potential differences between crayfish species in correlations between abundances of both branchiobdellids were observed in the locality with the sympatric occurrence of noble crayfish and signal crayfish; the abundance of bigger-sized *B. parasita* positively correlated to the abundance of *B. pentadonta* only in the signal crayfish, whilst this relationship may be negative in the big-sized noble crayfish densely infested by both species. Potential competition for space was assumed. The experiment in laboratory conditions revealed more intensive grooming in signal crayfish. The

results demonstrate the loss of overlooked biodiversity associated with the invasive species introduction.

Knowledge of how communities of aquatic invertebrates respond to anthropogenic changes in the environment can be useful, e.g., during the assessment of the implications of planned or currently active human interferences for cultural landscapes. This thesis will provide useful information for laboratory studies, of which the interpretation is often limited as they are carried out in artificial environments.

Czech summary

Bezobratlí představují významnou součást vodních ekosystémů, podílející se na řadě nezbytných ekosystémových služeb. Narušením stanovišť a druhově i funkčně rozmanitých společenstev může snadno dojít k jen těžko vratným změnám. Tato práce byla zaměřena na výzkum vztahů společenstev vodních bezobratlých ovlivněných lidskou činností. Byl pozorován obecně negativní vztah mezi gradientem kontaminace insekticidy a četností proudomilných bezobratlých na úrovni vyšších taxonomických jednotek (**Kapitola 2**), ačkoli larvy muchniček skupiny *Simulium vernum* a ploštěnky druhu *Polycelis nigra* vykazovaly pravděpodobné známky rezilience nebo rezistence vůči danému typu znečištění. Gradient hydrologického sucha nejlépe pozitivně koreloval s četností dospělců brouků (Coleoptera), ploštic (Heteroptera), lasturnatek (Ostracoda) a berušek vodních (*Asellus aquaticus*) a negativně pak koreloval s četností skupiny jepic (Ephemeroptera), pošvatek (Plecoptera) a chrostíků (Trichoptera); dále jen EPT. Na základě porovnání stavu společenstva v kontrolním odběrového úseku se stavy společenstev z úseků zasažených počáteční insekticidní otravou byl předpokládán nepříznivý synergický efekt hydrologického sucha a splachů ze zemědělsky obhospodařovaných ploch. Metody v moderním zemědělství lze považovat za jednu z největších hrozeb pro vodní bezobratlé kvůli (i) rostoucímu vysokému podílu zemědělsky obhospodařované půdy na celkové rozloze zemské souše, (ii) přímému a/nebo nepřímému zapojení zemědělských postupů do odstraňování a degradace unikátních vodních biotopů, (iii) potenciálnímu ovlivňování klimatických a hydrologických podmínek a (iv) kvůli masivní a plošné aplikaci látek vysoce toxických pro vodní organizmy.

Výsledky shrnuté v **Kapitole 3** ukazují klesající zaznamenaný počet druhů EPT (avšak nikoliv četnosti EPT jedinců) spolu s rostoucí kumulací antropogenních faktorů. Nicméně pouze společenstva jepic a obzvláště pošvatek vykazovala signifikantní úbytky, zatímco chrostíci vykazovali určitou rezistenci k předpokládaným nejsilnějším antropogenním faktorům. Tyto hlavní faktory zahrnovaly zatížení kovy (kadmium, olovo a zinek) s následným vyústěním městských vyčištěných a špatně vyčištěných odpadních vod s obsahem pesticidů, léčiv, organického materiálu ze splašků a nepochybně i řady dalších neanalyzovaných látek. Navzdory zvýšené četnosti chrostíků byly u čeledí Hydropsychidae, Limnephilidae a Rhyacophilidae pozorovány známky zhoršené kondice (malformace a přítomnost uhynulých kukel) v obou typech znečištění. Prostředí znečištěné odpadními vodami bylo osídleno signifikantně zvýšeným podílem pasivních filtrátorů a predátorů z řádu chrostíků. Tyto organizmy zjevně profitovaly z přísunu driftujících částic organického materiálu pocházejícího z odpadních vod, vysoké abundance potravních organizmů (například larev pakomárů – Chironomidae a berušek vodních) a stabilního proudění vody. Vidíme zde nutnost dále studovat reakce společenstev vodních organizmů na přísun vyčištěných odpadních vod, jelikož technologie čištění jsou v mnoha zemích světa stále zdokonalovány a je k dispozici poměrně málo publikovaných výstupů sledujících efekty zaváděných postupů na přirozené vodní ekosystémy.

Kapitola 4 odhaluje negativní působení nepůvodního raka signálního (*Pacifastacus leniusculus*) na původní komunitu potočnic (Branchiobdellidae). Dle našich výsledků může úplné nahrazení původního raka říčního (*Astacus astacus*) rakem signálním vést k vymizení sledovaných dvou druhů potočnic, *Branchiobdella parasita* a *B. pentadonta*. Na lokalitě se sympatrickým výskytem raka říčního a signálního byly rovněž pozorovány potenciální rozdíly mezi oběma raky v korelacích mezi četnostmi obou druhů potočnic. Četnosti větší potočnice *B. parasita* pozitivně korelovaly s četností menší *B. pentadonta* pouze u raka signálního, přičemž tento vztah může být až negativní u velkých hustě osídlených jedinců raka říčního. Byla tudíž předpokládána potenciální kompetice o životní prostor. Doprovodný laboratorní experiment ukázal, že rak signální se mechanicky zbavuje epibiontů ve vyšší míře ve srovnání s rakem

říčním. Výsledky tedy demonstrují potenciální ztráty v biodiverzitě nenápadných skupin organizmů vznikající introdukcí invazních druhů.

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Let, M., Grabicová, K., Ložek, F., Bláha, M., 2022. Bioconcentrations, depuration, shift in metabolome and a behavioural response in the nymphs of the dragonfly *Aeshna cyanea* (Müller, 1764) to environmentally relevant concentrations of methamphetamine. Aquatic Toxicology (under review).

Let, M., Ložek, F., Kouba, A., Buřič, M., Bláha, M., 2022. Signal crayfish as a threat for European ectosymbionts – overlooked biodiversity losses. Aquatic Sciences (under review).

Ložek, F., **Let, M.**, 2022. Effect cardiac pharmaceuticals on cardiac activity in post-embryonal stages of marbled crayfish *Procambarus virginalis* (in preparation).

Musil, M., **Let, M.**, Roje, S. Drozd, B., Kouba, A., 2022. Feeding in fear: feeding in crayfish is influenced by cues from conspecifics and/or a fish predator (manuscript).

Musil, M., **Let., M.**, Kouba, A., 2022. Predation effect of the three-spined stickleback, *Gasterosteus aculeatus*, on the first mother-independent juvenile stages of several invasive crayfish species (in preparation).

Let, M., Grabicová, K., Musil, M., Letová, H., Roje, S., Bláha, M., 2022. "Unwanted gift bearers": Distribution of pharmaceutical xenobiotics by aerial stages of aquatic insects from municipal waste waters into terrestrial environment (in preparation).

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Training and supervision plan during study

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Research department	2018–2022 – Laboratory of Freshwater Ecosystems of FFPW
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Ph.D. courses	**Year**
Applied hydrobiology	2019
Basic of scientific communication	2019
Biostatistics	2019
Modern regression methods	2019
Design and analysis of ecological experiments	2020
Ichthyology and fish taxonomy	2020
Aquatic toxicology	2020
Advanced regression methods	2020
English language	2021
Advanced ordination methods	2021

Scientific seminars	**Year**
Seminar days of FFPW	2018–2022

International conferences	**Year**
Let, M., Špaček, J., Kouba, A., Buřič, M., Bláha, M., 2019. Recovery of aquatic macroinvertebrate community in a small brook after poisoning by insecticides. 6th Fresh Blood for Fresh Water Conference. Book of Abstracts. April 23–27, 2019, Tihany, Hungary, p. 123.	2019
Musil, M., Roje, S., **Let, M.,** Kouba, A., 2022. Feeding in fear: feeding in crayfish is influenced by cues from conspecifics and/or a fish predator. 22nd International Conference on Aquatic Invasive Species. Book of Abstracts. April 18–22, 2022, Oostende, Belgium, p. 43.	2022
Ložek, F., **Let, M.,** Kouba, A., Buřič, M., Bláha, M., 2022. Signal crayfish as a threat for European ectosymbionts – overlooked biodiversity losses. 23rd Symposium of the International Association of Astacology. Book of Abstract. June 20–25, 2022, Hluboká nad Vltavou, Czech Republic, p. 91.	2022

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Inês Domingues, Ph.D. and João L. T. Pestana, Ph.D., University of Aveiro, Centre for Environmental and Marine Studies (CESAM), Portugal (1.5 months, Acute and Chronic Effect of Niclosamide on non-biting midges, Chironomus riparius, internship was terminated because of COVID-19)	2020

Pedagogical activities	**Year**
• Training of students of bachelor study, in range of 30 teaching hours	2019
• Supervisor of bachelor thesis "Influence of anthropogenic activities to communities of aquatic organisms in the upper drainage of the Litavka river" (student Jan Černý)	2019–2021
• Preparation of the collection of macroinvertebrates for lectures of Applied hydrobiology 2, in range of 30 teaching hours	2022
• Training of students of bachelor study, in range of 30 teaching hours	2022
• Leading of project entitled Preferences of the stream benthic macroinvertebrates to the specific types of microhabitats at International Summer School (student Cem Hasşerbetçi)	2022

Curriculum vitae

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