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CZU Fakulta životního prostředí

Determinace klíčových faktorů pro výskyt a vývoj jedinců ohrožených druhů sladkovodních mlžů – modelový druh *Margaritifera margaritifera*

Disertační práce

Autor: Ing. Vojtěch Barák

Školitel: doc. Mgr. Jan Růžička, Ph.D.

Konzultant: Mgr. Michal Bílý, Ph.D.

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Prohlášení

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Předmluva

Tekoucí a stojaté vody mě fascinovaly již od útlého dětství, kdy jsem spolu se svou mámou chodil pouštět lodičky na potok a házet žabky na rybníce na vesnici, kde jsme bydleli. Později jsem se dostal k problematice vodního hospodářství na střední škole v Plzni, kde zdejší ředitel dělal nábor do třetího ročníku a já jsem se spolu s několika dalšími spolužáky nechal zlanařit na obor Vodní stavby. Po úspěšném absolvování SŠ jsem pokračoval ve studiu na jedné z pražských vysokých škol, nicméně jsem časem dospěl k rozhodnutí ji opustit, neboť obor zaměřený na čistě „technickou vodu" mě zcela nenaplňoval. S čistým štítem a bez uznání jakýchkoli předmětů jsem poté přešel na Českou zemědělskou univerzitu, kde působím již více než deset let.

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Abstrakt

Velcí mlži (Unionoida) jako jádrová skupina sladkovodních mlžů jsou celosvětově ohroženými živočichy vzhledem ke svému komplexnímu životnímu cyklu a působení negativních abiotických faktorů, zejména znečištění vodního prostředí. Determinace klíčových faktorů pro výskyt a vývoj jedinců byla v této práci popsána pro modelový druh – perlorodku říční (*Margaritifera margaritifera* (Linnaeus, 1758)), především pro kritickou juvenilní fázi života. Ačkoli perlorodka říční patří mezi nejlépe prozkoumané druhy sladkovodních mlžů, habitatové požadavky raných vývojových stádií jsou dosud jen málo známy. Cílem této práce bylo zjistit nároky mladých perlorodek na životní prostor (**článek 1**) a jakost vody (**článek 3**), dále vytvořit protokol zaměřený na provádění in situ bioindikačních experimentů pro hodnocení vlivu abiotických faktorů na juvenilní mlže (**článek 2**), a vyhodnotit přímý vliv návštěvníků chráněného území s výskytem perlorodky říční (**článek 4**). Pozice juvenilů byla hodnocena pomocí síťových trubiček v říčním dně a ukázalo se, že mladí mlži preferují velice mělké vrstvy zóny hyporeálu: většinou se vyskytují v hloubce 2–3 cm pode dnem, přičemž přezimují v hloubce 3,0–4,5 cm. Protokol pro provádění terénních pokusů byl využit pro hodnocení vhodných stanovištních podmínek, přičemž byly zjištěny odlišné výsledky pro vývoj juvenilních mlžů při využití různých typů bioindikačních zařízení v prostředí volné vody a zóny hyporeálu. K nepříznivému vývoji mladých perlorodek v úseku se znečištěním vody (se zachycením epizodické události) došlo v důsledku kombinace zvýšené koncentrace amoniaku a dusitanů a sníženého obsahu kyslíku. Přímý vliv vodáků na jedince perlorodky říční byl hodnocen s využitím „falešných" mlžů (maket a modelů vytvořených ze schránek mlžů), přičemž rekreační splouvání může mít méně nepříznivé účinky oproti doprovodným aktivitám při pohybu v toku. Pomocí modifikovaných či nových metodických postupů byl potvrzen význam obsahu kyslíku ve vodě, typu substrátu a jakosti vody pro výskyt a vývoj juvenilních jedinců perlorodky říční. Tyto poznatky lze uplatnit v rámci záchranných programů velkých mlžů a hodnocení jejich biotopu, zejména v souvislosti s ochranou druhů a jejich habitatu. Mohou též přispět k nastavení opatření proti znečištění vodního prostředí a pochopení chování člověka při setkání s mlži a dalšími sladkovodními organismy.

Klíčová slova: Perlorodka říční, bioindikace, hloubková preference, epizodické znečištění, hyporeál, Teplá Vltava, Malše

Abstract

Freshwater mussels (Unionoida) represent a core group of freshwater bivalves and belong to the most endangered animals in the world due to their complex life cycle and negative effects of abiotic factors including water pollution. In this dissertation thesis, determination of key factors affecting mussel occurrence and development was described using a model species – freshwater pearl mussel (*Margaritifera margaritifera* (Linnaeus, 1758)) (FPM), especially critical juvenile phase of FPM. Although it is one of the most investigated species of freshwater bivalves, habitat preferences of young individuals remain poorly known. The aims of this thesis were: to find out spatial and water quality requirements of FPM juveniles (**Paper 1** and **Paper 3**), describe a protocol for performing in situ bioindication exposure tests (assessing the effects of abiotic factors on juvenile mussels) (**Paper 2**), and make visitor impact assessment in a protected area with FPM occurrence (**Paper 4**). The juveniles in mesh tubes were placed in the natural river bed and they showed a tendency to penetrate into very shallow hyporheic zone: they mostly prefer the depth of 2–3 cm (3.0–4.5 cm during wintering). The protocol for performing field experiments was followed to report site suitability for juvenile FPMs, and growth and survival differences were recorded using bioindication mesh/sandy cages in the free-flowing water and hyporheic zone. Adverse mussel development within the river section with impaired water quality (including episodic pollution) was characterized by multiple-stressor exposure with increased (total) ammonia and nitrite concentrations and decreased oxygen levels. Visitor impact on FPMs was assessed using fake specimens (i.e., concrete mussels and models made from real shells): the effects of recreational boating might be less detrimental than those of accompanying activities in the river channel. Modified or new methodical procedures were used for confirmation of an important role of oxygen level, substrate type and water quality for occurrence and development of juvenile FPMs. These facts should be considered within freshwater mussel action plans and biotope assessment, especially for species and habitat conservation purposes. They can also help in setting of water pollution control programmes and understanding human behavior during the contact with mussels and other freshwater species.

Keywords: Freshwater pearl mussel, bioindication methods, depth preference, episodic pollution, hyporheic zone, Teplá Vltava River, Malše River

Obsah

1. Úvod do problematiky

1.1 Sladkovodní ekosystémy

Vodní ekosystémy patří mezi jedny z nejohroženějších ekosystémů na celém světě v důsledku výrazného antropogenního tlaku. Degradace sladkovodního prostředí bývá spojována se ztrátou, modifikací či fragmentací habitatu pro volně žijící druhy, dále s nadměrným využíváním zdrojů včetně vody, znečištěním a šířením invazních druhů (Malmqvist and Rundle, 2002; Dudgeon et al., 2006; Strayer and Dudgeon, 2010; Vörösmarty et al., 2010; Collen et al., 2014; Darwall et al., 2018). Vzhledem k vysoké konektivitě dochází ke snadnému šíření hrozeb a jejich nepříznivých účinků z jednoho stanoviště na druhé (Dudgeon et al., 2006; Darwall et al., 2009). Vodní toky byly či nadále jsou negativně ovlivněny regulací svých koryt, protipovodňovou ochranou, budováním vodních elektráren, vnitrozemskou plavbou a vypouštěním (ne)čištěných odpadních vod. Významnou roli hrají také zásahy v povodí řek a potoků, často spojené se změnami land use (zahrnující intenzivní zemědělství, lesnictví, průmysl a rozvoj zástavby) (Malmqvist and Rundle, 2002; Allan, 2004; Gregory, 2006; Jarošová et al., 2012; Liedermann et al., 2014; Hauer et al., 2018). Uvedené lidské aktivity vyústily v krizi biodiverzity, kdy je téměř jedna třetina všech sladkovodních druhů ohrožena vymíráním, zejména v důsledku ztráty habitatu (Collen et al., 2014).

1.2 Sladkovodní mlži

Významnou součástí biodiverzity sladkovodních ekosystémů jsou mlži (Mollusca: Bivalvia), zejména hrabající zástupci řádů Unionoida (tzv. velcí mlži) a Veneroida, jejichž roli podrobně popisují Vaughn and Hakenkamp (2001). Tito měkkýši ovlivňují procesy ve vodě i dnovém sedimentu vzhledem k pozici na rozhraní obou prostředí. Z vodního sloupce dokážou při filtraci odstranit nejrůznější částice, naopak vylučují živiny a biodepozity (tj. fekální pelety a pseudopelety). Některé druhy také využívají deponovaného materiálu v sedimentu jako potravy, svůj význam mají dále procesy biodepozice (např. zvýšení obsahu organické hmoty, ovlivnění kyslíkových poměrů, potrava pro další organismy) (Hakenkamp and Palmer, 1999; Newell et al., 2002; Bruesewitz et al., 2008; Yuan et al., 2016) a bioturbace (hrabání mlžů vede ke zvýšení množství vody v sedimentu, homogenizaci substrátu či vstupu kyslíku) (McCall et al., 1979, 1995). Schránky mlžů slouží i jako habitat pro další organismy: právě měkkýši

se schránkou mohou přímo či nepřímo ovlivnit celé sladkovodní prostředí, a z toho důvodu jsou označováni jako tzv. ekosystémoví inženýři (Gutiérrez et al., 2003).

Sladkovodní mlži patří mezi nejohroženější taxonomické skupiny na celém světě (Strayer et al., 1999; Vaughn and Hakenkamp, 2001; Lopes-Lima et al., 2014, 2018b): 30 až 40 % druhů je buď (téměř) ohroženo vyhynutím, nebo již dokonce vyhynulo (Lopes-Lima et al., 2014; Böhm et al., 2021), což odráží obecný úbytek druhů a hrozby ve sladkovodních ekosystémech (Collen et al., 2014). Hlavní současnou hrozbou pro mlže v globálním měřítku je znečištění a také modifikace přírodního systému, dále pak vstup invazních druhů, nadměrné využívání a disturbance člověkem (Lopes-Lima et al., 2014; Böhm et al., 2021). Znečištění a modifikace přírodního systému (spolu s klimatickými změnami) patří také mezi budoucí hrozby (Böhm et al., 2021), a mlži tak představují významnou skupinu pro terénní monitoring (ASTM, 2013).

In situ bioindikace jsou často využívány v biologii ochrany přírody (více viz Armstead and Yeager, 2007), kdy jsou jedinci určitého druhu exponováni v daném prostředí s následným hodnocením jejich stavu. Tímto způsobem lze získat informace o vhodnosti stanovišť a kvalitě prostředí (jeho ekologickém stavu) pro ohrožené druhy (Pander and Geist, 2013; Pollard et al., 2017) včetně velkých mlžů (viz kap. 1.3; Buddensiek, 1995; Gum et al., 2011; Haag, 2012; Scheder et al., 2015; **Bílý et al., 2018 – článek 2**). K porozumění citlivosti mlžů vůči stresorům prostředí se často využívá „caged bivalve model" (CBM) (v četných studiích zaměřených hlavně na druhy Severní Ameriky; Salazar and Salazar, 2007), kdy jsou jedinci po vymezenou dobu uchováváni v zařízeních s mřížkou zakrývající otevřené části a zároveň umožňující vstup vody a potravy (Armstead and Yeager, 2007). Hlavní výhodu oproti monitoringu přirozených populací představují dobře definované časoprostorové podmínky, kdy nejsou kontrolovány pouze podmínky prostředí na daném stanovišti (Salazar and Salazar, 2007). Podobné terénní experimenty jsou často doporučovány pro provádění environmentálně realistických studií a hodnocení účinků vícenásobných stresorů (Burton et al., 2005; Connon et al., 2012; Ferreira-Rodríguez et al., 2019).

1.3 Velcí mlži

1.3.1 Význam

Velcí mlži jsou hlavním jádrem skupiny sladkovodních mlžů (Lopes-Lima et al., 2018b), jejichž význam spočívá v ekosystémových službách (benefitech pro člověka

odvozených z ekosystémů). Tyto služby lze rozdělit do těchto čtyř skupin: regulující, podpůrné, zajišťující a kulturní (Vaughn, 2018).

Mezi <u>regulující</u> služby náleží zejména biofiltrace a čištění vody. Jedinec přefiltruje až 40–50 l vody za den (Tankersley and Dimock, 1993; Ziuganov et al., 1994), přičemž rychlost filtrace je dána teplotou (Spooner and Vaughn, 2008) a objemem a zdržením vody (Strayer et al., 1999). Při vysoké hustotě velkých mlžů v tekoucích vodách může přefiltrovaný objem vody dokonce překročit denní průtok (Welker and Walz, 1998; Vaughn et al., 2004). Při filtraci dochází také k odstraňování kontaminantů z vody (Izumi et al., 2012; Ismail et al., 2014), nicméně vliv takové zátěže na samotné mlže není dosud podrobně znám (Vaughn, 2018).

<u>Podpůrné</u> služby zahrnují koloběh živin, modifikaci habitatu, environmentální monitoring a potravní sítě. Živiny jsou ukládány do tkání, schránky a fekálních pelet a pseudopelet, nejčastěji jsou pak vylučovány v rozpuštěné podobě (Strayer, 2014). Vylučování je stejně jako filtrační schopnost ovlivněno teplotními a průtokovými podmínkami (Atkinson and Vaughn, 2015; Vaughn, 2018). Modifikaci habitatu a vyšší výměnu živin mezi vodním sloupcem a sedimentem zajišťuje bioturbace, kdy jsou živiny přístupné pro ostatní organismy a dochází k ovlivnění potravních sítí („bottom-up" kontrola prostředí; Vaughn, 2018). Význam schránek jako možného habitatu byl již zmíněn v kap. 1.2, navíc spolu s tkáněmi mohou sloužit pro monitoring podmínek prostředí v blízké či vzdálené minulosti (Schöne et al., 2004; Newton and Cope, 2007). Fyziologické změny jedinců indikují změny prostředí v reálném čase (Goodchild et al., 2016; Hartmann et al., 2016), citlivá juvenilní stádia jsou také využívána v řadě ekotoxikologických testů (Wang et al., 2017).

Mezi <u>zajišťující</u> služby patří potrava pro další druhy (včetně člověka), výrobky ze schránek a lov perel; <u>kulturní</u> služby jsou spojovány s kulturní hodnotou pro některé národy a také s existenční hodnotou z hlediska ochrany přírody (Haag, 2012).

1.3.2 Ohrožení

Velcí mlži jsou výrazně ohroženou skupinou živočichů (Bogan, 1993; Richter et al., 1997; Lydeard et al., 2004; Strayer et al., 2004; Lopes-Lima et al., 2014, 2017, 2018b; Vaughn, 2018; Ferreira-Rodríguez et al., 2019), charakteristickou snižujícím se množstvím populací narůstajícího počtu ohrožených druhů (Strayer et al., 1999; Armstead and Yeager, 2007; Degerman et al., 2009) s nedostatečnou reprodukcí. Jedna

z hlavních příčin tkví v neobvyklém životním cyklu se zastoupením ryb (Lopes-Lima et al., 2014), jejichž žábry slouží pro vývoj parazitického larválního stádia (glochidií) (Modesto et al., 2018). Druhá příčina je pak spojena s působením negativních faktorů prostředí, jako jsou status hostitelských ryb, (ne)vhodnost habitatu, změny land use, výstavba přehrad (změny teplotního a průtokového režimu, migrační překážka pro ryby atd.), znečištění, klimatické změny a invazní druhy. Za největší nebezpečí pro velké mlže jsou považovány hlavně znečištění (Bogan, 1993; Richter et al., 1997; Strayer et al., 2004; Van Hassel and Farris, 2007; Haag, 2012; Lopes-Lima et al., 2014, 2017, 2018b; Ferreira-Rodríguez et al., 2019) a zvyšující se míra podílu nevhodných habitatů (Ferreira-Rodríguez et al., 2019).

1.3.3 Role znečištění

U velkých mlžů byla laboratorně zjištěna vysoká citlivost na přítomnost kovů, amoniaku a některých významných iontů (Wang et al., 2007, 2018; Nakamura et al., 2021), což platí zejména pro raná vývojová stádia (Eybe et al., 2013; Švanyga et al., 2013; Ferreira-Rodríguez et al., 2019). Bohužel jen málo informací o ekotoxikologii velkých mlžů je k dispozici mimo Severní Ameriku (Beggel et al., 2017; Kleinhenz et al., 2019; Nakamura et al., 2021). Další poznatky o citlivosti mlžů pocházejí většinou z terénního monitoringu přirozených populací, jako jsou změny velikosti populace, zvýšený úhyn jedinců či absence reprodukce (Van Hassel and Farris, 2007). V řadě případů není známa přesná příčina, ačkoli znečištění bývá podezřelým faktorem číslo jedna (např. Van Hassel, 2007; Gillis, 2012; Gillis et al., 2017). Přestože nedávné studie potvrdily možnost aplikace bioindikačních metod pro velké mlže (Bartsch et al., 2017; Černá et al., 2018), časoprostorová dynamika událostí a kombinace různých efektů ztěžuje zachycení účinků a/nebo případnou simulaci v laboratoři (Patnode et al., 2015), což platí obzvláště pro epizodické znečištění (**Barák et al., 2022 – článek 3**). Otázkou pak zůstává, jaká je míra nejistoty při nastavení environmentálních limitů pro ochranu mlžů (ASTM, 2013; Ferreira-Rodríguez et al., 2019).

Atributy jakosti vody jsou ovlivňovány změnou environmentálních parametrů (např. množství kyslíku a teplota vody; Hauer and Hill, 2007) a navíc často působí synergisticky, tudíž vymezení jednotlivých limitních hodnot se nejeví jako vhodné (BSI, 2017; Boon et al., 2019). Švanyga et al. (2013) uvádí, že prosperující populace perlorodky říční (viz kap. 1.4) se vyskytují v prostředí s nízkými koncentracemi

dusičnanů, nicméně zvýšené koncentrace amoniaku a s tím související hodnoty NO_3-N pod mezí detekce indikují zhoršené podmínky redukčního prostředí (Černá et al., 2018). Omezené množství kyslíku působí stres u velkých mlžů a tím pádem i jejich vyšší náchylnost vůči dalším stresorům (Armstead and Yeager, 2007). Pokud jsou již limitní hodnoty parametrů stanovovány, pak je třeba brát v úvahu, že mají na každém stanovišti jinou úroveň a váhu (Švanyga et al., 2013; Simon and Dort, 2014). Jako příklad lze uvést fosfor a amoniak, jejichž přirozené hodnoty mají značnou variabilitu v regionálním i lokálním měřítku (hodnoty v severní a západní Evropě bývají nižší než ve střední a jižní Evropě; BSI, 2017).

1.3.4 Ochrana

Determinace limitujících faktorů v životním cyklu ohrožených druhů je zásadní pro rozvoj strategie jejich efektivní ochrany a vývoj populace na daném stanovišti (Pickett et al., 1997), jak zmiňuje řada autorů i v případě velkých mlžů (Skinner et al., 2003; Österling et al., 2008; Geist, 2015; Quinlan et al., 2015; Lopes-Lima et al., 2017; **Bílý et al., 2018 – článek 2**). Kvantitativní studie podrobněji zkoumající hlavní hrozby odpovědné za jejich úbytek však chybí (Lopes-Lima et al., 2014).

Velcí mlži čelí obdobným hrozbám jako sladkovodní ekosystémy (viz kap. 1.1, 1.3.2), mnoho druhů je uvedeno na červeném seznamu IUCN. Některé z nich navíc mají význam jako druhy deštníkové (ochrana druhu vede k ochraně celého povodí) a klíčové (při příhodných podmínkách mění morfologicko-chemické parametry celého ekosystému) (Geist, 2010; Švanyga et al., 2013). Z uvedených důvodů lze velké mlže označit za cílovou skupinu pro ochranu ekosystémů sladkých vod.

1.4 Perlorodka říční (*Margaritifera margaritifera*)

1.4.1 Systematické zařazení

Na severní polokouli se v současnosti vyskytují zástupci řádu Unionoida ve dvou čeledích: Margaritiferidae a Unionidae. Podle nejnovější studie Lopes-Lima et al. (2018a) lze do první čeledi zařadit celkem 16 druhů, rozdělených do dvou podčeledí a čtyř druhů (*Gibbosula* Simpson, 1900, *Cumberlandia* Ortmann, 1912, *Margaritifera* Schumacher, 1816, *Pseudunio* Haas, 1910). V Evropě se vyskytují pouze dva druhy: perlorodka říční (*M. margaritifera* (Linnaeus, 1758)) a p. velká (*P. auricularius* (Spengler, 1793)) (Lopes-Lima et al., 2017, 2018a).

1.4.2 Status druhu

Perlorodka říční splňuje kritéria nejen pro druh deštníkový a klíčový, ale i vlajkový (populární druh pro aktivizaci zdrojů pro ochranu ekosystému) a také indikátorový (přítomnost druhu jako ukazatel nenarušeného povodí) (Geist, 2010; Švanyga et al., 2013). Bývá označována jako modelový druh pro ochranu mlžů (Lopes-Lima et al., 2014), obývající oligotrofní povodí, jež patří mezi nejvíce ohrožené ekosystémy v antropogenně ovlivněné (středo)evropské krajině (Švanyga et al., 2013). Degerman et al. (2009) jej v tomto smyslu popisují jako „tichého svědka" zhoršujícího se stavu (vodního) prostředí.

Jedná se o druh s holarktickým rozšířením (Severní Amerika a Eurasie; Böhm et al., 2021), jehož areál rozšíření v Evropě se táhne od Portugalska a Španělska přes Francii a Britské ostrovy a dále přes střední Evropu až po Skandinávii a severozápadní část Ruska (poloostrov Kola a Karélie) (Ostrovsky and Popov, 2011; Švanyga et al., 2013; Lopes-Lima et al., 2017). Od začátku 20. století došlo k velmi dramatickému úbytku populací napříč evropskými zeměmi a daný trend bohužel nadále pokračuje (Geist, 2010; Quinlan et al., 2015; Simon et al., 2015; Lopes-Lima et al., 2017). Tento druh je celosvětově ohrožený dle IUCN (Degerman et al., 2009) a kriticky ohrožený v Evropě (Cuttelod et al., 2011). Dvě třetiny všech známých evropských populací se dnes vyskytují ve Skandinávii (Degerman et al., 2009).

Ve střední Evropě byl zaznamenán populační pokles o více než 90 % (Araujo and Ramos, 2001; Degerman et al., 2009) a zbývající velice fragmentované populace mají problém s reprodukcí (Young et al., 2001; Geist, 2010). Výhled do budoucnosti ve středoevropských podmínkách přitom není podle různých predikčních modelů dobrý, naopak lze předpokládat zhoršení podmínek prostředí (Bolotov et al., 2018). V České republice populace vymřely na více než 95 % původního areálu rozšíření a poslední lokality hostí jen nepatrné zbytky původních milionových kolonií v ČR (Matasová et al., 2013; Švanyga et al., 2013; Simon et al., 2015), přičemž se jedná o druh kriticky ohrožený (Beran et al., 2017). Většina současných lokalit se přitom nachází při horní hranici původního areálu rozšíření v málo obydlených horských oblastech (Švanyga et al., 2013). Jde o podhůří Šumavy a Novohradských hor (Blanice a Teplá Vltava, resp. Malše) a oblast Trojmezí v Ašském výběžku (Lužní potok, Bystřina a Rokytnice) (Matasová et al., 2013; Švanyga et al., 2013; Simon et al., 2015, 2017).

1.4.3 Životní cyklus

Komplikovaný životní cyklus je hlavním vnitřním faktorem s nepříznivým vlivem na status perlorodky říční. Pohlavně dospělí jedinci vypouštějí larvy (glochidie) v létě či raném podzimu (BSI, 2017). Glochidie jsou pasivně unášeny proudem (<u>planktonní</u> fáze) a pro svůj další vývoj vyžadují lososovité ryby, na jejichž žábry se přichycují (<u>nektonní</u> fáze): v evropských podmínkách se jedná o lososa obecného (*Salmo salar* (Linnaeus, 1758)) a zejména pstruha potočního (*Salmo trutta fario* (Linnaeus, 1758)) (Young and Williams, 1984), jelikož tah lososa je velice často znemožněn vzhledem k překážkám vybudovaným na vodních tocích. Metamorfóza v juvenilní jedince je tepelně řízeným procesem (Hruška, 1992; Skinner et al., 2003; Švanyga et al., 2013; Simon et al., 2017), a trvá v závislosti na geografických podmínkách několik měsíců až jeden rok (Skinner et al., 2003; Bolland et al., 2010; BSI, 2017; Clements et al., 2018) (v jihočeských tocích cca 11 měsíců; Simon et al., 2017). Následně dochází k odpadnutí juvenilů z hostitele a mladé perlorodky se zahrabávají do říčního dna, kde tráví prvních několik let svého života v zóně výměny povrchové a podzemní vody (<u>hyporeální</u> fáze) (Buddensiek et al., 1993; Hruška, 1999). Juvenilní fáze bývá označována jako hlavní kritická fáze ve vývoji perlorodky říční (Buddensiek et al., 1993; Geist and Auerswald, 2007) a její délka se v řadě prací udává 5 až 10 let (Geist and Auerswald, 2007; Bolland et al., 2010; Švanyga et al., 2013; Scheder et al., 2015; Lopes-Lima et al., 2017). Pokud to podmínky prostředí umožňují, na povrchu dna se postupně objevují adultní jedinci (<u>bentická</u> fáze) (Degerman et al., 2009; Geist, 2010; Švanyga et al., 2013; BSI, 2017). Celková délka života běžně přesahuje 100 let (Ziuganov et al., 2000; Simon et al., 2017) a jedná se tak o jednoho z nejdéle žijících (bezobratlých) živočichů (Ziuganov et al., 1994; Degerman et al., 2009; Geist, 2010).

1.4.4 Juvenilové a prostorové preference

Prostorové nároky mladých mlžů představují klíčovou informaci pro ochranu druhu a jeho stanovišť (**Bílý et al., 2021 – článek 1**). Původně se předpokládalo, že žijí hluboko zahrabáni v dnovém substrátu (Bauer, 1988). S postupem času a rozvojem metod měření a vzorkování došlo ke změně tohoto předpokladu a dodnes se uvádí, že obývají prostředí mělkého hyporeálu (Buddensiek et al., 1993; Bauer and Wächtler, 2001) v hloubce do 5 až 10 cm (Geist and Auerswald, 2007; Švanyga et al., 2013; Moorkens and Killeen, 2014) (max. 20 cm; Quinlan et al., 2015). Vzhledem ke značné

variabilitě podmínek dna ve vzdálenosti pouhých několika cm (Malcolm et al., 2009, 2010) je nutná přesnější znalost hloubky zahrabání juvenilů (Quinlan et al., 2015).

Hruška (1999) při simulaci vniku nejmladších perlorodek do dna v průtočných akváriích zjistil, že se zahrabávají několik cm hluboko, nicméně dosud jen málo prací zkoumalo in situ období života juvenilních mlžů od jejich uvolnění z hostitele a zahrabání do sedimentu (**Bílý et al., 2021 – článek 1**). Vertikální migrace velkých mlžů je převážně studována u (sub)adultních jedinců (Amyot and Downing, 1997; Watters et al., 2001; Schwalb and Pusch, 2007). Autoři v několika studiích zkoumali chování juvenilních mlžů ještě před jejich zahrabáním do dna s ohledem na vliv smykového napětí (tj. měřítka síly tření způsobené vodou proudící kolem ponořeného povrchu; BSI, 2017) (Daraio et al., 2010; Schwalb and Ackerman, 2011; French and Ackerman, 2014). Data o chování a pohybu mladých perlorodek v prostředí říčního dna jsou však nedostatečná (**Bílý et al., 2021 – článek 1**; Hyvärinen et al., 2021). Ačkoli juvenilní jedinci jsou využíváni pro bioindikační hodnocení prostředí (viz kap. 1.4.7), dosud používaná zařízení buď neumožňují vertikální/horizontální migraci jedinců, nebo nelze popsat jejich případnou pozici na rozhraní voda-sediment. Za použití zařízení s možností pohybu mlžů a otevřeným horním koncem by bylo možné rozlišit jedince s preferencí nejsvrchnější vrstvy oproti těm, kteří opouštějí hyporeál a nechávají se splavovat dolů po proudu toku (**Bílý et al., 2021 – článek 1**).

1.4.5 Ekologické nároky

Nejen komplexní životní cyklus perlorodky, ale taktéž striktní nároky na prostředí mají významný podíl na zranitelnosti druhu vůči antropogenním změnám ve sladkých vodách v posledních 150 až 200 letech (Simon et al., 2015, 2017). Velcí mlži mají požadavky na teplotu a jakost vody stejně jako na substrátové a proudové podmínky (Strayer, 2008). Perlorodka říční je patrně jedním z nejlépe prozkoumaných druhů sladkovodních mlžů (Denic et al., 2022), nicméně některé aspekty jejích ekologických nároků jsou nedostatečně prozkoumány. Řada autorů uvádí, že patří mezi nejcitlivější sladkovodní organismy vůči změnám abiotických podmínek (Hastie et al., 2000; Geist, 2010; Taskinen et al., 2011), zejména znečištění prostředí (Araujo and Ramos, 2001; Geist, 2010; Denic et al., 2015).

Tab. 1. Přehled limitních hodnot pro vybrané ukazatele jakosti vody pro perlorodku říční

Dusičnanový N (NO₃-N)		Amoniakální N (NH₄-N)		Fosforečnanový P (PO₄-P)		Celkový P (TP)		Vápník (Ca)		Konduktivita* (κ)	
Limit (mg l⁻¹)	Zdroj	Limit (mg l⁻¹)	Zdroj	Limit (mg l⁻¹)	Zdroj	Limit (mg l⁻¹)	Zdroj	Limit (mg l⁻¹)	Zdroj	Limit (µS cm⁻¹)	Zdroj
$0,125$ **	Moorkens et al., 2007	$0,1$	Moorkens et al., 2000	$0,005$ **	Moorkens et al., 2007	$0,008$	Degerman et al., 2013	2	Bauer, 1988	40	Varandas et al., 2013
$0,125$	Degerman et al., 2009	$0,1$	Larsen, 2006	$0,03$	Bauer, 1988	$0,015$	Degerman et al., 2009	8	Švanyga et al., 2013	70	Bauer, 1988
$0,5$	Bauer, 1988	$0,1$ (NH_4^+)	Jungbluth, 2011	$0,03$	Oliver, 2000	$0,035$	Moog et al., 1998	10	Jungbluth, 2011	70	Reis, 2003
$1,0$	Oliver, 2000	$0,1$ (NH_4^+)	Švanyga et al., 2013	$0,06$	Moorkens et al., 2000	$0,035$	Jungbluth, 2011	10 ($CaCO_3$)	Oliver, 2000	80	Švanyga et al., 2013
$1,7$	Moorkens et al., 2000	$0,1$ (NH_4^+)	Varandas et al., 2013	$0,06$	Larsen, 2006	$0,035$	Švanyga et al., 2013			100	Oliver, 2000
$1,7$	Larsen, 2006			$0,10$	Varandas et al., 2013					100	Degerman et al., 2009
$2,0$ (NO_3^-)	Varandas et al., 2013									150	Moog et al., 1998
$2,5$ (NO_3^-)	Švanyga et al., 2013									150	Larsen, 2006
										200	Moorkens et al., 2000
										200	Jungbluth, 2011

* Vliv teploty vody

** Hodnota pro rozmnožující se populace

Perlorodka obývá oligotrofní vodní toky (Skinner et al., 2003; Morales et al., 2004; Švanyga et al., 2013; Simon et al., 2015, 2017; Lopes-Lima et al., 2017) s mírně kyselou vodou (pH <7, často v povodí s převahou žuly a ruly) (Bauer, 1988; Geist, 2010; Hauer, 2015). To odpovídá práci Geista (2010), kde autor popisuje výskyt druhu většinou v tocích s nízkým obsahem živin a vápníku. Příhodné geologické podmínky ve střední Evropě lze najít hlavně v oblasti Českého masívu, rozkládajícím se napříč Českou republikou, Německem a Rakouskem (Hauer, 2015; Flödl and Hauer, 2019). Situace není zcela jednoznačná, neboť autoři v různých evropských zemích udávají hodnoty pH až 8–9 v prostředí s výskytem perlorodky říční (Moorkens et al., 2000; Larsen, 2006; Jungbluth, 2011). V evropském standardu pro monitoring populací perlorodky říční a jejího prostředí (BSI, 2017) se navíc uvádí výskyt v širokém rozpětí typů prostředí (od malých oligotrofních řek po velké nížinné minerální vodní systémy) (přehled parametrů úživnosti a jejich limitních hodnot viz tab. 1, str. 17). To může souviset s nálezy odlišných růstových forem, jež jsou dány buď genetickou fixací, nebo lokální adaptací na méně vhodné podmínky (znečištění, eutrofizace a/nebo změny land use) (Matasová et al., 2013). Eutrofizace prostředí má nicméně nepřímý vliv na výskyt a vývoj druhu: zvýšený obsah živin vede k vyšší primární produktivitě v toku a vyššímu podílu organické hmoty, jejímž mikrobiálním rozkladem dochází ke spotřebě kyslíku (Patzner and Müller, 2001; Hauer and Hill, 2007; Strayer, 2008; Degerman et al., 2009; Švanyga et al., 2013) s možnou indukcí hypoxického stresu adultů (Lopes-Lima et al., 2017). Organická hmota se podílí taktéž na sedimentaci a kolmataci dna, vedoucí k vyčerpání kyslíku v prostředí obývaném juvenilními mlži (BSI, 2017). Organické znečištění prostředí souvisí se zvýšením hodnot BSK_5 (tj. biochemické spotřeby kyslíku) (BSI, 2017), jež by měly být obecně na nízké úrovni (např. Bauer, 1988; Oliver, 2000; Larsen, 2006; Švanyga et al., 2013), zejména pro úspěšný vývoj mladých mlžů (<1 mg $O_2 l^{-1}$; Moorkens et al., 2007).

Perlorodka je druh adaptovaný na chladné tekoucí vody (Geist, 2010), přičemž ve světle klimatických změn a různých scénářů globálního oteplování mají svůj význam vysoko položené toky jako lokální refugia (Bolotov et al., 2018). V literatuře se pro habitat perlorodky většinou uvádí teplotní rozsah 0 až 23 °C (Moog et al., 1998; Reis, 2003; Jungbluth, 2011; Švanyga et al., 2013; Varandas et al., 2013), resp. 0 až 25 °C

(Hastie et al., 2003b; BSI, 2017). Teplota vody nesmí být příliš nízká, neboť hrozí promrzání toku a limitování samočistících procesů (Simon and Dort, 2014) a snižuje se úživnost potravy (Švanyga et al., 2013; Simon et al., 2017), a musí být dostatečná pro přirozenou reprodukci druhu, jelikož ovlivňuje dozrávání a vyvrhování glochidií stejně jako zdárný průběh metamorfózy na hostitelských rybách (Hruška, 1992, 1999; Ziuganov et al., 1994; Hastie and Young, 2003; Scheder et al., 2011; Švanyga et al., 2013). Narůstající teplota má pozitivní vliv na přírůstky jedinců (Hruška, 1992), avšak rychlejší růst vede ke kratší délce života (Bauer, 1992; Ziuganov et al., 1994, 2000) a ztrátě reprodukčních let (BSI, 2017). Zvýšená teplota navíc snižuje rozpustnost kyslíku ve vodě a zvyšuje podíl toxického volného amoniaku (USEPA, 1986, 2013). S velmi vysokou teplotou vody souvisí období sucha, kdy se zmenšuje plocha pod vodou (Degerman et al., 2009; Moorkens and Killeen, 2014) a hrozí expozice na vzduchu a predace adultů (Sousa et al., 2018), stejně jako zvýšený úhyn juvenilů (Moorkens and Killeen, 2014). Některé perlorodkové toky či jejich části mohou také zcela vyschnout (Morales et al., 2004; Varandas et al., 2013; Sousa et al., 2018), a to i ve střední Evropě (Švanyga et al., 2013; Hoess and Geist, 2020; Denic et al., 2022).

Hloubka a rychlost proudění

Tradičně se perlorodkové toky označují jako „mělké" tekoucí vody, jak dokládá řada průzkumů napříč Evropou. Švanyga et al. (2013) udávají minimální hloubku 0,1 m, další autoři v nejnovější studii Denic et al. (2022) zmiňují 0,2 m. Výška vodního sloupce v úsecích s výskytem druhu většinou dosahuje 0,2 až 0,8 m (Hastie et al., 2000; Ostrovsky and Popov, 2011; Varandas et al., 2013). Běžně však byly perlorodky zaznamenány i ve větší hloubce: 1–3 m (Ziuganov et al., 1994; Hastie et al., 2000; Reis, 2003; Sousa et al., 2013), resp. až 5–6 m (Degerman et al., 2009). Vzhledem ke značné variabilitě mezi typy říčních habitatů neuvádí BSI (2017) žádné číslo pro vyjádření vhodného habitatu.

Podobná variabilita je spojena také s rychlostí proudu ve vodním toku (Ziuganov et al., 1994). Zatímco někteří autoři popisují jako vhodné „rychle" tekoucí vody (Hastie et al., 2003a, 2004; Skinner et al., 2003; Stoeckl et al., 2020), jiní udávají spíše toky s mírným prouděním vody (Ziuganov et al., 1994; Reis, 2003; Morales et al., 2004; Ostrovsky and Popov, 2011) s rychlostí min. 0,2 m s^{-1} při nízkých průtocích a většinou do 1 m s^{-1} za vysokého vodního stavu (Denic et al., 2022). Varandas et al. (2013) dokonce zmiňují výskyt i v pomalu tekoucích vodách. Přestože ani v tomto

případě není definována vhodnost habitatu dle BSI (2017), shoda panuje na tom, že průtok musí být dostatečně nízký pro zajištění stability substrátu, a přitom vysoký z hlediska kvality dnového prostředí (Álvarez-Claudio et al., 2000; Outeiro et al., 2008; Moorkens and Killeen, 2014; Scheder et al., 2015; Denic et al., 2022).

Substrátové poměry

Jak již bylo zmíněno, pro život perlorodky jsou významné tyto atributy substrátu: struktura (složení, jemný sediment na povrchu), kvalita (míra sedimentace, infiltrace jemného sedimentu, odolnost vůči penetraci) a stabilita (smykové napětí) (BSI, 2017).

Z hlediska velikosti zrna se uvádí jako vhodný písek (Reis, 2003; Ostrovsky and Popov, 2011) či štěrkopísek (Degerman et al., 2009; Ostrovsky and Popov, 2011; Sousa et al., 2013; Švanyga et al., 2013), a to zejména z důvodu snadného (za)hrabání mlžů. Někteří autoři k písku a/nebo štěrku přidávají ještě menší kameny (Álvarez-Claudio et al., 2000; Outeiro et al., 2008; Moorkens and Killeen, 2014; Stoeckl et al., 2020), jež zlepšují fyzikální stav dna toků a pozitivně ovlivňují gradienty fyzikálně-chemických parametrů v prostředí hyporeálu (Dahm et al., 2007; Geist and Auerswald, 2007). Nejčastěji se však za optimální dnové podmínky považují takové plochy nebo kapsy vyplněné pískem a/nebo štěrkem, jež jsou navíc obklopeny většími kameny zvyšujícími stabilitu prostředí (Hastie et al., 2000, 2001, 2003a; Geist and Auerswald, 2007; Jung et al., 2013; Varandas et al., 2013; Hauer, 2015; Quinlan et al., 2015; BSI, 2017; Boon et al., 2019). Stejnozrnný nestabilní štěrk či bahno naopak představují nevhodný typ substrátu (Degerman et al., 2009; BSI, 2017; Boon et al., 2019). Jak však upozorňují Denic et al. (2022), stabilita prostředí může být dostačující pro adultní mlže, kdežto mladí jedinci mají navíc zvýšené požadavky na množství jemnozrnného materiálu v souvislosti s obsahem kyslíku (viz kap. 1.4.6).

Kyslík

Množství rozpuštěného kyslíku ve volné vodě je relevantní pro adultní perlorodky (obývající rozhraní voda-sediment), zatímco pro juvenilní jedince (zahrabané ve dně) má větší význam nasycení vody v hyporeálu (Quinlan et al., 2015). Obecně se udává vysoká míra nasycení okolo 100 % bez velkých výkyvů (Oliver, 2000; Reis, 2003; Švanyga et al., 2013; BSI, 2017). Koncentrace O_2 by měla dosahovat min. 7,6 mg l^{-1} (Jungbluth, 2011), někteří autoři uvádějí dokonce až 9 mg l^{-1} (Moorkens et al., 2000; Varandas et al., 2013): většina hodnot je však vztažena k prostředí volné vody, nikoli

k podmínkám pode dnem. Nárůst teploty a zvýšený metabolismus organismů může kyslíkové poměry v toku zhoršit, jak upozorňují Hoess and Geist (2020).

<u>Souhrn</u>

Ekologické nároky perlorodky říční lze shrnout následujícím způsobem: teplota a pH v rozsahu hodnot, dostatečně vysoké množství kyslíku, dostatečně nízké množství živin (N, P) a jemného sedimentu (Skinner et al., 2003). Odhad limitních hodnot těchto a dalších atributů nicméně vychází z přítomnosti/absence druhu na dané lokalitě, resp. statutu dané populace (Araujo and Ramos, 2000; Moorkens et al., 2007; Degerman et al., 2009; Švanyga et al., 2013; Varandas et al., 2013), přičemž všechny perlorodkové habitaty ve střední Evropě jsou více či méně poškozeny a reprezentují spíše podmínky tolerovatelné než optimální (o problematice nastavení environmentálních limitů více viz kap. 1.3.3) (Denic et al., 2022). K uvedeným nárokům na prostředí je ještě třeba přidat potravní detrit (jemný rozložený organický materiál z helokrenních pramenišť, rhizosféry psárkových luk či ponořených vodních makrofyt), jehož vznik a transport závisí na procesech v celém povodí (Švanyga et al., 2013).

1.4.6 Juvenilové a abiotické faktory

Habitatové požadavky raných vývojových stádií perlorodky říční jsou dosud jen málo podrobně prozkoumány (Quinlan et al., 2015; Lavictoire et al., 2016). Mladé perlorodky mají obecně vyšší nároky na podmínky prostředí než adultní jedinci (BSI, 2017). Vyžadují kvalitní biotop splňující řadu požadavků, jako jsou teplota vody a její fyzikálně-chemické parametry (zejména obsah O_2), substrátové poměry a přítomnost úživného zdroje potravy (Geist and Auerswald, 2007; Tichá et al., 2012; Švanyga et al., 2013; Simon et al., 2017). Často chybí přímé terénní důkazy o významu a vlivu těchto faktorů na výskyt a vývoj juvenilů, jejichž přežití slouží též jako bioindikátor kvality habitatu (Geist and Auerswald, 2007). V textu níže jsou uvedeny poznatky o ekologických nárocích mladých jedinců dle struktury uvedené v kap. 1.4.5.

<u>Jakost vody</u>

Eutrofizace vody má nepříznivý vliv jak na růst, tak na přežití juvenilů napříč zástupci druhů velkých mlžů (Buddensiek, 1995; Constable et al., 2003; Strayer, 2008; Degerman et al., 2009; Eybe et al., 2013; Hua et al., 2013; Haag et al., 2019). Jen málo podrobností je k dispozici pro perlorodku (Taskinen et al., 2011), ačkoli mladí mlži

jsou obecně citliví na degradaci jakosti vody (viz kap. 1.3.3). Nedávná studie Belamy et al. (2020) upozorňuje na nesoulad mezi daty z laboratorního a terénního prostředí pro různé parametry jakosti vody a jejich ekotoxikologické limity pro přežití jedinců druhu. Navíc chybí údaje o vlivu epizodického znečištění (**Barák et al., 2022 – článek 3**), jelikož podmínky v laboratoři (kontinuální expozice vzorkům vody či konstantní koncentrace jedné látky) neodpovídají občasné, resp. pulzní expozici v přirozeném vodním prostředí (Connon et al., 2012).

Teplota vody

Nárůst teploty vody má pozitivní vliv na růst juvenilních jedinců velkých mlžů včetně perlorodky říční (Buddensiek, 1995; Bartsch et al., 2003; Lange and Selheim, 2011; Denic et al., 2015; Haag et al., 2019). Velmi vysoká teplota však může mít negativní vliv z hlediska (sub)letální tolerance mladých mlžů, jak potvrzuje řada experimentů v laboratorních podmínkách (Dimock and Wright, 1993; Pandolfo et al., 2010; Ganser et al., 2013; Archambault et al., 2014). Jak bylo uvedeno v kap. 1.4.5, rozsah hodnot pro výskyt perlorodky je značně široký, nicméně nedávné velmi teplé roky vedly i ve střední Evropě ke zvýšení teploty vody v letním období blížící se předpokládanému hornímu limitu (Černá et al., 2018). Navíc může docházet ke zhoršení kyslíkového režimu a umocnění vlivu toxického znečištění (viz kap. 1.4.5).

Hloubka a rychlost proudění

Jak již bylo uvedeno v kap. 1.4.5, pro hloubku vody a rychlost proudu toku nejsou definovány žádné jednoznačné limitní hodnoty. Hloubka vody má však nepřímý vliv na teplotu, rychlost proudu a úroveň O_2 (Johnson et al., 2001). Proudové podmínky navíc ovlivňují míru sedimentace a pohyb živin v toku a tím pádem i kyslíkový režim v sedimentu (Moorkens and Killeen, 2014) i ve volné vodě.

Substrátové poměry

Pro úspěšný vývoj juvenilů hraje zásadní roli množství jemného sedimentu, jež závisí na vstupu anorganického materiálu a produkci organické hmoty přímo v toku (Moorkens, 2018). Autoři se shodují, že dno toků by nemělo být vystaveno procesu nadměrné sedimentace, neboť jemný materiál omezuje jeho prostupnost (Geist and Auerswald, 2007; Scheder et al., 2015; Hoess and Geist, 2020; Stoeckl et al., 2020) a tím pádem i vstup kyslíku (Geist and Auerswald, 2007; Quinlan et al., 2015; Stoeckl et al., 2020). Výměna vody na rozhraní vodního sloupce a dnového říčního sedimentu

mimo zajištění prokysličeného prostředí příznivě ovlivňuje také transport živin a odpadních produktů (Geist and Auerswald, 2007; Bolland et al., 2010; Quinlan et al., 2015; Scheder et al., 2015).

Kyslík

Stejně jako nadměrné množství živin, i chudé kyslíkové poměry mají nepříznivý vliv na růst a přežití juvenilních mlžů (Sparks and Strayer, 1998; Beckvar et al., 2000; Strayer, 2008; Degerman et al., 2009). Hodnoty nasycení okolo 100 % bývají běžně zaznamenány v tekoucích vodách a nejsou limitujícím faktorem v prostředí volné vody dle práce Černé et al. (2018). Význam prokysličeného dna pro mladé perlorodky byl již zmíněn výše, nicméně i dobré dnové podmínky se mohou proměňovat kvůli průtokovým změnám (Quinlan et al., 2014). Nejnovější laboratorní experimenty ukazují, že epizodické hypoxické podmínky mohou vést až k absolutnímu úhynu juvenilních jedinců (Hyvärinen et al., 2022). Detekce biologicky významných událostí nízkého obsahu kyslíku může pomoci k pochopení dynamiky O_2 (Quinlan et al., 2015).

1.4.7 Juvenilové a in situ bioindikace

Pro zjištění vlivu abiotických faktorů na výskyt a vývoj perlorodky říční lze využít in situ bioindikačních experimentů a principu CBM, jež byly charakterizovány v kap. 1.2. Jednoduchá metoda spočívá v přesném změření rychle rostoucích juvenilů na začátku a na konci experimentu (tj. před instalací zařízení v toku a po jejich vyjmutí z vody), přičemž kromě hodnocení přírůstků lze také získat údaje o úmrtnosti jedinců (Simon et al., 2017). Růst jedinců v přirozených podmínkách musí být dostatečně rychlý pro předehnání koroze, jež narušuje schránku mlžů (BSI, 2017; Simon et al., 2017). První podrobnosti o využití destičkových izolátů ve volné vodě a hodnocení růstu a přežití juvenilních perlorodek po několikatýdenní expozici v tocích přináší Buddensiek (1995). Později byly vyvinuty další typy zařízení nejen pro perlorodku říční: např. pískové klícky (boxy vyplněné pískem; Hruška, 1999, 2002), průtočná sila založená na principu upwellingu (Barnhart, 2006) (s pohybem vody ve směru zezdola nahoru; Dahm et al., 2007) nebo další zařízení (souhrn viz Gum et al., 2011). Otázkou však zůstává, jak metodicky provádět práci s juvenily a experimentálními zařízeními a jak efektivně hodnotit stav jedinců v rámci testů (**Bílý et al., 2018 – článek 2**).

Nedávné studie potvrdily možnost využití in situ bioindikačních experimentů pro testování jakosti vody, teploty a využitelnosti dostupné potravy (Lange and Selheim,

2011; Švanyga et al., 2013; Denic et al., 2015; Scheder et al., 2015), jež jsou navíc doprovázeny měřením fyzikálně-chemických parametrů prostředí a/nebo kvalitativní analýzou potravní složky. Jakost vody je dávána do souvislosti zejména s přežitím juvenilů, zatímco teplota a kvalita potravy mají hlavní vliv na jejich růst (Lange and Selheim, 2011). Hodnocení kvality a úživnosti potravního detritu je však efektivnější spíše ex situ v laboratorních podmínkách (kvůli eliminaci vlivu teploty v přirozeném prostředí) (Švanyga et al., 2013; Simon et al., 2017). Nicméně terénní experimenty jsou využívány stále nedostatečně, což platí zejména v případě hodnocení účinků znečištění vodního prostředí (**Barák et al., 2022 – článek 3**). Často se provádí v prostředí vodního sloupce (viz výše citované práce), jen vzácně v zóně hyporeálu (Černá et al., 2018). Parametry volné vody jsou uváděny jako slabé prediktory kvality habitatu pro juvenily žijící ve dně (Geist and Auerswald, 2007; Stoeckl et al., 2020), nicméně jak uvádí Strayer (2008), při posuzování vhodnosti dané lokality pro daný druh je třeba nejprve zkoumat teplotní režim a jakost vody a teprve poté podmínky dna. Vzhledem k výměně na rozhraní voda-sediment navíc musí hodnoty atributů v pórové vodě (v zóně hyporeálu) odpovídat hodnotám ve volné vodě (Moorkens et al., 2000). Z toho důvodu lze na volně tekoucí povrchovou vodu nahlížet jako na referenční prostředí pro analýzy pórové vody (Buddensiek et al., 1993).

1.4.8 Hrozby

Vnější hrozby s nepříznivými účinky na životní cyklus perlorodky a její nároky na prostředí lze rozdělit do tří hlavních kategorií: (1) nedostatek hostitelských ryb, (2) znečištění vody a (3) změny hydromorfologie toků (nedostatek vhodného substrátu kvůli úpravám toků či zanášení dna jemným materiálem a snížení úrovně O_2 kvůli změnám land use v povodí). V minulosti hrál významnou roli také lov perel, dnes se k uvedeným hrozbám přidávají také invazní druhy a klimatické změny (Bauer, 1988; Ziuganov et al., 1994; Geist et al., 2006; Geist and Auerswald, 2007; Degerman et al., 2009; Geist, 2010; Taeubert et al., 2010; Cosgrove et al., 2016; BSI, 2017; Flödl and Hauer, 2019; Hoess and Geist, 2020). Nepříznivé účinky mohou být znásobeny při současném působení více stresorů (Lopes-Lima et al., 2017).

Obdobně jsou popisovány hrozby pro druh v České republice: (1) kvalita a kvantita hostitelských ryb (nevhodný rybářský management), (2) znečištění (např. eutrofizace a režim nakládání s odpadními vodami), (3) regulace toků a výstavba nádrží (změny

průtokového a splaveninového režimu), (4) změny land use (zalesnění části povodí a ochlazení toku, degradace potravních zdrojů apod.), (5) erozní činnost (vstup jemného materiálu do toku) a (6) turismus (rekreační aktivity typu splouvání) (Matasová et al., 2013; Švanyga et al., 2013; Simon et al., 2015, 2017).

Jak již bylo uvedeno, přímý vliv člověka byl v minulosti spjatý zejména s lovem perel. Dnes lze rozlišovat jak cílený kontakt (na základě záznamů nedávné pytlácké aktivity spojené se sběrem jedinců včetně ilegálního lovu perel; Simon et al., 2017; Stoeckl et al., 2020), tak náhodné setkání (vodáctví a pohyb návštěvníků v toku při brodění či koupání; **Barák et al., 2022 – článek 4**). Interakce mlžů s člověkem při aktivitách typu rekreačního splouvání vodních toků mohou způsobovat disturbanci jedinců druhu, jak uvádí také BSI (2017).

1.4.9 Specifické postavení ČR

<u>Výskyt</u>

Lokality s výskytem druhu se ve velké míře nacházejí v přeshraničních oblastech, což je případ také povodí Malše a Teplé Vltavy (Simon et al., 2015).

Horní tok Malše má přirozenou dynamiku toku, zachovalý povodňový režim a polopřírodní nivu (Simon and Dort, 2014). Ačkoli zde lze nalézt jen rozptýlenou a nepočetnou populaci perlorodky říční, pouze na Malši a na dolní Blanici byly nedávno zjištěny známky ojedinělé přirozené reprodukce (Simon et al., 2015). Jakákoli lokalita s doloženými záznamy úspěšného rozmnožování je významná pro udržení dlouhodobé životaschopnosti populace druhu (Cosgrove and Hastie, 2001), nicméně vzhledem k nálezu max. desítek mladých jedinců zde nelze předpokládat nárůst početnosti či zlepšení věkové struktury (Simon et al., 2015, 2017). Problémy na Malši představují množství erozních splavenin a nestabilita dnových sedimentů, nízká teplota vody v horní části a eutrofizace v dolní části toku (negativní vliv zemědělského hospodaření a zejména bodového komunálního znečištění; **Barák et al., 2022 – článek 3**) (Švanyga et al., 2013; Simon and Dort, 2014).

Teplá Vltava představuje unikátní systém středoevropské říční nivy: jde o horskou meandrující řeku severského charakteru s přirozeným korytem s hustými dnovými porosty makrofyt a pravidelnými záplavami. Celý tok pak obklopují četná rašeliniště, mrtvá ramena, vlhké louky a lesní společenstva (Švanyga et al., 2013; Černá et al., 2018; Křenová, 2018). Oligotrofní řeka s vysokou jakostí vody, prokysličeným dnem

a potravní nabídkou (detritem z vodních makrofyt) může poskytovat vhodné habitaty pro výskyt dospělých i juvenilních mlžů (Matasová et al., 2013; Švanyga et al., 2013; Černá et al., 2018; **Bílý et al., 2021 – článek 1**). Ve středoevropské krajině nenajdeme žádnou srovnatelnou řeku této vodnosti s nízkou trofií a specifickými fyzikálně-chemickými parametry pro život perlorodky říční (Švanyga et al., 2013). Přes všechna pozitiva byla na Teplé Vltavě zaznamenána pouze rozptýlená a málo početná populace druhu bez přímého důkazu přirozené reprodukce (Matasová et al., 2013; Švanyga et al., 2013; Simon et al., 2015; Černá et al., 2018). Hlavní problémy zahrnují nedostatek hostitelských ryb (kvůli vysazování konkurenčních druhů a tahu ryb z vodní nádrže Lipno), regulace přítoků jako potenciálních biotopů pro mlže/ryby a v neposlední řadě (intenzivní) turistickou návštěvnost toku (rekreační splouvání; Švanyga et al., 2013).

Značný nárůst intenzity splouvání Teplé Vltavy na území NP Šumava na přelomu 20. a 21. století (Švanyga et al., 2013; Křenová and Kindlmann, 2015; Křenová, 2018) vedl k poškození porostů dnových makrofyt za nízkých průtoků (Zelenková, 2008). Riziko přímého kontaktu člověka a mlžů za uvedených podmínek bylo pravděpodobně vysoké (Lorenz et al., 2013; Švanyga et al., 2013), bohužel nebylo blíže hodnoceno. Splouvání Teplé Vltavy je regulováno od roku 2004 (Simon and Kladivová, 2006). V roce 2009 došlo ke zpřísnění podmínek (Diviš, 2009), přičemž od roku 2012 musí být většina lodí doprovázena odborným průvodcem. V hlavní oblasti výskytu perlorodky říční platí nejpřísnější režim splouvání (tj. denní doba a čas, typ plavidla (kánoe a kajak), minimální hladina vody, počet lodí za hodinu, povinný průvodce pro skupiny a nutná registrace včetně platby poplatku; více viz Barák, 2017). Intenzita splouvání se snižuje: místo dřívějších více než 12 000 plavidel (2005–2006) dnes řeku splouvá 2–4 000 lodí ročně (Křenová, 2018). Ačkoli značný pokles počtu návštěvníků může vést k vyšší úrovni turistiky (Bednar-Friedl et al., 2012), znalosti o přímém vlivu regulovaného splouvání na perlorodku říční stále chybí (**Barák et al., 2022 – článek 4**). Rekreační splouvání je spojeno s dalšími aktivitami, jako např. brodění v toku a disturbance dna (Cole and Landres, 1995), přičemž zde hrozí negativní vliv sešlapu dna s výskytem velkých mlžů (Calcagno et al., 2012) stejně jako manipulace s jedinci (Ohlman and Pegg, 2020). Schránky perlorodky mohou být náchylné k mechanickému poškození (Bílek, 2013), avšak reakce populací druhu na antropogenní disturbance jsou jen málo prozkoumány (Ziuganov et al., 2000).

Odchov

V České republice byla vyvinuta unikátní (tzv. česká) metoda polopřirozeného odchovu perlorodky říční (autor: J. Hruška). Odchov jedinců na přirozené potravě se podařilo dovést až do věku okolo 15. až 20. roku života (Švanyga et al., 2013; Simon et al., 2017), přičemž ČR byla prvním evropským státem s odchovnou stanicí (Geist, 2010). Odchovaní juvenilové již byli na některých tocích včetně Malše (v horní části v období ještě před nálezy jedinců z přirozené reprodukce) a Teplé Vltavy vysazeni do volné přírody v rámci propopulačních opatření (Simon et al., 2015). Ačkoli by měla převažovat spíše revitalizace habitatu tekoucích vod, odchov mlžů může být nástrojem „last minute" udržení evolučního potenciálu cenných populací (Gum et al., 2011). Jelikož v rámci záchranného programu (Švanyga et al., 2013) je ročně generováno tisíce jedinců z polopřirozeného odchovu, právě in situ bioindikační experimenty zmiňované napříč tímto textem mohou poskytnout odpověď na otázku, kam mladé mlže „vypustit" (**Bílý et al., 2018 – článek 2**).

2. Cíle práce a výzkumné otázky

Na základě vymezených dosud málo probádaných oblastí výzkumu perlorodky říční byly definovány následující cíle a otázky.

(**I**) Zjistit prostorové preference juvenilních jedinců v přirozeném prostředí říčního dna (**Bílý et al., 2021 – článek 1**):

Mají mladí jedinci tendenci v experimentálních zařízeních (sondičkách) setrvávat či je opouštět?

Pokud tam setrvávají, upřednostňují horní vrstvy dna (do 10 cm hloubky)?

Liší se jejich hloubková preference v závislosti na sezónních podmínkách?

(**II**) Vytvořit protokol pro provádění in situ bioindikačních testování s juvenilními jedinci v oligotrofních tocích (včetně instalace zařízení a vyhodnocení testů; **Bílý et al., 2018 – článek 2**):

Liší se vývoj mladých jedinců v závislosti na typu experimentálního zařízení a jeho umístění (na základě reprezentativních výsledků)?

(**III**) Vyvinout a otestovat robustní metodu pro in situ hodnocení vlivu bodového znečištění na juvenilní jedince (**Barák et al., 2022 – článek 3**):

Liší se vývoj mladých jedinců mezi poškozeným a referenčním horním/dolním úsekem toku?

Jaké hlavní indikátory komunálního znečištění mají pozitivní/negativní vliv na exponované jedince?

Jaká jsou doporučení v oblasti ochrany sladkovodních mlžů při využití CBM pro druhy obývající tekoucí vody s bodovými zdroji znečištění?

(**IV**) Vyhodnotit přímý vliv návštěvníků (vodáků) chráněného území (NP Šumava) na perlorodku říční pro zlepšení managementu ochrany druhu (**Barák et al., 2022 – článek 4**):

Liší se interakce mezi vodáky a mlži v závislosti na abiotických podmínkách?

Jaké je typické chování vodáků v důsledku interakcí (zejména s ohledem na typ stanoviště)?

Jaká intenzita mechanické disturbance při provozování vodáckého sportu vede k poškození schránky (modelu) mlže?

3. Soubor předkládaných článků

ČLÁNEK 1: Hloubková preference juvenilních jedinců perlorodky říční (Bílý et al., 2021) – „uveřejněný" (J_{imp})

Occurrence depth of juvenile freshwater pearl mussels (*Margaritifera margaritifera*) in a river bed tested by experimental mesh tubes

Michal Bílý, Ondřej Simon, **Vojtěch Barák**, Václava Jahelková

Podíl autora: Významně jsem se podílel na zpracování a vizualizaci dat. Revize rukopisu proběhly ve spolupráci s Michalem Bílým.

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FRESHWATER MOLLUSCS

Occurrence depth of juvenile freshwater pearl mussels (*Margaritifera margaritifera*) in a river bed tested by experimental mesh tubes

Michal Bílý · Ondřej Simon · Vojtěch Barák · Václava Jahelková

Abstract Sediment depth preference was tested on 1-year-old freshwater pearl mussel juveniles. Mesh tubes allowing vertical migration with a possibility to leave hyporheic space, filled with 1–2-mm granulometry size substrate and populated with groups of juveniles, were placed in the natural river bed of the Teplá Vltava River, Czech Republic. The depth position of the juveniles was recorded after 1- and 2-month exposure during the summer and after 8 months including wintering. The juveniles showed a tendency not to stay on the sediment surface, but to penetrate into a very shallow depth, and to accumulate and stay 2–3 cm deep in the substrate in the summer period. No juvenile was found below 8 cm sediment depth, neither in the summer nor after wintering. In the experimental tubes, various survival rates (0–100%) were recorded in close relation to substrate oxygenation development at the tested sites. Oxygen decrease

was accompanied by juveniles trying to escape the hyporheal.

Keywords Hyporheic zone · Sediment · Occurrence depth · Vertical migration

Introduction

The survival and successful development of early unionoid juveniles is crucial for population viability (Bauer, 1988; Geist & Schopf, 2005). Therefore, determining juvenile living space preferences is also key information for species and site conservation. Generally, it is assumed that juvenile post-parasitic mussels occupy similar habitats to adults (Neves & Widlak, 1987; Strayer, 2008) and that upper layers of hyporheic zones constitute the microhabitats for juveniles (Buddensiek et al., 1993). Yet, there is very little work examining the period after unionoid juveniles detach from a host fish and burrow into the substratum in situ (French & Ackerman, 2014). Juvenile unionoids have frequently been used in toxicology studies (Newton & Bartsch, 2007; Ganser et al., 2013; Kleinhenz et al., 2019), mostly in North America, but only one experiment (Bartsch et al., 2003) used in situ design in hyporheic conditions (for review see Farris & Van Hassel, 2007). Vertical migration of unionoid mussels in rivers has been

M. Bílý (✉) · O. Simon · V. Barák · V. Jahelková
Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Suchdol,
165 00 Prague, Czech Republic
e-mail: bilym@fzp.czu.cz

studied with adult or subadult specimens (Amyot & Downing, 1997; Watters et al., 2001; Perles et al., 2003; Schwalb & Fusch, 2007) but very rarely with juvenile mussels (Yeager et al., 1994). Some studies describe the behaviour of juvenile mussel just before burrowing and the effect of river bed shear stress (Daraio et al., 2010; Schwalb & Ackerman, 2011; French & Ackerman, 2014).

Data about juvenile behaviour and habitat preference of the Freshwater Pearl Mussel (FPM) *Margaritifera margaritifera* (Linnaeus, 1758) itself are very scarce. Since pioneer Buddensiek´s work (Buddensiek et al., 1993), FPM juveniles have been used for bioindication assessment of riverine free-flowing water (for review, see Gum et al., 2011) or rarely in hyporheic condition (Černá et al., 2018). All these experiments used Buddensiek plates, which exclude vertical or horizontal migration of juveniles. Hruška sandy cages filled with a layer of selected sand only represent bioindication units, giving the possibility of vertically or horizontally movement (Bílý et al., 2018; Černá et al., 2018), but this method cannot describe a juvenile's position on the sediment–water interface. Very valuable results have come from studies testing the influence of condition (e.g. sediment grain size) on optimal FPM juvenile growth and survival in culture systems (Lavictoire et al., 2016), as well as the release of post-parasitic stages into experimental in situ receptor sites (Moorkens, 2018). Nevertheless, the substrate depth which juvenile FPM prefer in a natural river bed has remained unknown until now.

Any identification of juvenile life space is impossible if it is based on natural occurrence records because finding mussel juveniles up to several millimetres in body size is very uncommon (Neves & Widlak, 1987). In this situation, a solution can be experimentally placing juveniles into a small closed space representing a river bed segment, enabling the juvenile's free movement, and allowing their position to be identified.

For such types of experiment, obtaining a sufficient number of animals is a problem. Experiments are not feasible with single individuals, so experimental groups are necessary. Although breeding programmes are able to produce thousands of semi-naturally reared FPM juveniles annually (Švanyga et al., 2013), every small quantity of such juveniles represents an extremely valuable resource; hence, any experiment with expected loses should be carefully considered.

Therefore, we took the opportunity to use several hundred juveniles reared and designated especially for in situ bioindication in 2014 and 2015 (Černá et al., 2018).

In this paper, we present the results of a pilot in situ experiment with FPM juveniles focused on hyporheal depth preference. Vertical cylinder devices were used, with the possibility of juvenile vertical movement, including the possibility of emerging onto the river bed surface. After a methodical ex situ experiment confirming the designed method was applicable, a field experiment was carried out in 3 steps. The central one was an exposure in the summer for 1 and 2 months, with the aim of finding out at what substrate depth the individuals settle. The study was done on one of the best preserved Central European mountain rivers—the Teplá Vltava (Šumava National Park, Czech Republic), which is also one of the FPM localities with high conservation potential (Matasová et al., 2013; Simon et al., 2015).

Methods

Experiment locality and selection of sites

The field experiments were carried out on a 100-m-long stretch ('Experimental Meander') of the Teplá Vltava River, located 3 km above the confluence with the Studená Vltava River. It is a part of the core FPM occurrence zone of the Vltava River catchment (Matasová et al., 2013; Simon et al., 2015). Previous studies showed this locality as one of the most favourable for juvenile survival and growth within the catchment (Černá et al., 2018). Matasová et al. (2013) list subadult mussel presence in sites close to the Experimental Meander and the findings were also confirmed in 2019 (unpublished). The river is about 15 m wide at this stretch and it is wadable year-round during normal water flows. The river bed composition is very varied. Stones and gravel, sand-gravel, and sand are present (ISO 14688-1:2002). During low water flow events, small temporal rapids can occur. Discontinuous areas of macrophytes are present (especially *Myriophyllum* sp. and *Batrachium* sp.).

The experimental equipment was placed in sites with discontinuous gravel substrate (up to 60 mm size) with a coarse sand fill (up to 2 mm). The water depth of the sites was from 0.15 to 0.4 m at the time of

Fig. 1 Tube installation technique. Schematic drawing of tube ▶ installation into the river bed using a special basket (**a**), detail of tube before final arrangement of bed surface (selected material inside and two mesh sizes are visible) (**b**), and detail of tube after final arrangement of bed surface (**c**)

(a)

(b)

(c)

equipment placing, allowing accessibility during an exposure. As dissolved oxygen (DO) concentration is a crucial factor for juvenile mussel survival in hyporheic conditions (Chen et al., 2001, Roley & Tank, 2016), the sites were established after previous mapping of oxygen conditions to maximize suitability for juveniles. The hyporheic water was extracted using a modification of hyporheic water sampler (Trulleyová et al., 2003; Dahm et al., 2007; Hauer & Lamberti, 2007) and DO measured in the field with a pocket oximeter (INSA). Only microhabitats where oxygenation regularly overlapped 70% in 10 cm of sediment depth were chosen for the experiments.

Experimental animals, mesh tubes and substrate

Juveniles of 1 + year of age (born in the year preceding the experiment) with shell length ranging from 0.5 to 1.0 mm and belonging to a local conservation unit (Geist & Kuehn, 2005; Simon et al., 2015) were used for the experiments. They originated from the Action Plan for the FPM in the Czech Republic (Švanyga et al., 2013). Before our experiments, they had successfully completed a 1-month bioindication exposure in the target Vltava River zone (Černá et al., 2018), thereby confirming their viability. After the experiments, all the survivors found were returned to the breeding programme.

The experimental devices were tubes 3 cm in diameter and 12 or 17 cm in height (see "Experiment design" section for details). The outer layer of the tube was made from stainless steel net of 1.00 mm mesh density. The inner tube layer was made from 0.25 mm nylon mesh (Bílý et al., 2018), making the tube wall impassable for juveniles. Only the upper tube mouth stayed open (Fig. 1b), so the juveniles can escape the experimental tube. This measure is very important for the experiment design, even at the cost of losing some juveniles. Otherwise, there would be confusion between juveniles really preferring the topmost surface layer and those which leave the hyporheal but are not washed away if the upper tube mouth was closed.
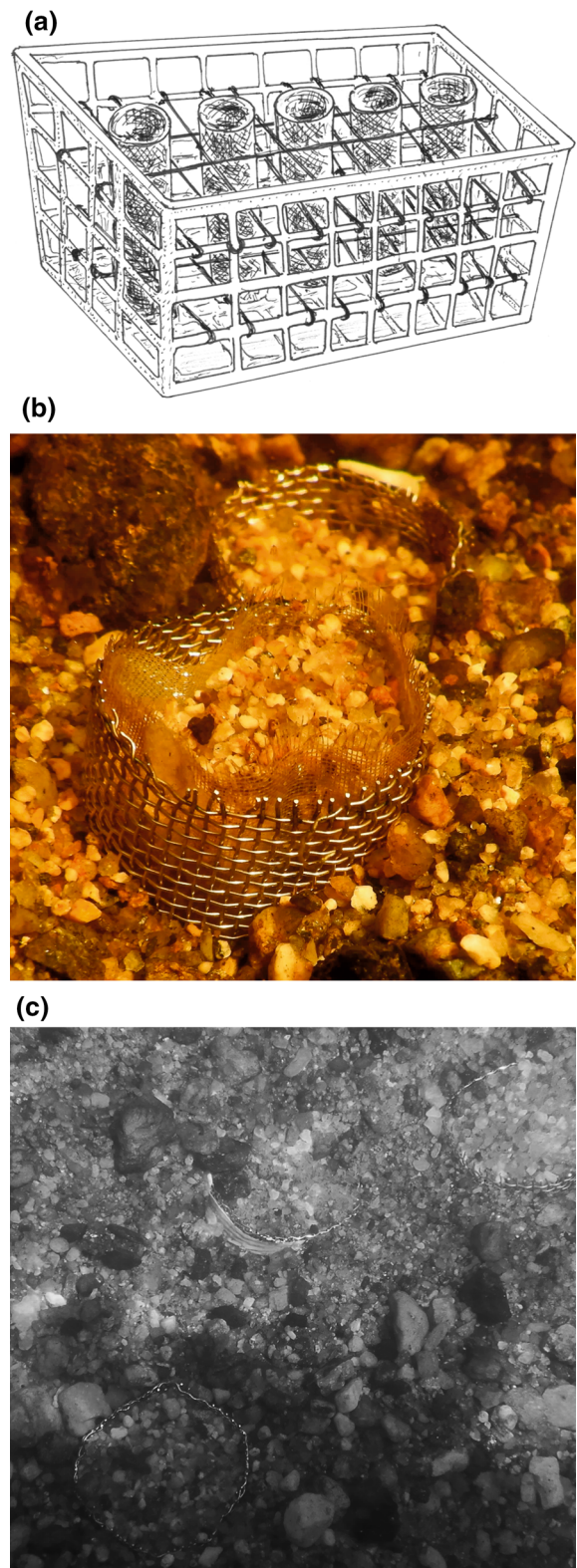
The tubes were filled with a size-fractioned sand substrate originated from the river bed surface of the experiment locality. A sand grain size of 1.0–2.0 mm was used, which was successfully tried within the Action Plan for the FPM in the Czech Republic, especially for bioindication (Bílý et al., 2018; Černá et al., 2018) and reported as suitable for juveniles (Lavictoire et al., 2016). In addition, this grain size makes it relatively easy to search for juveniles under a stereomicroscope. To minimize changes in natural biofilms on grain surface, the sand was collected and the grain size was separated maximally 48 h before tube placing in the field. Beforehand, the fractioned sand was stored at 14°C, submerged in a container with original river water.

Tube installation technique in the field

The tubes were filled with the fractioned sand substrate up to 1 cm below the tube upper edge. They were stored in a vertical position, completely submerged in a container with river water and were transported to the locality in this position. Immediately before tube installation into the field, a defined number of juveniles were placed on the substrate surface. Consequently, they were covered with a 1-cm-thick layer of fractioned sand, so the remaining space in the tube was filled. This 1-cm-thick layer of sand protects the juveniles from being quickly washed away after tube installation into the river.

The tubes were placed into the river bed in sets of 3 or 5 pieces. Every set was positioned in a special plastic basket with a sparse grille and large holes and of the same high as the tubes (Fig. 1a). The inner space of the basket was separated by horizontally positioned plastic ropes forming a bilayer square network (3 × 3 cm). Using this network, the tubes can be placed in a vertical position, without mutual contact. The longer edge of the basket and thus the row of tubes was situated in a direction across the river channel.

Firstly, the basket was placed into a pre-excavated hole in the river bed. The upper edge of the basket corresponded to the surrounding bed cover as well as the upper edge of the tubes, which were placed into the basket subsequently. During manipulation, it is appropriate to preserve the open upper mouths of the tubes with an elastic membrane to prevent substrate or juvenile loss due to the river current. Lastly, the remaining basket space between the tubes was filled with a bed substrate originated from the location of basket installation and the elastic membrane on the tubes was removed. Although the substrate inside the tubes was specially prepared, the tubes were surrounded by an original bed substrate and exposed in natural flowing hyporheic water (Fig. 1c).

After a defined time (weeks/months), the tubes were taken out from the baskets. They were protected by an elastic membrane on their upper mouth (to prevent substrate or juvenile loss) and transported to a laboratory (in a nearby field research station) while submerged in river water in a field thermo box. The transport took a maximum of 45 min. Due to slight but permanent disturbance of tube condition during transport (mechanical shocks, slight deflections from position, change of surrounding water movement), a limitation or even elimination of juvenile movement could be expected.

Laboratory procedures and results evaluation

In the laboratory, the substrate column from each tube was immediately divided into depth layers of about 0.75 cm (1.0 cm in the methodical ex situ experiment). The tube was positioned vertically, its upper (open) mouth downwards and put on a small Petri dish of diameter 3.2 cm. Using a squeeze bottle and with fine tapping, the substrate was very carefully moved down until the Petri dish was full. The full volume of this Petri dish represented approximately 0.75 cm of the substrate column. Consequently, the tube mouth was closed with a thin plastic plate, slid from the side, and the tube was moved on the next Petri dish and the process was repeated. The wet sand becomes a compact, adhesive matter, presenting a complicated route between the grains, so there was no danger of juveniles sinking between depth horizons during the separation process.

Finally, a set of small Petri dishes containing the separated substrate of about 0.75-cm-thick layers was ready. Each separated layer was moved to a bigger Petri dish (10 cm diameter) enabling the substrate grains to be spread. The dishes were filled with river water and stored in a thermo box until observation. Consequently, the layers were carefully observed under a stereomicroscope in small portions. Juveniles (alive and dead) were counted and categorized as occurring in the observed substrate depth layer. For maximum accuracy of the results of the main

experiment, the actual volume of each processed substrate layer was measured and the actual height of the individual layers was back-calculated with an accuracy of 1 mm. For final juvenile depth distribution evaluation, histograms were made with a unit of 1.5 cm.

Additional measurements

Each in situ-placed basket was equipped with two temperature data-loggers (HOBO Onset) recording temperature every hour during the exposure time at depths of 3 and 10 cm (5 and 15 cm in an introductory experiment). From the same depths, two silicone hoses of 4 mm diameter were led above the river bed surface. They enabled us to check the oxygenation of hyporheic water at any time during the experiment; this method was used with regard to the need to not disturb the substrate structure in close proximity of the tubes, even at the cost of any inaccuracies of DO measurement relating to water sample abstraction (Quinlan et al., 2014). The lower (collecting) end of the hose was protected against clogging by two layers of net (external 100 μm, internal 40 μm). The upper end was extendable above the water surface and served for sample extraction with a syringe (Rulík et al., 2000). After extracting the hose volume, the next water volume was used to immediately measure DO and water conductivity using a field oximeter and conductometer (INSA, WTW).

DO and water conductivity were also measured in free-flowing water; in addition, water depth and current velocity were measured at the sites.

Experiment design

A preliminary methodical ex situ experiment (when tubes with juveniles were exposed for 24, 48 and 72 h in laboratory conditions) showed juvenile findability (often 100%) and their tendency to penetrate from substrate surface deeper (Online Resource 1). Then, 3 steps of the field experiment were followed.

Step 1: introductory experiment (first wintering)

This part had three aims: (i) to try the possibility of in situ tube application; (ii) to try long-time juvenile survival (including wintering) in the tube condition

and the tendency to escape/remain in the tubes; and (iii) substrate depth horizon preference of the juveniles in the field. Six tubes of 17 cm height were placed in the river bed in two triples using two baskets. Five of the tubes were designed standardly while the sixth one lacked the inner mesh layer so it enabled horizontal juvenile escape. 25–26 juveniles were positioned into each tube (together 153 specimens). Pairs of continual thermometers and extracting hoses were applied into 5- and 15-cm tube depth. The tubes were placed on October 10th 2014 and collected after 8 months of exposure on the 6th of June 2015 together with a spot oxygen measurement.

Step 2: main experiment

320 juveniles were positioned in 20 tubes (16 specimens each). After the introductory experiment experience, tubes 12 cm high were applied with pairs of continual thermometers and sucking hoses at 3 and 10 cm depth. The tubes were placed at four sites (A, B, C, D) at 15–40 cm water level depth. Each site had one basket with five tubes on the 30th of July 2015. We anticipated similar conditions for the tubes in one basket; nevertheless differences, i.e. diversification between the sites, were supposed. After the first 3 days, the baskets were inspected, their position was adjusted if necessary for stability, and the first oxygen control measurements were taken. Six measurements took place during 55 days of tube exposure, with a range of 6–17 days. The first 8 tubes (2 from each site) were collected after a month (26 days) exposure on August 25; the resulting gaps were newly filled with river bed substrate. Except for one tube from site A and one from site C, the remaining tubes (10) were collected after about 2 months (55 days) of exposure on September 23.

Step 3: supplementary wintering experiment

Because site A had good oxygenation and stable conditions for 2 months as well as zero mortality of juveniles after 1 month, this site was selected for the supplementary wintering experiment. On the 23rd of September 2015, the last remaining tube from site C was relocated into a basket at site A near the local remaining tube, using a gap from one of the collected tubes; the remaining gap in this basket was filled with river bed substrate. The basket was newly aligned with

the surrounding river bed surface. The new pair of wintering tubes was left at site A all winter and collected on the 4th of April 2016.

Results

Introductory experiment (first wintering)

After the 8 months of exposure (October 2014–June 2015), 51% findability (mean) was recorded from the five standardly constructed (= down and laterally impassable) tubes (Table 1). Nevertheless, only 4 specimens (in 2 tubes representing 2 baskets) were found alive (Fig. 2a), while the other 61 specimens were identified as empty shells. Mostly the shells were in very good condition (Fig. 2b), rarely only a thin transparent periostracum was retained (Fig. 2c). All the findings ranged from about 1 to 8 cm of substrate depth, with the maximum concentration between 3 and 4.5 cm (Fig. 3a), remarkable especially in 2 tubes with findability over 60% (Online Resource 2). Only the completely passable tube was found to be juvenile-free. Oxygen values on the day of tube sampling (June the 6th) were favourable at 5 cm substrate depth (8.3 and 9.9 mg l^{-1}, which corresponded to 87 and nearly

100%) and adequate in 15 cm (5 and 7.8 mg l^{-1}, representing 52 and 82%). Nevertheless, a decrease is assumed during the exposure. Temperature oscillated strongly, especially in spring, and a depth gradient between the shallower and deeper hyporheic layer is visible, especially during the coldest periods in December/January and February (Online Resource 3).

Main in situ experiment

Oxygen conditions differed between sites A, B, C and D over time during the 2 months of exposure, probably relating to free-flowing water level oscillation. Although the first water level decrease only represented about 2 cm in the studied sites (Fig. 4b), it caused the emergence of several m^2 of river bed just upstream from site B and a thus a change from running surface water to standing at this site. Connected with that, hyporheic oxygen concentration decreased strongly at site B (ca 4–5 mg l^{-1} at 3 cm and even ca 2 mg l^{-1} at 10 cm depth of hyporheal) (Fig. 4a). All the other sites showed favourable concentrations, mostly not falling under 8 mg l^{-1} at 3 cm depth, except a short decrease at site C when the horizon of 3 cm became oxygen poorer than the horizon of 10 cm (7 vs 10.5 mg l^{-1}, Fig. 4a). Conductivity (Online

Table 1 Overview of juvenile numbers in evaluated tubes

Experiment step	Number of evaluated tubes	Input N	Found			Alive			Mortality of found individuals %
			N	Mean%	SD %	N	Mean%	SD %	
Introductory experiment (first wintering)	**5**	**128**	**65**	*51*	*18*	**4**	*3*	*4*	*94*
Main experiment									
Site A	4	64	58	*91*	*11*	58	*91*	*11*	*0*
Site C	4	64	49	*77*	*9*	48	*75*	*7*	*2*
Site D	5	80	37	*46*	*28*	34	*43*	*26*	*8*
SUM (A, C, D)	**13**	**208**	**144**	*69*	*27*	**140**	*67*	*27*	*3*
Site B	2	32	17	*53*	*	0	*0*	*	*100*
SUM (A, B, C, D)	**15**	**240**	**161**	**	**	**140**	**	**	**
Supplementary wintering experiment	**2**	**32**	**18**	*56*	*	**17**	*53*	*	*6*

Total numbers (N), mean percentual values (mean %) with standard deviations (SD %) for a tube, and mortality of found individuals (%) are given for individual tube sets. Rows marked SUM describe summary results for individual tube sets within the main experiment

Bold indicate summary numbers

Italic indicate statistical values

*SD was counted only if number of evaluated tubes > 3

**These evaluations were not carried out due a big difference at site B
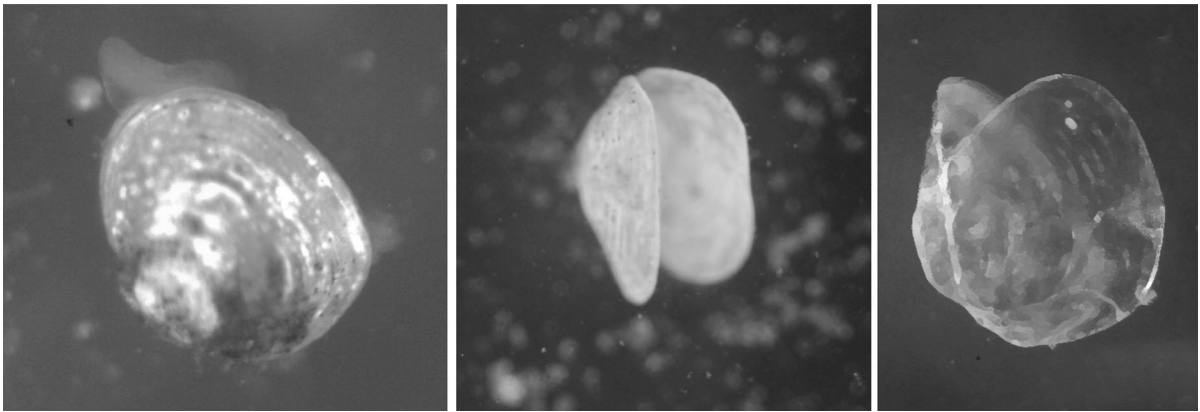
Fig. 2 Juvenile findings from the introductory experiment (first wintering). A live specimen (**a**), a well-preserved empty shell (**b**) and a mostly decomposed empty shell (**c**)
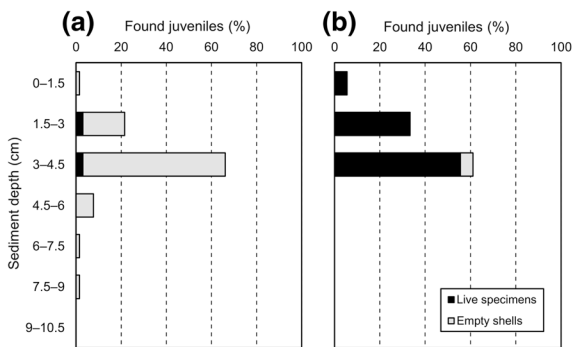

Fig. 3 Depth distribution (%) of juveniles found within the introductory experiment (first wintering, Oct 2014–Jun 2015, 5 tubes) (**a**), and the supplementary wintering experiment (Sep 2015–Apr 2016, 2 tubes) (**b**). Live specimens: black bars, empty shells: grey bars

Resource 4) and temperature measurements (Fig. 5) also illustrate differences between the sites. Site B showed the highest temperature difference between surface and hyporheic horizons, smaller differences occurred at sites A and C (with different courses), while temperature was practically without depth differences at site D, signalling a permanent exchange of surface and hyporheic water. This corresponds with flow velocity differences at the sites (Fig. 4c) and also with oxygenation changes, when the episodical oxygen decrease C (Fig. 4a) signalled a break in hyporheic and surface water exchange during the second half of the exposure (Fig. 5b).

In 15 evaluated tubes, 161 juveniles including 140 survivals were found from 240 input animals (Table 1). Findability and survival were very different between sites. Site A showed over 90% findability,

even reaching 100% in one tube (Online Resource 2) and complete survival of founding specimens in all tubes. 77% findability with minimal mortality (1 dead specimen) was recorded at site C and only 46% findability with 8% mortality from site D, where the findability variation between tubes was the highest (Online Resource 2). In contrast, site B showed total mortality. Findability of the dead specimens was 53% after the first month but very low (1 specimen in 1 tube) after the next month of exposure. Therefore, only the one-month exposed tubes were evaluated for juvenile depth distribution from site B and the site was excluded from evaluation of live specimen findability, which represented 67% for sites A, C, and D in both evaluating periods (AUG, SEP) together. Almost the same live specimen findability (68%, 67%) in the two periods (Online Resource 2) represents a very important result.

The recorded depth distribution of live juveniles showed a very remarkable accumulation between 2 and 3 cm of sediment depth, where around half of found specimens were situated (Fig. 6). Their numbers dropped strongly in both up and down directions. The findings below 6 cm were unique and the deepest specimen found was recorded at 8 cm depth. Evaluated on a 1.5 cm scale beginning at the substrate surface, 57% of juveniles were found in a zone of 1.5–3 cm.

Like findability, depth distribution also did not show any significant differences between the two months (Fig. 7), so the difference between the sites could be evaluated independently of the exposure time. Sites A, C and D showed a similar distribution
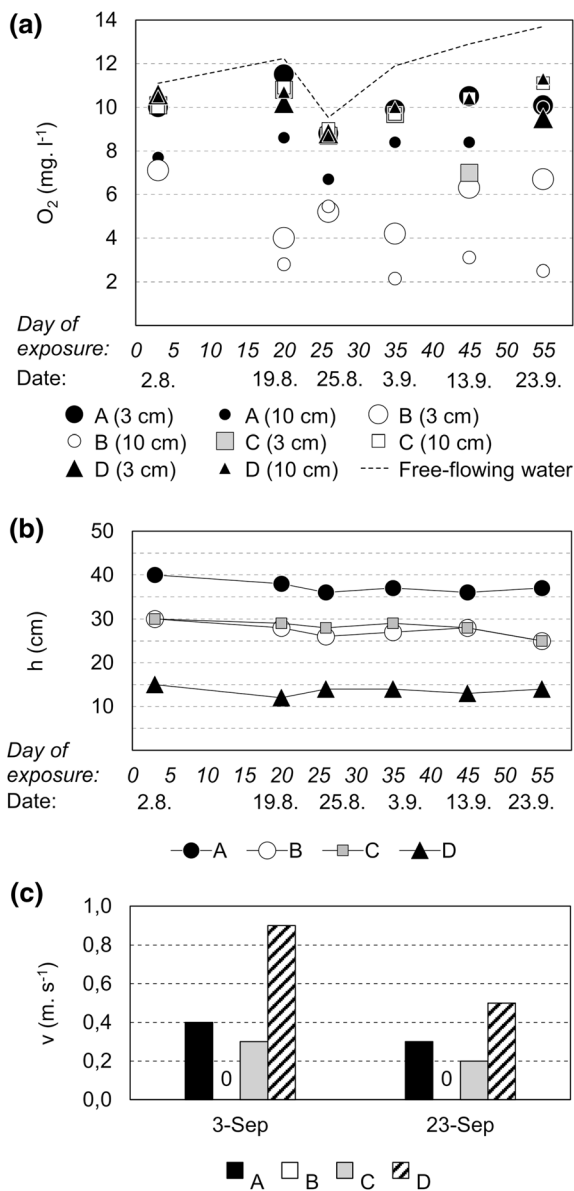
Fig. 4 Oxygen concentration changes (mg l^{-1}) in free-flowing water and sediment depths 3/10 cm (**a**), free-flowing water level depth changes (cm) (**b**) and flow velocity (m s^{-1}) 5 cm above the riverbed (**c**) at four sites A–D during the main experiment (Aug–Sep 2015). **a** Free-flowing water: dashed black line; site A (3/10 cm): large/small black circle, site B (3/10 cm): large/small white circle, site C (3/10 cm): grey/white square, site D (3/10 cm): large/small black triangle. **b** Site A/B/C/D: black circle/white circle/grey square/black triangle. **c** Site A/B/C/D: black/white/grey/black and white bar

(Fig. 8), which is not very different from the mean (Fig. 7), with the maximum between 1.5 and 3 cm. Nevertheless, an increased occurrence of juveniles in

the subsurface layer (to a depth of 1.5 cm) is noticeable at site D. In contrast, site B showed a clear maximum of dead specimens (nearly 80%) in the subsurface layer.

Supplementary wintering experiment

The hyporheic water temperature relationships were similar to the first wintering experiment. The 10-cm-deep hyporheal showed smaller oscillations comparing to the 3 cm one, especially in early spring when temperature amplitudes increased in free-flowing water and the difference between 3 and 10 cm hyporheal can represent a whole 1°C (Online Resource 3). The oxygen concentration was favourable in both depth horizons on the day of sampling (4th of April 2016). Juvenile findability was 56% with the comparable specimen numbers (8, 10) in both the tubes and with only one dead specimen found. Juvenile depth distribution showed a maximum between 3 and 4.5 cm depth (Fig. 6) and was very similar to the first wintering experiment (Fig. 3).

Discussion

Basic findings

The juveniles were never distributed randomly and every time they inclined to an accumulating occurrence. The occurrence maxima clearly point to a preference for a certain sediment depth, namely 2–3 cm within the main (summer) experiment. This depth is not easy to define precisely because the river bed surface is uneven and the actual depth of the shallow hyporheic zone is disputable (Hauer & Lamberti, 2007). Nevertheless, live juveniles only rarely occurred in the subsurface layer up to 1.5 cm depth, so it seems evident that they regularly penetrated deeper after their installation into 1 cm. On the other hand, their numbers decreased significantly below 3 cm depth. A similar type of unionoid burrowing behaviour with a shallow sediment horizon preference was described by Yeager et al. (1994), when newly metamorphosed juveniles (180–240 μm) of *Villosa iris* (Lea, 1829) burrow after 20 min, but not deeper than 1 cm in sandy sediment in a laboratory test. Upper interstitial as a suitable microhabitat for young mussels (especially FPM) was already expected
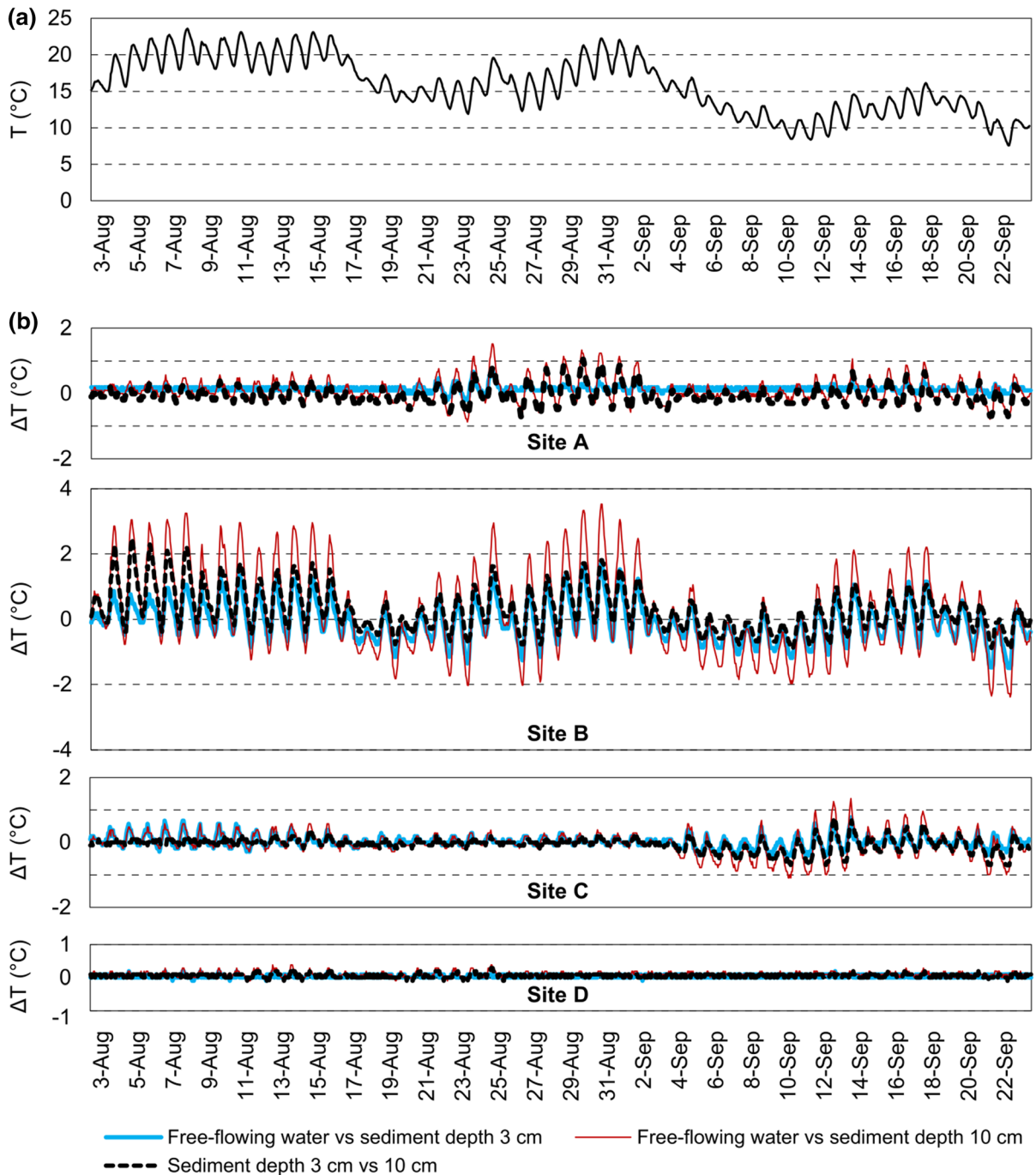
Fig. 5 Temperature changes (°C) in free-flowing water (**a**), and temperature differences between free-flowing water and sediment depths 3/10 cm at four sites A–D (**b**) during the main experiment (AUG–SEP 2015). **b** Free-flowing water vs sediment depth 3 cm: solid light blue line; free-flowing water vs sediment depth 10 cm: solid dark red line; sediment depth 3 cm vs 10 cm: dotted black line. Data-loggers: HOBO Onset (temperature records every 1 h)

by Buddensiek et al., (1993), especially due to a high rate of exchange between the free water body and the interstitial water and due to higher oxygen

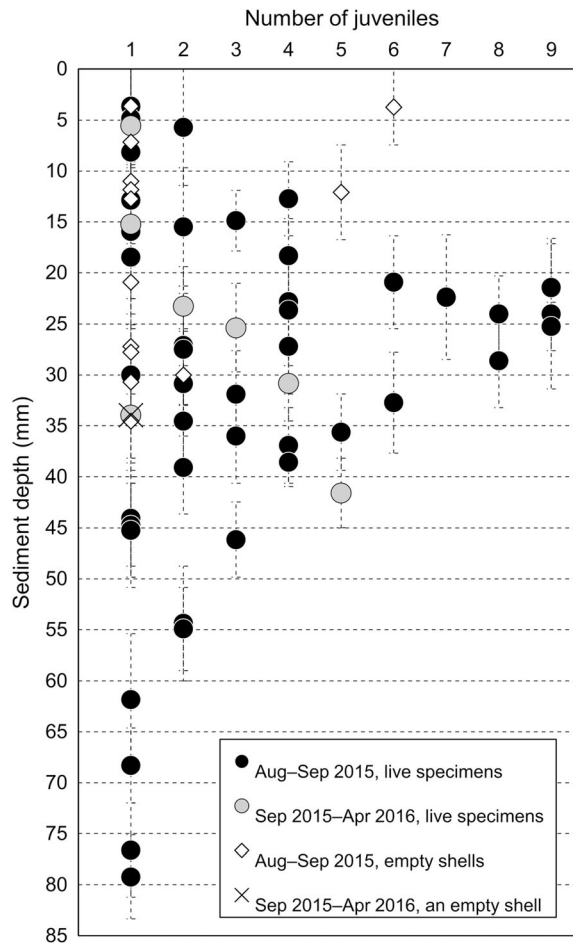concentration in the first 3 cm depth in comparison with deeper horizons.

Fig. 6 Depth distribution of ALL juveniles found in the individual sediment layers within the main experiment (Aug–Sep 2015, 16 tubes) and the supplementary wintering experiment (Sep 2015–Apr 2016, 2 tubes). Data from layers with at least one found juvenile are described. Vertically: the marks indicate the medium depth of the sediment layer, i.e. the most probable depth of juvenile(s) occurrence. The line segments indicate the thickness of the layer, i.e. a maximum theoretical range of juvenile(s) occurrence. Summer: live specimens: black circle, empty shells: white rhomb. Winter: live specimens: grey circle, empty shells: black cross

Fig. 7 Depth distribution (%) of LIVE juveniles found after 1 and 2 months (grey and black bars) within the main experiment (Aug–Sep 2015). Number of evaluated tubes: 6 = (2× A, 2× C, 2× D) after 1 month, 7 = (2× A, 2× C, 3× D) after 2 months. No live specimens were found at site B

The identical design of live specimen depth distribution after 1 and 2 months (Fig. 7) suggests that, after taking a suitable depth position, juveniles stay there as long as conditions permit. No doubt they are not trying to go deeper. Although only some parts of the year were captured by the experiments, and although the experimental unit (tube) number was low, it seems that FPM juveniles commonly do not submerge deeper than 8 cm substrate in the conditions at our experimental sites. As the Teplá Vltava River
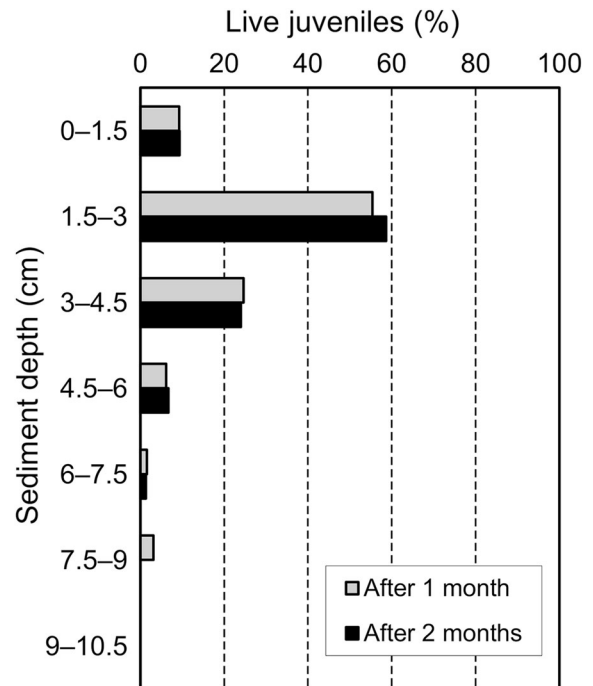
(especially the experimental meander) has favourable FPM localities (Černá et al., 2018), where a well-oxygenated hyporheal is present, we can assume that this occurrence depth limit would not be significantly deeper elsewhere, especially in rivers with steeply decreasing hyporheic oxygen. On the other hand, the preferred sediment depth of 2–3 cm could vary between rivers and also between localities within a river, depending on local substrate composition.

Permanent juvenile occurrence just on a sediment surface is not supposed. Wash away and bed shear stress (Ackerman & Hoover, 2001) are probably the main factors affecting non-burrowing juvenile mussels. On the other hand, it could also be a positive factor enabling juvenile mussel escape from locally bad conditions after active digging out. It has been shown that drift intensity can be influenced by juveniles themselves thanks to a wide range of foot and valve movements changing the probability of sedimentation (Schwalb & Ackerman, 2011; French & Ackerman, 2014).
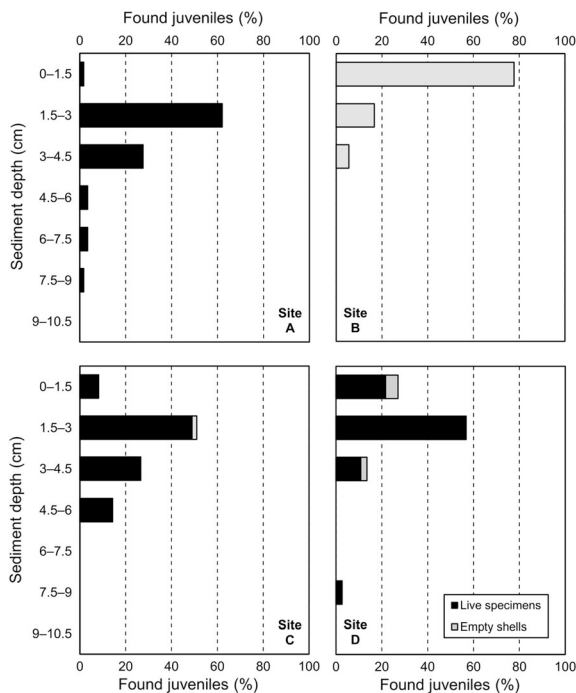
Fig. 8 Depth distribution (%) of ALL juveniles found at four sites (A–D) within the main experiment (Aug–Sep 2015). Number of evaluated tubes: 15 = (4× A, 2× B, 4× C, 5× D). Live specimens: black bars, empty shells: grey bars

Effects of microhabitat variability

Even though it was not the primary experimental intention, the four sites of the main in situ experiment represented very different hyporheic conditions within one short river stretch, with different flow velocity and hydrochemical behaviour. This variability was reflected in juvenile depth position, survival and tendency to stay on site. Although the last two variables are not always distinguishable, several patterns are visible even in our small dataset. Site A seems to be an example of an optimal and stable environment, with a high ratio of survival where the juveniles have a tendency to persist and are able to winter. Site C was similar, but with different temperature and oxygen fluctuation regimes. Site D can be interpreted as an oxygen-stable but mechanically-unstable environment; variation between tubes was highest, as well as juvenile losses—probably caused by escaping. Although the temperature conditions (Fig. 5b) are typical for downwelling at this site (Hauer & Lamberti, 2007), the live juveniles showed a tendency to accumulate in the subsurface layer here

more than at other sites. As a river bed depth of 30–40 cm from the water level is reported as optimal for young mussels (Moorkens & Killeen, 2014), it may be that site D (maximum 15 cm deep) is too shallow for juveniles and they have a tendency to leave it.

Site B was an example of an environment where oxygenation decreased unpredictably, probably very quickly somewhere between the first and second check, caused by water level decreasing under a depth critical for this site. Moving up and escaping from the tubes was probably the juveniles' reaction to this change. If such a sudden oxygen decrease affected free-living juveniles, a quick rise to the river bed level would be the only way to survive. No horizontal movement could help juveniles to find better conditions if water stagnation affects an area of several square metres. The effort to escape seems to be the reason for dead specimen accumulation in the subsurface substrate layer. They represent juveniles which were not on the surface at the time, while the more successful ones could leave the tube space. By the time of the second tube set collection from this site (about 6 weeks after the assumed oxygen depression), all the remaining empty shells would probably have dissolved.

Wintering

After the first 8 months of exposure, the majority of juveniles were found dead. Nevertheless, the shells were mostly well preserved, so the time of death must have been close to tube collection. There is a lack of published results about 0.5–2 mm juvenile FPM shell decomposition rates, but unpublished data (Rambouskóvá, 2015) suggest complete shell degradation after 8 weeks, depending on substrate quality and sediment saturation of calcium. In addition, an experiment with juvenile newly excysted mussels (Lavictoire, 2016) proved that it can take less than 2 months for a shell to lose the calcium layer completely. A shell in good conditions, indicated as degradation class 1, represents a recently dead juvenile (Lavictoire, 2016).

Therefore, juvenile survival during the majority of the exposure time and death shortly before the end of the experiment due to sudden oxygen decrease seems to be the most probable interpretation of our finding. Nevertheless, other causes are not excluded (predation, parasites, or other health problems). In contrast, a very high survival ratio (only one dead specimen from

18 found) was recorded from the second 8 months of exposure. This reflects generally stable and suitable hyporheic conditions at site A throughout the experiment.

Slightly deeper sediment (3–4.5 cm) where most wintering specimens were concentrated compared to summer exposure is not very conclusive due a small n. Nevertheless, it could be interpreted as descending a little deeper, where the temperature is more stable and farther from zero.

The 50% animal loses both in the first and in the second wintering period could be caused by natural juvenile mortality in the hyporheal, which can reach tens of per cent annually in the Vltava catchment semi-natural breeding programme (Švanyga et al., 2013). In similar experiments with *Lampsilis cardium* (Rafinesque, 1820) using caged juveniles with a vertically change position possibility, the mean survival was 47% in 3 cm hyporheal, while 86% in free water exposures (Bartsch et al., 2003). On the other hand, a number of escapers through the tube surface layer is also a probable cause of loses because the tube conditions are semi-natural and juveniles may look for a more optimal environment during or after wintering. Zero juveniles in the completely passable tube allowing horizontal movement of juveniles (during the first wintering) reflects this possibility.

Key outcomes

The experiments showed a tendency for juveniles to penetrate into some sediment depth. The depth preference of 2–3 cm is apparently not random and could be a result of two contrast pressures. A shallow position is safer for suitable oxygen concentration; apart from that it allows juveniles to escape from sediment and drift to another place if oxygen conditions are getting worse. In contrast, a deeper position represents better safety for rinsing from the substrate and consequent passive drifting. It seems that juveniles prefer a substrate layer as shallow as possible, which still deep enough to not threaten their position stability. By what senses the juveniles know their current depth is a question. In every case, a very shallow hyporheal proves to be a favourable environment for FPM juveniles. This fact should be considered within species reintroduction programmes.

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ČLÁNEK 2: Provádění bioindikačních in situ experimentů s juvenilními jedinci perlorodky říční (Bílý et al., 2018) – „uveřejněný" (J_{imp})

Bioindication testing of stream environment suitability for young freshwater pearl mussels using in situ exposure methods

Michal Bílý, Simona Němčíková, Ondřej Simon, Karel Douda, **Vojtěch Barák**, Bohumil Dort

Podíl autora: Významně jsem se podílel na zpracování protokolu jako hlavní části metodického článku a na natáčení doprovodného video materiálu. Revize rukopisu proběhly ve spolupráci s Michalem Bílým.

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Video Article

Bioindication Testing of Stream Environment Suitability for Young Freshwater Pearl Mussels Using *In Situ* Exposure Methods

Michal Bílý[1], Simona Němčíková[1], Ondřej P. Simon[1,2], Karel Douda[3], Vojtěch Barák[1], Bohumil Dort[1]

[1]Faculty of Environmental Sciences, Czech University of Life Sciences Prague

[2]T. G. Masaryk Water Research Institute

[3]Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague

Correspondence to: Michal Bílý at bilym@fzp.czu.cz

Abstract

Knowledge of habitat suitability for freshwater mussels is an important step in the conservation of this endangered species group. We describe a protocol for performing *in situ* juvenile exposure tests within oligotrophic river catchments over one-month and three-month periods. Two methods (in both modifications) are presented to evaluate the juvenile growth and survival rate. The methods and modifications differ in value for the locality bioindication and each has its benefits as well as limitations. The sandy cage method works with a large set of individuals, but only some of the individuals are measured and the results are evaluated in bulk. In the mesh cage method, the individuals are kept and measured separately, but a low individual number is evaluated. The open water exposure modification is relatively easy to apply; it shows the juvenile growth potential of sites and can also be effective for water toxicity testing. The within-bed exposure modification needs a high workload but is closer to the conditions of a natural juvenile environment and it is better for reporting the real suitability of localities. On the other hand, more replications are needed in this modification due to its high-hyporheic environment variability.

Video Link

The video component of this article can be found at https://www.jove.com/video/57446/

Introduction

The exposure of experimental organisms *in situ* with the subsequent evaluation of their condition is one possible way to get information about the environmental quality and (especially) the site suitability for a species. Within animals, such a bioindication is applicable primarily for small invertebrates which are able to live in a limited bounded space. Young stages of bivalves (Bivalvia) are one such suitable organism group[1].

Bivalves of the family Unionidae are a very important component of aquatic ecosystems[2]. However, these species are often critically endangered, especially in streams and rivers. Some of them are characterized as 'umbrella species' whose conservation is closely related to the conservation of the whole stream biotope and which require a comprehensive approach[3]. These animals have a life cycle associated with many environment components, from water chemistry[4,5] to changes in the populations of fish which serve as mussel larvae hosts[6]. Because mussel juveniles often represent a critical phase of the mussel life cycle, the site suitability for their development at this stage is crucial for a successful species population development in a locality.

The freshwater pearl mussel (FWPM, *Margaritifera margaritifera;* Unionida, Bivalvia) is a critically endangered bivalve occurring in oligotrophic European streams. Their numbers have fallen drastically during the 20th century across the occurrence area. It seems that the current decline in species reproduction in the majority of the central European populations is primarily caused by very low to zero survival of juveniles during the first few years of their life. It is assumed that juvenile FWPMs live for many years in the shallow hyporheic zone[7], of which the conditions and their variability are still not well described. Moreover, until their second year of life, the juveniles only have a dimension of up to about 1 mm, so they are very difficult to find in large volumes of sediment under natural conditions[8]. Therefore, experiments with captive juveniles are necessary for the study of their ecology.

Within the Czech Action Plan for Freshwater Pearl Mussel[9], there are thousands of juveniles rising every year from a semi-natural breeding program. Nevertheless, there is a question of which localities and habitats are suitable for successful population support by these juveniles or for eventual species reintroduction. *In situ* bioindications present a way of finding the answer.

Despite the fact that inconsistent survival rates of juvenile mussels in exposure cages were observed in some earlier works that questioned the suitability of juvenile mussels as bioindicators[10], several recent studies have confirmed the applicability of juvenile exposure methods for water

quality testing[11,12,13]. Additionally, it has been demonstrated that several factors need to be considered when interpreting the results of these particular studies, such as the stock origin[14] and the persisting effects of larval conditions[15].

The question arises of how to install experimental juveniles in tested localities and how to most effectively evaluate their condition. The first rigorous application of *in situ* exposure methods with juvenile FWPMs was published by Buddensiek[16]. Juvenile FWPM individuals were kept in sheet cages, exposed in the free-flowing water of streams, and their survival and growth were quantified after several weeks of exposure. The approach was originally developed as a semi-artificial breeding method, but the author also highlighted its applicability for the assessment of habitat requirements and water quality. Although the FWPM juvenile survival is naturally very low on a scale of months/years and only a very small number of animals will survive, the survival rate can be a good marker of the environmental effect on a scale of several weeks[16]. Over years of research, exposure methods were developed further to hold experimental juvenile mussel in-stream habitats and to evaluate their growth and survival rates; these include sandy boxes[17], mussel silos based on an upwelling principle[18], and various other exposure cages (summarized by Gum and colleagues)[11]. Because juveniles occur naturally in shallow hyporheic zone[7], the application of experimental devices within the stream bottom is very desirable.

In our article, we describe the use of two exposure devices for FWPMs: i) modified Buddensiek sheet cages ("mesh cages") also enabling bioindication testing in hyporheal conditions; and ii) Hruška sandy boxes ("sandy cages"). The protocol describes the application of both methods in open water and hyporheic conditions (*i.e.*, four variants of exposure are described). The methods were gradually modified and expanded over more than 15 years of application within the Czech Action Plan for Freshwater Pearl Mussel[9] and verified by a set of experiments.

Protocol

1. Mesh Cage

Note: See **Figure 1**.

1. **Prepare material**
   1. Prepare the material for the in-laboratory part of the experiment: ~1 - 2 L of river water per mesh cage, mesh cages (1 main plastic body, 2 plastic covers, 2 sheets of special technical sieves with 340 µm pores, 4 bolts and 4 nuts per cage), pliers, a spanner, Pasteur pipettes, a strainer, a digital camera, a trinocular dissecting zoom stereo microscope, a calibration grid (microscope equipment), 5 Petri dishes of 50 mm diameter, beakers, 2 plastic dishes (~25 cm x 15 cm x 3 - 5 cm), and a plastic box.
   2. To perform the hyporheal installation, prepare a rubber hose and a 100-µm-pore mesh, and a squirt bottle. For the construction of the device, see **Supplementary File 1: S.1. Mesh cages construction**.

2. Assemble the bottom and central part of the mesh cages. Assemble the part of the cage that holds the individuals. Insert one plastic cover first, then one sheet of the plastic sieve, and finally the main body on top. Use four bolts to secure it.
3. **Prepare biological material**
   1. Put the mesh cage into the plastic dish containing river water. Ensure that the chambers are half full. Take the FWPM juveniles (see **Supplementary File 1: S.6. Biological material**) out of the thermally-insulated box and put them in the Petri dish.
      Note: Ensure that sudden temperature changes do not exceed ~2 °C.
   2. Using a squirt bottle and strainer, sift through the juveniles to clear the detritus.

4. Set up the microscope and camera. Perform a calibration of the instruments (see **Supplementary File1: S. 5. Microscope and phototechnics**). Place a Petri dish containing a little water under the microscope.
5. **Put the juveniles into cages (experimental laboratory work)**
   1. Use a Pasteur pipette to remove one individual from a Petri dish and carefully place it in the Petri dish under the microscope.
   2. Check the individual's fitness by looking into the eyepiece (~40X magnification).
      Note: "Good" fitness signifies that the individual moves, rotates from side to side, pushes the foot out of the shell, *etc*. Remove dead or low fitness individuals with a Pasteur pipette and place them in a separate Petri dish (FWPM juveniles with an opened shell, no movement, the foot is not pulled out, a fragmented shell, juveniles who float uncontrollably in the water, a visible decomposition of the shell, partial decalcification).
   3. Take two photographs of an FWPM individual showing good fitness using a constant magnification of ~80X. See **Supplementary File 1: S.5. Microscope and phototechnics**. Save the photos.
      Note: For a good measurement of its length, the juvenile must be laid lengthwise (lateral view). The main goal is to take a high-quality picture of the maximum shell length good enough to enable a picture analysis afterward.
   4. Insert the juvenile into the appropriate chamber in the cage as soon as the pictures are taken. Record the numbers of the pictures and the chamber.
   5. Repeat this step with each individual for all the used chambers in the mesh cage.
      Note: see **Supplementary File 1: S.1. Mesh cages construction**.
   6. Once all the used chambers have pearl mussels, put the plastic sieve on the cage, then gently put the plastic cover on and secure all parts together with the nuts.
   7. In the case of an installation into a hyporheic zone, pass one of the hose ends through one of the chambers and fix it in this position, then take the anti-clogging mesh and bind it on the bottom end (see **Supplementary File 1: S.1. Mesh cages construction**).

6. **Store juveniles**
   1. Put the cage into the plastic box with the river water, so that the juveniles are fully immersed, and keep it in the thermobox. Before the installation, let the juveniles adapt to the *in situ* river water temperature at the place of installation (gradual cooling, max. 5 °C in 24 h).

7. **Install mesh cages**

1. Prepare the field material including the mesh cages with the juveniles, steel spikes, bolts and metal nuts, a spanner, field temperature dataloggers (see **Table of Materials** and **Supplementary File 1: S.4.2. Water measurement**), a string, a camera, the field protocol, a hammer, and a spade.
2. Transport the FWPM juveniles to the site in a field thermobox (insulated box), keeping a stable water temperature with variations < ~2 °C. Put the thermobox with the mesh cages into the river on the site to let the juveniles adapt to the local environmental conditions (pH, conductivity, *etc.*).
3. Install the mesh cage.
    1. Remove the mesh cage from the field thermobox. Provide it with two steel spikes and fasten the field datalogger. Anchor the cage into a habitat with conditions typical for FWPMs in the study area (*e.g.*, at the edge of the main stream flow, not in direct water flow, not in standing water, not in direct sunlight).
        1. For open water, using a pair of the steel spikes, fix the cage to the river bottom; lay it on its side and level with the river bottom, downstream at an angle of 45° to the river flow, towards the center of the river. The lower horizontal edge should be about 10 - 15 cm above the river bottom surface. Maintain a minimum distance of 2 m between each cage at one locality (see **Supplementary File 1: S.4. Cages maintenance**).
        2. For the hyporheic zone, dig the cages into the river bottom in a perpendicular landscape position, perpendicular to the stream of water, so that the upper horizontal edge of the cage is parallel to the river bottom surface and the chambers are located at the hyporheic depth which should be tested. Take out the upper end of the rubber hose above the bottom surface for the possibility of water sampling during the experiment (see **Supplementary File 1: S.4.2. Water measurement**).
        Note: It is recommended to perform regular checks and maintenance on the cages (see **Supplementary File 1: S. 4. Cages maintenance**).

8. Uninstall the cages and transport the juveniles after the exposure. For this, pull the cages out of the water, clear them of fine sediment as well as of drifted material and put them into the field thermobox filled with river water. Transport the cages immediately to the laboratory and start the mortality and growth rate evaluation.
   Note: See **Supplementary File 1: S.3. Exposure duration**. In the case of a temperature difference of more than 5 °C between the cages and the laboratory environment, it is first necessary to let the temperature equalize.
9. Evaluate the experiment by checking the life/fitness of each juvenile (see steps 1.5.2 and 1.5.3) and take 2 images of each live juvenile in a Petri dish using a constant magnification of ~80X. Record the fitness and the numbers of the pictures and chambers.
10. **Complete the experiment (common to all methods)**
    1. Perform the measurements in image analysis software. Use image analysis software for the body size determination of every evaluated juvenile on both the input images (step 1.5.3) and on the output images (step 1.9). Use the maximum total shell length recorded in both photographs as body size values in both input and output.
    2. Insert the measured values into the table processor and calculate the growth increment (%) for each surviving juvenile.
    3. Estimate the survival rate (%) per mesh cage using the ratio of the number of surviving individuals to all experimental individuals in the mesh cage.
    Note: After the experiment, return the survivors to the breeding program
    (see **Supplementary File 1: S.6. Biological material**).

2. Sandy Cage

Note: See **Figure 2**.

1. **Prepare material**
    1. Prepare the material for the in-laboratory part of the experiment: 2 Petri dishes (diameter ~8.5 cm), Pasteur pipettes, a strainer, 25 L of river water, a plastic box, sieves (mesh size 1 and 2 mm), a big plastic box (25 L), a sandy cage (see **Supplementary File 1: S.2. Sandy cages construction**), a digital camera, a trinocular dissecting zoom stereo microscope, a calibration grid (microscope equipment), sorted river sand from the study area (see step 2.1.3), and the protocol. See **Table of Materials** and **Supplementary File 1: S. 2. Sandy cages construction**.
    2. Prepare the material for the isolation process: round containers (1 for each cage plus 1 extra), 2 Petri dishes (diameter ~14 cm), a Pasteur pipette, magnifying glasses, and 1 L of river water.
    3. Sift the river sand through a 2-mm sieve and then through a 1-mm sieve to get a grain size of 1 - 2 mm. Dry the sand and save it in a dry form until required.

2. Take the juveniles (see **Supplementary File 1: S.6. Biological material**) out of the thermobox and put them in the Petri dish. Using a squirt bottle and strainer, sift through the juveniles to clear the detritus.
3. Set up the microscope and camera (see **Supplementary File 1: S.5. Microscope and phototechnics**).
4. **Put juveniles into cages (experimental laboratory work)**
    1. Place the sandy cage in the plastic box. Scatter the sorted sand (see step 2.1.3) up to one-third of the height of the sandy cage. Pour water into the box. Ensure that the sand surface is about 10 mm below the water level. Insert the sandy cage into the 25 L box of river water and expose it to the same temperature as the juvenile FWPMs (see **Supplementary File 1: S.6.2. Storage of the biological material**) for 12 h. Avoid any exposure of the sand to sunlight.
    2. Take the Petri dish with the prepared FWPM juveniles.
    3. Check the individuals' fitness by looking into the eyepiece (see step 1.5.2).
    4. Perform the photographic documentation as follows. Take one picture of all individuals discovered (see step 1.5.3) and choose 10 of the largest individuals. Alternatively, take pictures of all juveniles together with low magnification (~40X) for a bulk evaluation and choose the 10 largest individuals. Save all the photos and record their numbers.

      5. Using a squirt bottle, move the FWPM juveniles into the prepared sandy cage.

5. **Store juveniles**
   1. Put the cage into the big plastic box with river water so that the cage is fully immersed and keep it in the thermobox. Let the juveniles adapt to the *in situ* river water temperature (gradual cooling, max. 5 °C for 24 h) before the installation.

6. **Install sandy cages**
   1. Prepare the material for the field installation: sandy cages, a ~25-L field thermobox, a flat stone (minimal weight 1 kg), a net (mesh size 10 x 10 mm), a squirt bottle, field temperature dataloggers (see **Table of Materials** and **Supplementary File 1: S.4.2. Water measurement**), a spade, and the field protocol.
   2. Transport the cages with the juveniles to the site in the field thermobox, keeping a stable water temperature (~2 °C change). Put the field thermobox with the sandy cages into the river at the field site to let the FWPM juveniles adapt to the local environmental conditions (pH, conductivity, *etc.*).
   3. Install the sandy cages into habitats with conditions typical for FWPMs (*e.g.*, at the edge of the main stream flow in a meander, not in direct water flow, not in standing water, not in direct sunlight).
      1. For open water, fasten the sandy cages to a flat stone using a net and place it on the river bottom. Ensure that the larger side of the cage forms an angle of 45° with the flow.
      2. For Hyporheal, dig the cages into the river bottom perpendicular to the flow of water so that the cage lid is level with the river bottom surface.
      Note: It is recommended to perform regular checks and maintenance on the cages (see **Supplementary File 1: S. 4. 1. Site checks**).

7. **Uninstall cages and transport juveniles after exposure**
   Note: see **Supplementary File 1: S.3. Exposure duration**.
   1. Pull the cages out of the water, clear them of drifted material and put them into the field thermobox filled with river water.
   2. Transport the cages to the laboratory and start the mortality and growth rate evaluation.
      Note: In the case of a temperature difference of more than 5 °C between the cages and the laboratory environment, it is necessary to let the temperatures equalize.

8. **Separate FWPM juveniles from sand**
   1. Prepare a round container with a water depth of 50 mm (for each cage separately) and one extra round container. Transfer the sand from the cage into the round container. Use a swirling motion to wash out the lighter particles into an extra container.
   2. Sample the content from this container gradually and search for juveniles step-by-step using a Pasteur pipette and a magnifying glass. Put the juveniles in the Petri dish using the Pasteur pipette. Repeat this step until the last juvenile has been found and then another 10x after the first negative finding. After each wash step, add clean river water to the original container with sand.
      Note: Especially after the first washing out, properly examine the content and clean it of ballast such as fine sediment and other alluvia.

9. **Evaluate the experiment**
   1. Check the fitness of each juvenile (see steps 2.4.3 and 1.5.2) and count the number of survivors.
   2. Take a picture (see step 2.4.4.) of each individual separately, although this means there is no clear identity of each individual. Alternatively, take bulk photos and choose a subset of the 10 best-grown individuals from the final results.
      Note: Both possibilities have a similar reporting value. Possibility 1 has a limitation of a higher workload but also the benefit of the highest photo magnification and thus also greater accuracy.

10. **Complete the experiment**
    1. Perform measurements in image analysis software. Complete the experiment as done in the mesh cages (see step 1.10) with the following exception: do not evaluate the growth rate (%) of each juvenile but evaluate the group as a whole in the sandy cage experiment.
       Note: After the experiment, the survivors should be returned to the breeding program
       (see **Supplementary File S.6.1. Selection of a biological material**).

Representative Results

The four bioindication methods (open water sandy cages, within-bed sandy cages, open water mesh cages, and within-bed mesh cages) were applied to investigate the environment condition suitability for FWPMs in the upper Vltava River Basin (Bohemian Forest, Czech Republic). This river represents one FWPM residual locality within central Europe[19]. Here, we present a specially selected set of results illustrating the most important aspects of the four methods. Further details are described in a comprehensive study by *Černá et al.*[13].

The river environment was studied at two levels:

(I) A longitudinal river profile was represented by main stream localities (sites A - E) and tributaries of different pollution stages (sites R and V). The localities were tested both by sandy cages and by mesh cages installed in free-flowing water. In addition, a gravel hyporheic zone was tested by within-bed sandy cages in localities B, C, and D.

(II) A hyporheic environment was tested in the selected locality C. The suitability of different substrates (sand, gravel, stones) was tested by within-bed mesh cages.

The growth rate and survival rate of >1-year-old juveniles (see **Supplementary File 1: S.6. Biological material**) were tested. The experiment was carried out to its full extent in the summer of 2014 and was repeated to a smaller extent at some localities in the summer of 2015. Within level (I), 2 - 6 sandy cages with a minimum of 100 juveniles and 6 (2014) or 4 (2015) mesh cages with 6 juveniles were applied at each locality tested by the appropriate method. Within level (II), 7 mesh cages with 6 juveniles were installed in every tested environment. The exposure time was one month for the mesh cages and three months for the sandy cages.

The statistical analysis was conducted in R, version 3.1.0[20]. Kruskal-Wallis, Kruskal-Nemenyi, and Wilcoxon-Mann-Whitney tests were used. For data with a normal distribution, linear or quadratic regression was carried out.

The localities can be clearly distinguished based on the growth rate in the open water mesh cages despite the high within-cage variability, even in different growth-favorable periods (**Figure 3**). In the more growth-favorable exposure in 2015 (growth rate 19.3 - 41.8%), a significant trend was discovered in the longitudinal profile where the growth rate increased downstream (Kruskal-Wallis test, $p < 0.001$). Importantly, the survival rate was equivalently high in both seasons (from 83%) (**Figure 4A**).

On the other hand, the open water sandy cages showed a different trend between the main stream localities in 2014: the growth rate increased downstream from locality A (52%) through the middle locality C (153%), and thereafter decreased again until locality E (46%) (a quadratic regression of absolute growth values: $r^2_{adj} = 0.77$, $F_{2,13} = 25.66$, d.f. = 16, $p < 0.001$). This trend was also confirmed in 2015 when the greatest growth rate was recorded at the middle locality C again. Also, the absolute growth rate values did not differ much between 2014 and 2015. On the other hand, the survival rate differed between the years, being much higher in 2015 (from 48% to 72%) than in 2014 (about 25%) (**Figure 4B**).

An effect of two different exposure methods is also clearly visible in the polluted tributary (locality V). The sandy cages exposed here during the three months showed 0% survival, while an 83% survival rate with some growth was recorded by open water mesh cages exposure here during the 30 days.

Results from the within-bed sandy cages illustrate different conditions in the hyporheic environment in comparison to open water in the relevant localities. The growth rate was always lower in the hyporheal sites than in open water, and the survival rate was much more variable (from almost 50% to 0%, **Figure 4B**).

A study of hyporheic microhabitats using the within-bed mesh cages showed a significant effect of the substrate composition on juvenile survival. The best conditions were recorded from the oxygen-saturated stony bottom (a survival rate close to 100%) while the worst (a < 40% survival rate) were indicated in poorly oxygenated sand where a very high variability in surviving was also detected. Hyporheic water oxygenation, which was repeatedly measured during the experiment, explains this trend (**Figure 5**).
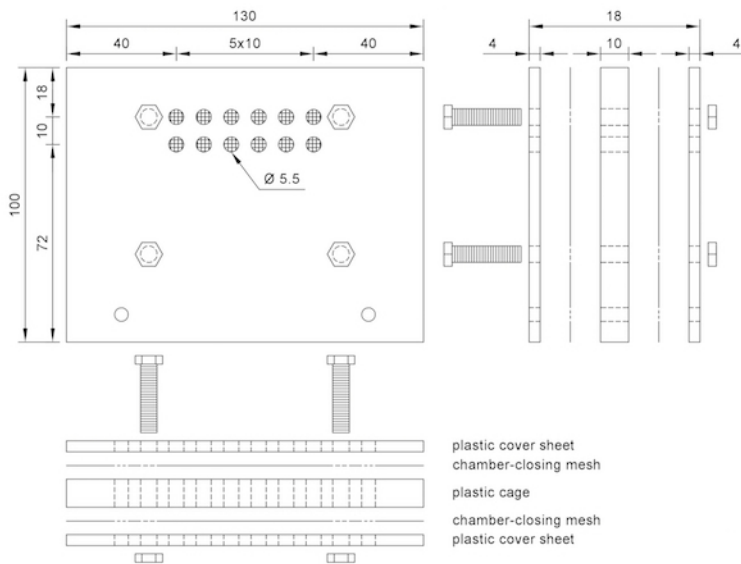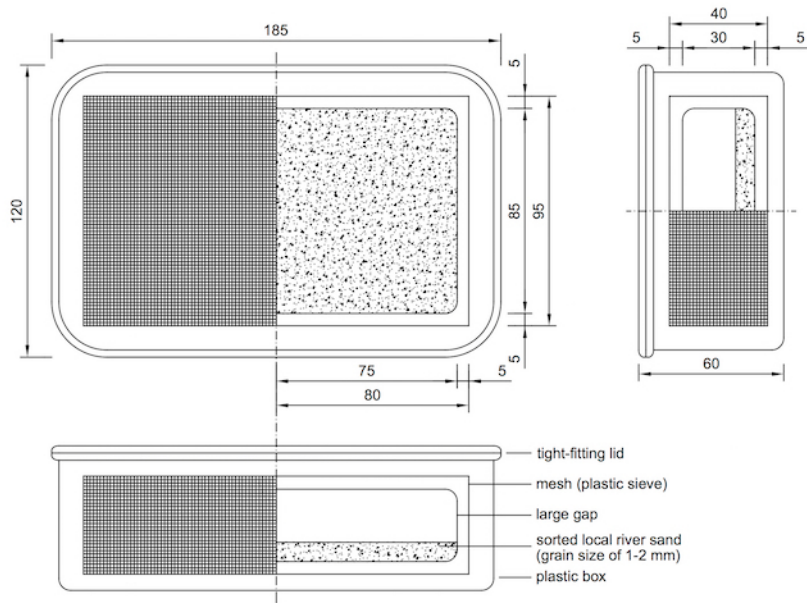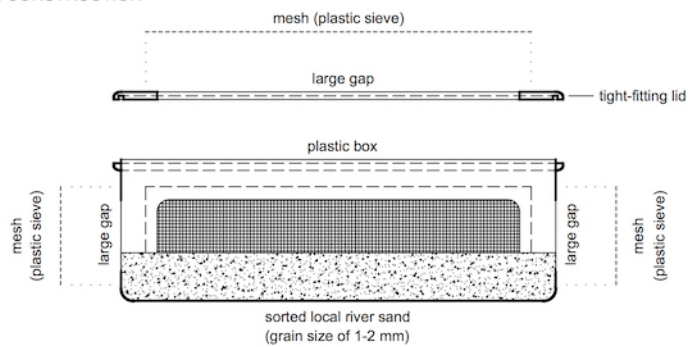

Figure 1. Bioindication mesh cage with individual chambers. See **Supplementary File 1** for further details. Please click here to view a larger version of this figure.
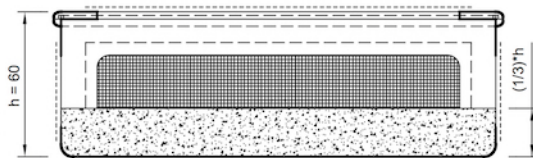
Figure 2. Bioindication sandy cage. See **Supplementary File 1** for further details. Please click here to view a larger version of this figure.
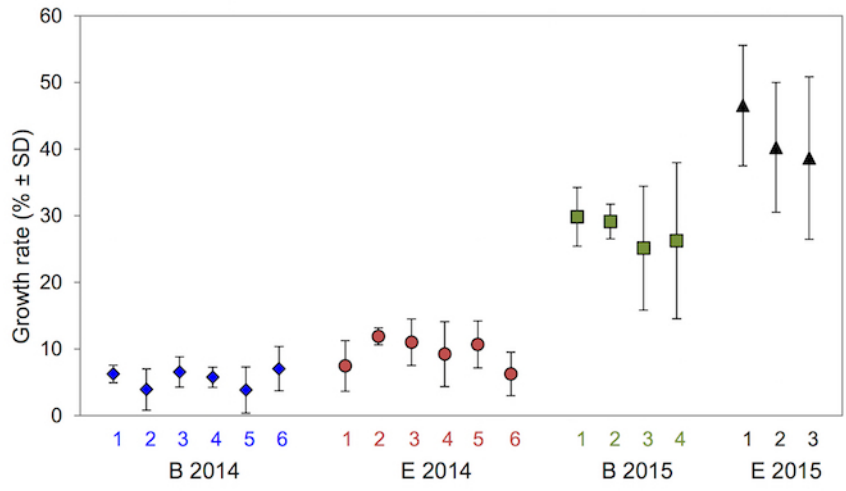
Figure 3. Individual variability in juvenile growth rate recorded by open water mesh cages in localities B and E during two seasons.
The means and standard deviation are described for every mesh cage. The values are based on the measurement of 6 juveniles (or 4 - 5 juveniles if the mortality rate > 0%) in every mesh cage.

Figure 4. Example results from a field bioindication experiment with mesh and sandy cages. (**A**) This panel shows example results from a field bioindication experiment with mesh cages. A total of 6 localities (B, C, D, E, R, and V)within the Vltava River catchment were tested on 2 separate occasions (in 2014 and 2015). The exposure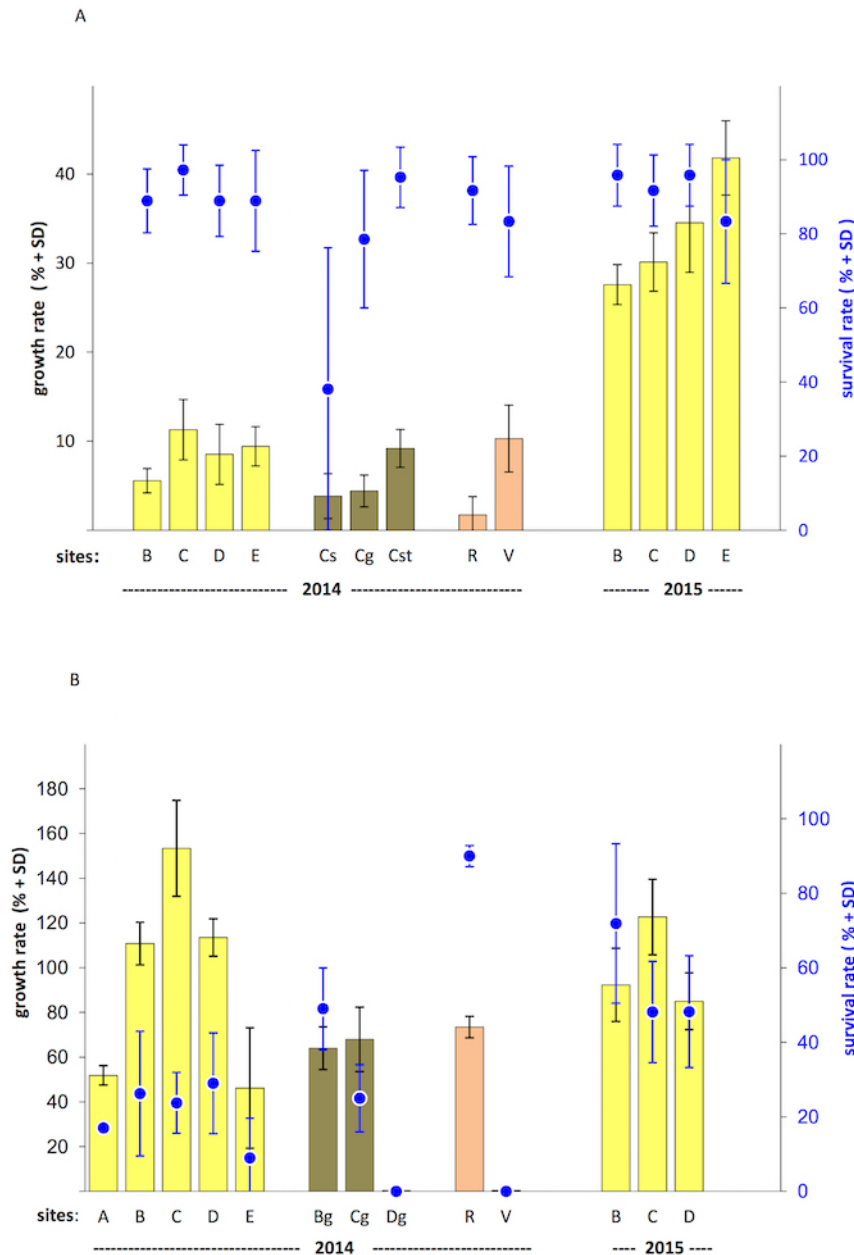 time was 30 days during the summer season. The Localities B - E represent (in order) a longitudinal profile of an approximately 20-km stretch of the river's main stream. Localities R and V represent profiles of 2 tributaries. Capitals mark the same locality both in panel (**A**) and (**B**). All localities were tested with open water mesh cages. In addition, locality C was also tested using within-bed mesh cages installed in 3 different types of river bed (Cs = sand, Cg = gravel, Cst = stones) in 2014. The cages were installed in 4 - 7 replications at every site. 6 freshwater pearl mussel juveniles of 1+ year old were used per mesh cage. The average growth rates are marked for the 3 largest individuals (3 MAX) from every tested mesh cage (columns, left axis) and the average survival rate per mesh cage (blue points, right axis). (**B**) This panel shows example results from a field bioindication experiment with sandy cages.A total of7 localities (A, B, C, D, E, R, and V)within the Vltava River catchment were tested over on 2 separate occasions (in 2014 and 2015). The exposure time was 3 months during the summer season. Sites A - E represent (in order) a longitudinal profile of an approximately 30-km long stretch of the river's main stream. Sites R and V represent profiles of 2 tributaries. Capitals mark the same locality both in this and in the previous panel. All localities were tested with open water sandy cages. In addition, localities B, C, and D were also tested using within-bed sandy cages installed in gavel river bed substrate (Bg, Cg, and Dg) in 2014. The cages were installed in 2 - 4 replications at every site. At least 100 freshwater pearl mussel juveniles were present in every sandy cage. The average growth rate for the 10 largest individuals (10 MAX) from every tested sandy cage (columns, left axis) and the average survival rate per sandy cage (blue points, right axis) are marked. Please click here to view a larger version of this figure.
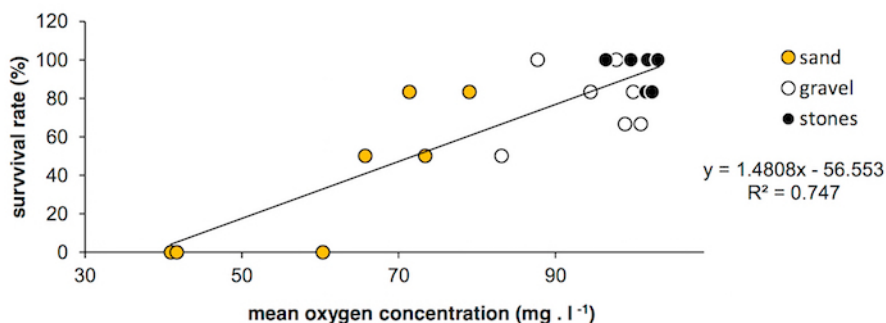
Figure 5. Oxygen saturation. This panel shows the relationship between minimal values of oxygen saturation over 30 days of mesh cages exposure and the surviving rate per cage in within-bed mesh cages exposed in different bed microhabitats in 2014. Please click here to view a larger version of this figure.

locality	2014		2015	
	3-month exposure of sandy cages	1-month exposure of mesh cages	3-month exposure of sandy cages	1-month exposure of mesh cages
A	13.9	-	-	-
B	14.4	13.4	13.9	17.5
C	15	13.8	14.4	18.3
D	15	13.8	14.3	18.3
E	15.5	14	-	18.7
R	13.5	12.8	-	-
V	14	13.2	-	-

Table 1. Average surface water temperature (°C) at the localities during exposure in 2014 and 2015.

Discussion

Exposure time:

Even one-month exposed mesh cages show a visible growth reflecting differences between localities (**Figure 3**), so they are very usable for the quick and easy detection of a locality characterization. Nevertheless, the relevance of the results depends on the short-term state of the conditions, which can oscillate. In particular, short rainfall events can play a role. In contrast, unpredictable episodic pollution may not always be recorded. In locality V (**Figure 4A**), water chemistry analysis detected a short wave of strong ammonium increase[13]. This was probably responsible for the mortality in the three-month exposed sandy cages but did not affect the 30-day exposed mesh cages.

Temperature fluctuations can also affect short-term exposure results. The one-month average temperature during mesh cage exposure differs between the years (**Table 1**). The growth rate also varies where higher temperatures were accompanied by higher growth rates (Kruskal-Wallis test $p < 0.001$). On the other hand, the average water temperature at the same localities during the three-month sandy cage exposure was very similar in both years (**Table 1**) and the growth rate did not differ significantly (**Figure 4B**).

Benefits and failings of the described methods:

An open water exposure is relatively easy to perform but is of limited value for habitat bioindication. The method of open water mesh cages is relatively old[16] and has been repeatedly used with minor modifications[10,11,12,13,21,22,23]. Nevertheless, these cages are not limited by oxygen, whose deficiency is probably responsible for many juvenile deaths in hyporheic conditions. Thus, open water mesh cages can show good development even in localities with increased mortality and a declining growth rate in open water sandy cages (locality E) or a 100% death rate in within-bed sandy cages, as at locality D in 2014 (**Figure 4B**). Apparently, the open water mesh cages show locality growth potential, but this may not be realistic as it is dependent on the real availability of hyporheic microhabitats within a locality. Because the open water mesh cages have the ability of high survival (**Figure 4A**), even up to a 100% survival rate[13], they can serve well for the bioindication of chronic toxicity (or acute toxicity if it is expected at a given time). Also, they can be a useful food source presence testing to some extent.

As a new and uncommon method, the open water sandy cages better simulate hyporheic habitat conditions. Movement of the juveniles between sand grains is possible in this apparatus, which helps to reduce the biofilm growth on the juvenile shell. A hyporheic oxygen deficiency can be caused by the activity of microbes colonizing the sand grains; this effect can also partially occur in cages placed above a river bottom. Nevertheless, due to the necessary periodic cleaning of clogging drifting material from a cage, fine sediments are also removed and thus the conditions are changed in comparison to the natural hyporheic habitat. So, the growth rate can also be considered as locality growth potential in open water sandy cages. However, this is closer to real locality suitability than in open water mesh cages. Therefore, the longitudinal growth rate gradients recorded by sandy cages (**Figure 4B**) also seem to be more plausible and indicate a more suitable river stretch. Moreover, in sandy cages, the possibility of juveniles and subadults breeding up to sexual maturity is verified[9], so sandy cages can serve as a safe breeding and biomonitoring method simultaneously.

Sandy cages and mesh cages placed in the within-bed position are closest to the real conditions in a shallow hyporheal. By allowing a juvenile's movement, sandy cages, in particular, offer them both a vertical and horizontal gradient of several centimeters in scale. This ability to move could be very important for escaping from temporary oxygen-deficient micro-zones. This possibility is absent in the within-bed mesh cages. Therefore, a relatively high number of bioindication units is necessary, because the hyporheic conditions are very variable[13,24] (**Figure 5**) and losses due to an unsuitable location are common.

In summary, the bioindication methods used in this research correspond with presumed juvenile natural conditions in the following order:
1. open water mesh cages,
2. open water sandy cages,
3. within-bed mesh cages,
4. within-bed sandy cages.

The workload per unit increases in the same order. Moreover, the juvenile number required for a statistical testing of the results obtained increases in within-bed exposures too. It seems that the within-bed sandy cages represent a more expensive but accurate bioindication method. This new method needs more testing in the future and comparison with other types of hyporheic studies based on the piezometer measurements[25,26]. In particular, there is a need to study the degree of similarity using a direct probe measuring physicochemical conditions in the cages and in the surrounding hyporheic environment.

The number of individuals measured in one cage:

Compared to mesh cages, it is not possible to measure the growth increment of specific juveniles in sandy cages as there is no information on which individual from the input set is which one in the output. It is necessary to work with an average value. If counted for all the individuals, this value can be very low due to a number of very slowly growing specimens; however, a couple of individuals can grow very quickly (growth jumpers). Such uneven growth is typical for mussels[27]. The growth variability among juveniles rises with increasing exposure time and big differences can occur, especially in growth-favorable seasons. Also, a long exposure leads to major mortality rates in the mesh cages (for a review see Lavictoire, Moorkens, Ramsey, Sinclair, and Sweeting[28]), so we can work with a significantly lower number of individuals at the end of the experiment compared to the input juveniles' set. Measuring only the several best-growing juveniles is a possible method.

The experience of FWPM breeding within the Czech Action Plan for Freshwater Pearl Mussel[9,29], as well as the results from experiments on sea bivalves[30,31], suggests that growth-deficient juvenile bivalves have a high mortality rate, and there is only a negligible chance of their living to maturity. In contrast, growth jumpers have a higher survival rate and they are crucial for a population recovery. The parameter 10 MAX (the 10 most-quickly growing individuals) takes the growth jumpers into account and can increase the informative value of the experiment, even if high mortality takes place (**Figure 4B**, season 2014). It should be noted that the growth estimate obtained by this method cannot be a false positive. It may only be slightly underestimated because many of the largest juveniles at the end of the experiment would have grown a little bit more in this case. Also, the workload is less if only 10 individuals are evaluated. Similarly, a measurement of three maximally growing individuals (3 MAX) proved to be appropriate in mesh cages, eliminating the influence of slowly growing, non-perspective individuals, which could bias the real image of site growth potential.

Disclosures

The authors have nothing to disclose.

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ČLÁNEK 3: Hodnocení vlivu vícenásobného bodového znečištění na juvenilní jedince perlorodky říční (Barák et al., 2022) – „uveřejněný" (J_{imp})

Experimental quantification of multiple point-source pollution effects on juvenile freshwater mussels in a stream exposure gradient

Vojtěch Barák, Václava Jahelková, Ondřej Simon, Michal Bílý, Karel Douda

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RESEARCH ARTICLE

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Experimental quantification of multiple point-source pollution effects on juvenile freshwater mussels in a stream exposure gradient

Vojtěch Barák[1,2] | **Václava Jahelková**[1] | **Ondřej Simon**[1] | **Michal Bílý**[1] |
Karel Douda[2] (ID)

[1]Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Praha Suchdol, Czech Republic

[2]Department of Zoology and Fisheries, FAFNR, Czech University of Life Sciences Prague, Praha Suchdol, Czech Republic

Correspondence
Karel Douda, Department of Zoology and Fisheries, FAFNR, Czech University of Life Sciences Prague, Kamýcká 129, Praha Suchdol, 165 00, Czech Republic.
Email: k.douda@gmail.com

Abstract

1. Population declines in freshwater mussels (Unionida) resulting from failed recruitment, together with episodic water pollution potentially leading to juvenile mussel mortality, are often suspected (but rarely well documented), even in the most strictly protected aquatic areas.

2. The aim of this study was to test a robust design for an in situ investigation of the water pollution effects on juvenile freshwater pearl mussel (FPM, *Margaritifera margaritifera*) in the protected Malše River (Czech Republic), which acts as a model system. A 30-km river reach was delineated to include a section with previously recorded episodic multiple point-source pollution events, as well as downstream and upstream control sections. Juvenile FPMs in open water mesh cages were exposed to river water at 15 sites in 42 exposure units (342 juveniles in individual chambers), and their survival and shell growth were assessed after a 33-day period of low-flow and high-temperature conditions. The main physicochemical indicators related to municipal water pollution were measured weekly.

3. The study results confirmed significantly impaired water quality indicated by episodic changes in total ammonia nitrogen (up to 1.037 mg L^{-1}), total phosphorus (up to 0.516 mg L^{-1}) and oxygen saturation (down to 75.0%) in the impaired section. While the growth of FPM (corrected for temperature effects) was significantly increased (shell length increment: 32.7 ± 9.7% – impaired, 26.4 ± 10.0% – control sections; mean ± SD), the survival (mean ± SD) of juveniles was significantly decreased in the impaired section (86.4 ± 12.5%) compared with the control sections (97.4 ± 5.7%).

4. This study has demonstrated the capacity of applying a caged bivalve model at a fine spatial scale within a longitudinal river profile to document the impacts of water pollution. The methodology applied here may provide a useful tool for assessing and improving the level of water quality management and is applicable in FPM action plans and stream biotope conservation in general.

1 | INTRODUCTION

The effects of contaminants are traditionally considered one of the main reasons for the global declines in freshwater mussels (Ferreira-Rodríguez et al., 2019). Numerous studies place mussels among the most sensitive organisms to a variety of contaminants, especially metals, ammonia and major ions (Wang et al., 2007; Wang et al., 2018; Nakamura et al., 2021), and their early life stages may be particularly vulnerable (Eybe et al., 2013; Ferreira-Rodríguez et al., 2019).

Knowledge regarding mussel sensitivity is typically based on monitoring changes in population size, changes in distribution, increased mortality of individuals, absence of natural reproduction, changed physiology, or the detection of sudden die-offs, in combination with experimental toxicant exposures under laboratory conditions (Van Hassel & Farris, 2007). Nevertheless, there are a number of cases where the exact causes cannot be identified, despite water pollution being the main suspect (Van Hassel, 2007; Gillis, 2012; Gillis et al., 2017), as highly variable spatiotemporal dynamics and combined effects make it difficult to capture these events in the field and simulate them in the laboratory (Patnode et al., 2015). Subsequently, a lack of evidence creates uncertainty in setting environmental limits for the protection of this animal group (ASTM, 2013a; Ferreira-Rodríguez et al., 2019).

Water quality degradation seems to be the main factor in declining populations of unionacean mussels (Strayer et al., 2004), including the critically endangered freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) (FPM). FPM is an ideal target species for aquatic ecosystem conservation as it is considered an umbrella, indicator, flagship and keystone species (Geist, 2010). However, populations have severely declined in distribution and abundance throughout their range (Lopes-Lima et al., 2017). In surviving populations, a lack of recruitment is considered the main challenge because the juvenile period is thought to be a critical life stage of FPM (Geist, 2010; Gum, Lange & Geist, 2011). Understanding the specific habitat requirements of early life stages is vital for their conservation (Quinlan et al., 2015; Lavictoire et al., 2016). Nevertheless, there is a lack of information on the relationship between habitat conditions and the success of juvenile FPM development (Lavictoire et al., 2016; Bílý et al., 2021). Studies on the effects of water pollution on FPM are rare (Taskinen et al., 2011); a recent study shows that there is still a significant discrepancy between field and laboratory data for different substances in terms of ecotoxicological limits for the survival of FPM (Belamy et al., 2020).

In situ exposure with subsequent evaluation of species performance is often used in conservation biology (Armstead &

Yeager, 2007 and references therein) and provides valuable information on the environmental quality and site suitability for endangered species (Haag, 2012; Pollard et al., 2017), including FPM (Buddensiek, 1995; Gum, Lange & Geist, 2011; Bílý et al., 2018). Recent studies have confirmed the applicability of these bioindication methods for freshwater mussels at various spatial scales (Bartsch et al., 2017; Černá et al., 2018; Bílý et al., 2021), and several authors (Haag, 2012; Ferreira-Rodríguez et al., 2019; Haag et al., 2019) recommend using field exposures to investigate the multiple-stress effects in natural settings. Nevertheless, this method is still relatively underused and has only rarely been used to determine the effects of episodic sources of water pollution. A declining FPM population (Malše River, Czech Republic) reportedly disturbed by the impact of municipal pollution from point sources (NCA CR, 2013; Simon & Dort, 2014) was studied here, but there is a lack of data documenting these effects owing to the inherent episodicity of pollution events.

The objective of this study was to develop and test a robust experimental design for the in situ investigation of the effects of multiple point-source pollution on juvenile freshwater mussels, particularly FPM. The specific goals were to: (i) compare FPM survival/growth within impaired and control river sections; (ii) quantify the relationships between the main indicators of municipal pollution measured at weekly intervals and the results of field exposures; and (iii) provide recommendations for researchers and practitioners in the field of freshwater bivalve conservation to use caged models for the conservation of species living in streams and rivers impaired by point source pollution.

2 | METHODS

2.1 | Study area

The Upper Malše/Maltsch River, with a transboundary river basin (Czech Republic–Austria, Central Europe), represents a relatively well-preserved ecosystem of (sub)mountain rivers with natural flow dynamics, flood regimes and seminatural floodplains (Simon & Dort, 2014). The approximately 40-km-long river reach was designated as a Special Area of Conservation (CZ0314022 Horní Malše and adjacent AT3115000 Maltsch) under the European Habitats Directive, which then forms part of the Europe-wide Natura 2000 network (Council of the European Communities, 1992). Findings of all FPM life stages have been confirmed in the river (Simon & Dort, 2014), although the FPM population with a low total number of individuals ($\sim$440 visible individuals) and a low proportion of subadult individuals ($\sim$10%) is scattered in the river (Simon et al., 2015). The Czech Action Plan for FPM conservation (NCA CR, 2013) lists this

river as promising for the long-term occurrence of viable populations because of evidence of active natural reproduction: juveniles were found only in two Czech rivers with FPM occurrence (Simon et al., 2015). However, the Malše River is supposed to be significantly affected by local human pressures (see the aforementioned water quality deterioration).

The river reach for this study covers the main occurrence of FPM in the river and is situated between the confluence with the Felberbach stream and the city of Kaplice (stream order IV (river km (RKM 62–78) and V (RKM 47–62); Strahler, 1957) at an altitude of 540–660 m above sea level. Its width and depth range from 3 to 10 m and from 0.3 to 0.8 m, respectively, during low-flow conditions. Meadows and pastures with forest patches are typical features of local floodplains (NCA CR, 2013). The long-term average annual discharge is 2.14 m^3 s^{-1} for profile Kaplice situated at the end of the study river reach. During the field experiment (July to August 2019; see Sections 2.4 and 2.5), discharge ranged from 0.35 to 3.05 (median 0.65) m^3 s^{-1}. Records showed four periods of higher flow (with maxima of 0.87, 1.53, 2.47 and 3.05 m^3 s^{-1}; see Supporting Information 1). All data were obtained from the Flood Forecasting Service of the Czech Hydrometeorological Institute Prague (CHMI, 2019).

2.2 | Experimental design

The modified gradient design method (Salazar & Salazar, 2007) was applied by delimitation of the impaired and both the upstream and downstream control river sections (Rogers et al., 2018). Available literature data (Simon & Dort, 2014; Simon & Kladivová, unpublished data) and preliminary water quality screening performed in 2018 (Supporting Information 2) indicated the presence of potential anthropogenic water pollution sources from a small tributary and a municipal sewage treatment plant (using secondary activated sludge biological treatment processes with (de)nitrification, 900 equivalent

inhabitants) in the central part of the FPM occurrence. Four study sites were situated within this impaired section: IMP-1 (0.15 km downstream of the confluence with a small tributary) and IMP-2 to IMP-4 (0.4, 1.2 and 1.5 km downstream of the plant outfall; RKM 62–66, Figure 1). Within each of the two control sections, five and six sites were located upstream (CUP-1 to CUP-5 – RKM 66–78) and downstream (CDN-1 to CDN-6 – RKM 47–62) of the impaired section, respectively (Figure 1). Long-term monitoring of a sewage treatment plant situated in the upstream control section did not show any significant effect on water quality and therefore was not investigated in the present study. All the sites studied were chosen based on water chemistry data 2017–2018 (see above), and the distance between them ranged from 0.3 to 6.2 (river) km (see Supporting Information 3).

2.3 | Experimental animals

Juvenile FPMs used for field exposures were recruited from a seminatural breeding programme (Czech Action Plan for FPM; NCA CR, 2013) with a Blanice River mussel population as a glochidia source. Individuals in age cohort 1 + (7–8-month-old juveniles; within the second growth period of the post-parasitic stage) were used. The juvenile size before exposure ranged from 1,008 to 2,545 μm (mean ± SD 1,682 ± 287 μm; see Supporting Information 4). At the end of the experiment, all surviving juveniles were returned to the breeding programme.

2.4 | In situ exposure

Juvenile FPMs were exposed to river conditions in open water mesh cages with individual cylindrical chambers measuring 10 mm (height) × 5.5 mm (diameter), formed by a bore in plexiglass material and closed by a polyamide fine 340-μm mesh (modified from
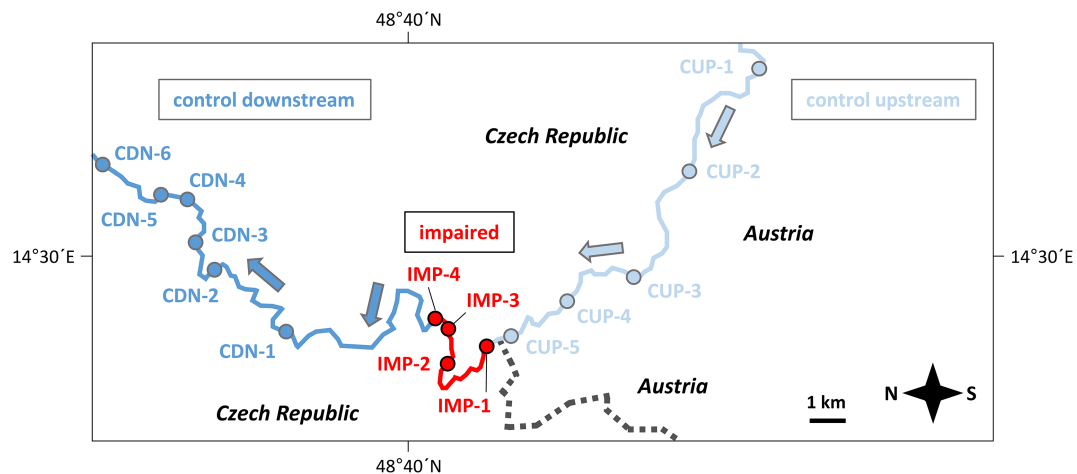

FIGURE 1 Map of sites in the Malše River study area (control downstream section – light blue (sites CDN-1 to CDN-6); impaired section – red (sites IMP-1 to IMP-4); control upstream section – pale blue (sites CUP-1 to CUP-5))

Buddensiek, 1995; Bílý et al., 2018). Two to four replicated cages (22–24 individually held juveniles) per site were used, resulting in a total of 342 juveniles housed in 42 cages at 15 experimental sites. This number of cages/mussels could be (un)installed in 1 day, so the first/last date of exposure was the same for all mussels at each site. The installation of bioindication mesh cages in open water conditions followed the protocol described by Bílý et al. (2018). All juveniles were individually photographed under a microscope (magnification of 80×) and placed into chambers. After installation in the field, the cages were checked and cleared of drifted material (i.e. debris; after 14 days; NCA CR, 2013). The exposure lasted for 33 days (July to August 2019), which has been demonstrated to be a suitable exposure duration for site characterization in terms of FPM juvenile growth and survival (Bílý et al., 2018). At the end of the exposure period, juveniles were examined and again photographed, and their size measurement was performed in ImageJ software (Abràmoff, Magalhães & Ram, 2004). The shell length increment (% of the initial size, referred to here as 'growth') for each surviving juvenile and survival (% per cage) were used as test endpoints. Because of the high temperature dependency of juvenile mussel growth (Bartsch et al., 2003; Černá et al., 2018; Haag et al., 2019), analyses were performed for both temperature-corrected and uncorrected growth data. For the former analysis, the effect of temperature was removed (as a covariable) from the final model (see Section 2.6) using a regression relationship of temperature and shell growth from the whole dataset.

2.5 | Water physicochemistry and chemical analyses

The main physicochemical parameters related to municipal pollution were chosen as indicators of the degree of site disturbance in this study. These are nitrogen forms, total phosphorus (TP), dissolved oxygen (DO), conductivity and suspended solids, together with pH and calcium. Five surface water samples were taken at each experimental site at intervals of 6–8 days during the 33-day experiment (July to August 2019). Surface water samples were collected from a depth of 10 cm in the thalweg into 1-L PE bottles; all sites were sampled in a downstream order. Samples were transported immediately to the laboratory in a thermally insulated box with ice packs. Nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), ammonium nitrogen (NH_4-N), pH, calcium (Ca), TP and total suspended solids dried at 105°C (TSS) were quantified at the environmental laboratory of T. G. Masaryk Water Research Institute, Prague (Czech Republic) according to the respective basic European or international standards (CSN EN ISO 10304-1 2009–10, CSN EN 26777 1995–10, CSN ISO 7150-1 1994–07, CSN ISO 10523 2010–03, CSN EN ISO 11885 2009–10, CSN EN ISO 6878 2005–03, CSN EN 872 2005–10). Ammonia nitrogen (NH_3-N) and total ammonia nitrogen (TAN as the sum of NH_4-N and NH_3-N) were calculated using the reported temperature and pH data. All TAN data (including those from previous studies cited in Section 4.3) were normalized to pH 7.0 and

temperature 20°C (for all equations, see USEPA, 2013). Furthermore, in situ conductivity (corrected to 25°C; κ_{25}) and DO measurements were taken five times at the sampling sites. For those measurements, a calibrated mobile multimeter (Multi 3620 IDS) with two measuring probes (TetraCon 925 for κ_{25} and FDO 925 for DO; WTW GmBH, Germany) was used. Water temperature was continuously measured by temperature data loggers (HOBO 64 K Pendant, UA-001–64, Onset Computer Corporation, USA) fastened to exposure cages at each site.

2.6 | Statistical analysis

To investigate the differences in juvenile growth between river sections, two separate linear mixed models with arcsine-transformed proportional shell growth as the response variable were used. The fixed effects were river section (Model A: impaired × control downstream × control upstream, Model B: impaired × control) and site position (RKM); the identity of site (15 levels) and cage (42 levels) were used as random effects in both models. Models with original and temperature-corrected data were run separately (see Section 2.4). Pairwise t tests were used for post hoc comparisons.

Because of generally high survival, Fisher's exact test with post hoc pairwise tests was used to compare the mortality of the juveniles among the river sections using the total number of survivors and deaths in individual sections. The linear mixed-effects regression models were fitted in R version 3.6.0 (R Core Team, 2020) using the lmer function of the lmerTest package (an extension of the lmer function from the lme4 package; Bates et al., 2015), which allows an approximation of the degrees of freedom. Hypothesis testing of the explanatory variables was performed using likelihood ratio tests with type III ANOVA and the Satterthwaite method for the approximation of degrees of freedom using the ANOVA function in the lmerTest package after checking the normality of data by visual inspection and the Shapiro–Wilk test.

Stream sections were screened for potential differences in water quality indicators using the non-parametric Kruskal–Wallis test. When the results of the Kruskal–Wallis test were significant, pairwise comparisons were performed according to the two-tailed unpaired Wilcoxon Mann–Whitney test and adjusted for multiple comparisons using the Bonferroni method.

3 | RESULTS

3.1 | Juvenile growth and survival

The growth (not corrected for temperature) of juvenile FPM was 30.8 ± 10.1% (mean ± SD) in the impaired stream section over the 33-day exposure period, indicating that at the end of this period the mussels were 30% larger than their initial size. The growth of mussels within the control downstream section of 30.7 ± 12.0% (mean ± SD) was comparable with that in the impaired section; however, the

control upstream section showed a lower growth of 21.2 ± 9.5% (mean ± SD; Figure 2a; Supporting Information 4). The growth of juveniles increased incrementally in the downstream direction (on average 8.8% for every 10 km of river distance; see Supporting Information 5). In terms of the growth of juvenile FPM, the compared models resulted in a significant stream section factor in Model A (three stream sections, $P < 0.05$) but not in Model B (two stream sections, $P > 0.05$) for the temperature-uncorrected data. The site position factor (RKM) was significant in both models without temperature correction (both $P < 0.01$). Post hoc comparisons showed that both the impaired and downstream control sections differed significantly (both $P < 0.001$) from the control upstream section but were not significantly different from each other.

The temperature-corrected growth of juvenile FPM was 32.7 ± 9.7% (mean ± SD) in the impaired stream section. However, the growth of mussels was 27.8 ± 8.2% (mean ± SD) and 25.2 ± 11.1% (mean ± SD) in the control upstream and downstream sections, respectively (Figure 2a; for site-specific data see Supporting Information 5). In the case of temperature-corrected data, the stream section factor was significant in both Models A ($P < 0.001$) and B ($P < 0.05$). This time, the effect of RKM was not significant in Model B ($P > 0.05$), which was eliminated after the temperature correction. Post hoc comparisons showed that both the upstream ($P < 0.01$) and downstream ($P < 0.001$) control sections differed significantly from the impaired section in Model A, and there were no differences between control sections.

The juvenile survival in the impaired stream section was 86.4 ± 12.5% (mean ± SD), while it was 96.9 ± 6.5% (mean ± SD) and 97.8 ± 4.9% (mean ± SD) in the upstream and downstream sections, respectively (Figure 2b; Supporting Information 4), and often reached 100%. Fisher's exact test with post hoc pairwise tests showed that the impaired section had significantly higher mortality than both the upstream ($P < 0.05$) and downstream control sections ($P < 0.01$).

3.2 | Water quality

All the indicators of high organic loads showed highly elevated values in the impaired section. Numerical values were significantly higher within the impaired section than in both control sections in the case of TAN, NH_3-N, NH_4-N and NO_2-N (and lower in the case of DO; $P < 0.05$, Figure 3; Supporting Information 3). The adverse effects of water pollution on the studied receiving environment were mitigated downstream of the impaired section by self-purification processes in the river. There was also a significant increase in P and TSS in the impaired section compared with the upstream control. However, despite the observed decrease in downstream parameters, there was no longer a difference between the impaired and control downstream sections.

The complementary set of parameters was either not elevated at all in the impaired section or showed an increasing trend in the longitudinal profile (numerical values increase downstream: NO_3-N, T, κ_{25}, Ca, pH) in accordance with the assumptions of the river continuum gradient (Vannote et al., 1980) (see trends and P values in Figure 3; Supporting Information 3).

In terms of the occurrence of extremely high values potentially at (in)direct risk to FPM, maximum concentrations of TP and TSS (0.516 and 150 mg L^{-1}, respectively; see Section 4.5; Supporting Information 3) could be related to a single event recorded during the first period of higher flow conditions (see Section 2.1). The mean ammonium/(total) ammonia concentration within the impaired section was higher than the maximum values in the control sections (Figure 3). A unique event (combined sewer overflow at the sewage treatment plant: TAN 1.037 mg L^{-1} and DO 75.0%; see Supporting Information 3) was the main reason for that increase. However, even if those specific data are not taken into account, there are still increased levels of TAN, NH_3-N and NH_4-N in the impaired section, indicating a persistent effect of point-source pollution on water chemistry. A similar trend was found for NO_2-N levels (Figure 3); however, this trend was
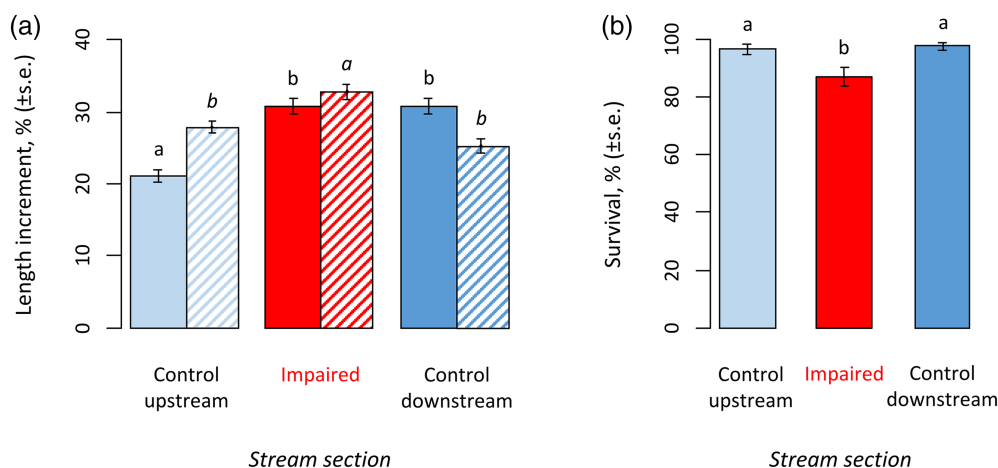
FIGURE 2 Growth and survival in juvenile FPM deployed in various sections of the Malše River over a 33-day experiment (July to August 2019); n = 4–6 sites per section, 2–4 replicated mesh cages (total of 22–24 juveniles) per site. (a) Shell growth calculated for each surviving juvenile (length increment as a percentage of the initial size; mean and SE). Hatched bars with temperature corrected values. (b) Survival calculated per cage (mean and SE). Different letters (a, b; those italicized for temperature-corrected growth data) indicate significant differences among groups ($P < 0.05$).

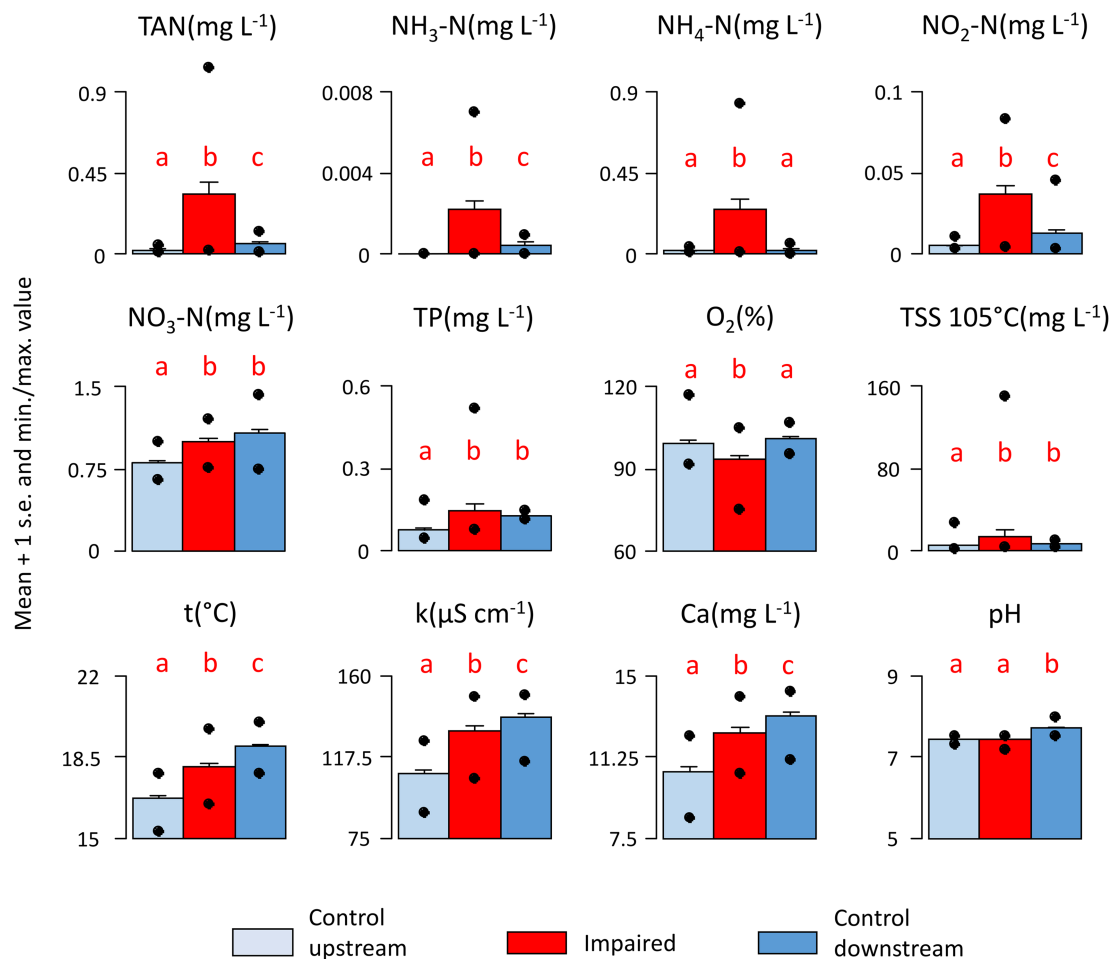**FIGURE 3** Physicochemical properties of the surface water (mean and SE; points represent minimum and maximum values) in various sections of the Malše River over a 33-day experiment (July to August 2019); $n = 20$–30 samples/measurements per section. TAN (total ammonia nitrogen) data were normalized to pH 7.0 and 20°C. Different letters (a, b, c) indicate significant differences among groups ($P < 0.05$). *Note*: Conductivity is labelled as 'k' here.

not as evident because the event caused higher nitrite concentrations even within the control downstream section.

4 | DISCUSSION

This study has demonstrated a new experimental approach that applies a caged bivalve model at fine spatial scales within a longitudinal river profile. Multiple point-source pollution effects on juvenile freshwater mussels were experimentally quantified, as a significant increase in mortality and a slightly elevated growth of FPM were found within the impaired river section. Several potential stressors (total ammonia and nitrite, low DO levels) were found to be elevated and were probably the main reasons for adverse mussel development.

4.1 | Experimental design

The in situ caged mussel approach allows environmentally realistic studies to be performed and assesses the impact of multiple-stressor

exposure (Burton et al., 2005; Armstead & Yeager, 2007; Gillis et al., 2017). The gradient design with field-deployed mussels has been successfully applied to upstream–downstream assessment (Salazar & Salazar, 2007; Nobles & Zhang, 2015; Patnode et al., 2015). In line with the study by Rogers et al. (2018), it is shown that a combination of multiple caging units and multiple sites in individual sections allows more reliable impact detection. However, shorter river reaches with a higher number of sites were investigated here (132 vs. 31 km in length, eight (2/4/2) vs. 15 (5/4/6) sites within the control upstream/impaired/control downstream section) to obtain an even more detailed result and the possibility of comparing individual sections (see Supporting Information 5). It helps to characterize patterns of mussel condition and/or environmental factors in greater detail, which provides an opportunity to implement a potential gradient of reference conditions (within control sections).

The potential limitations of in situ exposure tests include the type and placement of field cages, effects of transportation and handling, and security against floods or vandalism (Burton et al., 2005; Armstead & Yeager, 2007; ASTM, 2013a). In addition, in situ exposure of mussels is typically accompanied by water quality monitoring. More

frequently than once a month sampling events increase the likelihood of detecting extremes in pollutant concentrations (Goudreau, Neves & Sheehan, 1993). In the present study, using weekly sampling, problems in sewage treatment plant function causing elevated TAN concentrations and decreased DO levels were more likely to be identified. Continuous logging probes are expensive and not available for many parameters, so their application is limited at present (Quinlan et al., 2015).

4.2 | In situ exposure (growth and survival) and water quality

Mesh cages situated within impaired and control downstream sections showed higher mean growth than those at upstream sites. This was accompanied by higher water temperature; differences in mean daily values during exposure reached 1.5°C. The positive dependency of mussel growth on temperature has already been demonstrated (Bartsch et al., 2003; Černá et al., 2018; Haag et al., 2019); thus, removing the effect of temperature allowed the detection of the expected effect of other water quality variables. Černá et al. (2018) performed in situ exposure tests with FPM 1+ in the Vltava River Basin (Czech Republic). The authors found mean growth ranging from 28 to 42% in the open water mesh cages within a longitudinal river profile (summer 2015, 30-day exposure, mean water temperature 17.5–18.7°C). The results of the present study are comparable with their findings (growth of 24–44% within river reaches with similar temperature conditions; see Supporting Information 3, 4).

Based on temperature-adjusted results, it can be assumed that the increased growth of FPM in the impaired section was caused by elevated nutrient status (Figures 2a, 3) rather than water temperature. Stream nutrient enrichment is assumed to affect mussel growth positively owing to an increase in food availability associated with increased nutrient loading (Buddensiek, 1995; Strayer, 2008; Strayer, 2014). Similar to the findings reported here, in the field study by Bartsch et al. (2003), ammonia concentrations in the Saint Croix River (USA) were positively related to the growth of juvenile *Lampsilis cardium* at 4 and 28 days (but unrelated at 10 days). As already proposed by Salazar & Salazar (2007) and Strayer (2014), ammonia may function as a nutrient (at lower concentrations) and contaminant (at higher concentrations). However, in general, eutrophication is believed to have an adverse impact on mussel growth (Buddensiek, 1995; Strayer, 2008; Degerman et al., 2009; Haag et al., 2019).

As hypothesized, the survival of FPM within the impaired section was lower than that at the control sites (73–95% and 91–100%, respectively). It corresponded to increased (total) ammonia levels and decreased oxygen levels (Figures 2b, 3). The adverse impact of eutrophication and poor oxygen conditions on the survivorship of young mussels has been shown previously (Buddensiek, 1995; Sparks & Strayer, 1998; Strayer, 2008; Degerman et al., 2009; Eybe et al., 2013; Černá et al., 2018).

4.3 | Potential role of ammonia and the current protective criteria

Freshwater mussels are among the most sensitive taxa to ammonia, and such information is essential for the environmental regulation and biological conservation of mussel populations (Nakamura et al., 2021). In the field study by Bartsch et al. (2003), ammonia concentrations did not explain the mortality of juvenile *L. cardium*. Patnode et al. (2015) found higher survival of caged juvenile *Epioblasma torulosa rangiana* and lower ammonium ion levels at sites located further downstream of two treatment facilities in the Allegheny River (USA). Nevertheless, they suggested that the main toxicant was chloride rather than ammonium. Černá et al. (2018) recorded a malfunction of the sewage treatment plant (secondary treatment with the same treatment processes as described in Section 2.2) causing high ammonium ion levels (NH_4^+ of 1.22 mg L^{-1}) and 100% mortality of FPM in sandy cages (i.e. plastic boxes filled with sand; Bílý et al., 2018). In the present study, only 27% mortality and a related maximum NH_4^+ value of 1.01 mg L^{-1} were recorded (see Supporting Information 3, 4). However, Černá et al. (2018) did not present data on unionized ammonia NH_3, although it has been demonstrated to be much more toxic (Mummert et al., 2003; Haag, 2012; Strayer, 2014). Moreover, oxygen deficient microzones in the sandy cages may have contributed to the observed mussel mortality (Bílý et al., 2018).

(Water-only) toxicity exposures with juvenile mussels under laboratory conditions are typically conducted for 96 h (ASTM, 2013b). Recently, two species closely related to FPM have been tested. Wang et al. (2017) found EC50s (median effective concentrations) of 7.7–8.4 mg TAN L^{-1} for 6–10-day-old *Margaritifera falcata*. LC50s (median lethal concentrations) ranged from 11.5 to 16.7 mg TAN L^{-1} for 1-day-old *Pseudunio auricularius* (Nakamura et al., 2021). TAN concentrations (0.020–1.037 mg L^{-1} measured at weekly intervals) and mortality of FPM (5–27% over the 33-day exposure period) were found within the impaired section of the Malše River (see Supporting Information 3, 4). The maximum TAN concentration is very close to the safe environmental level of 1.15 mg TAN L^{-1} (equal to 10% LC50; Mummert et al., 2003; Nakamura et al., 2021).

The Environmental Protection Agency (EPA) criteria for ammonia (USEPA, 2013) consider data for freshwater mussel species. Neither the maximum concentration criterion (1-h average = 17 mg TAN L^{-1}) nor the continuous concentration criterion (CCC = 30-day rolling average = 1.9 mg TAN L^{-1}) was reached in the present study. The highest ammonium ion levels found (NH_4^+ of 1.08 mg L^{-1}) were much higher than the guide value for Czech salmonid waters (0.04 mg L^{-1}; Government of the Czech Republic, 2006) and habitat requirement for FPM (0.1 mg L^{-1}; British Standards Institution (BSI), 2017). However, the NH_4^+ concentration is an acceptable surrogate for TAN only when the pH and water temperature are known (see Section 2.5).

The time of mussel death remains unknown. Juveniles may close their valves in response to episodic pollution, which might confer a temporary advantage in limiting exposure to toxic substances (Valenti

et al., 2005). However, the results from the study by Wang et al. (2007) indicate that (2-month-old) juveniles might not be able to avoid exposure to ammonia. In the present study, not only elevated total ammonia levels but also associated reduced DO levels could adversely affect freshwater mussels (Cooper, Bidwell & Cherry, 2005) ('multiple-stressor exposure'; see Section 4.1).

4.4 | Potential role of oxygen and the current protective criteria

A high oxygen supply in the water column and pore water (for buried juveniles) is an important characteristic of mussel habitat quality (Geist & Auerswald, 2007; Stoeckl, Denic & Geist, 2020), especially for stream species. Therefore, eutrophication-associated reduction in DO and related mussel development should be investigated (Geist & Auerswald, 2007; Lopes-Lima et al., 2017). In the field study by Bartsch et al. (2003), the authors described a positive relationship between DO and juvenile mussel (L. cardium) survival. Later, it was confirmed for FPM by Černá et al. (2018). In the case of zero mortality, oxygen saturation always exceeded 85%, whereas a DO level below 65% was the main reason for the death of all juveniles (deployed in within-bed mesh cages). These results emphasise the crucial importance of oxygen in juvenile FPM survival, which agrees with the findings of the present study as well as with those of a recent study by Bílý et al. (2021).

The oxygen requirements of mussel species are known mainly from laboratory testing (Dimock & Wright, 1993; Sparks & Strayer, 1998; Chen, Heath & Neves, 2001). In the latter study, the authors found that the oxygen regulation ability appears to be related to the conditions a species normally experiences in its habitat type. For Villosa iris living in well-oxygenated river and stream riffles (just like FPM), DO >6 mg L^{-1} should ensure its relatively unchanged aerobic metabolism.

The EPA criteria for oxygen (USEPA, 1986) recommend a 1-day minimum threshold of 5 mg L^{-1} (7-day mean value of 6.5 mg L^{-1}), while the Czech guide standard is 7 mg O$_2$ L^{-1} (Government of the Czech Republic, 2006) (all values for salmonid waters). Concentrations measured in the Malše River were either higher or very close to those specific levels (Supporting Information 3). BSI (2017) suggests that DO saturation in FPM rivers should be high (i.e. near 100%). Fluctuations in DO level within the impaired section (see Supporting Information 3) were found not to correspond to that. However, detailed information on FPM DO tolerances is lacking (Quinlan et al., 2015).

Reductions in DO may be caused by nutrient loading but also by high temperature (Sparks & Strayer, 1998). The maximum water temperature for FPM should not exceed 23–25°C (Varandas et al., 2013; BSI, 2017). Higher values in the Malše River were recorded only within the control downstream section (see Supporting Information 3). Thus, a decrease in DO in the impaired section was not associated with high temperature but with increased ammonia concentrations (Černá et al., 2018).

4.5 | Potential role of other pollutants

In addition to increased total ammonia and decreased DO concentrations, elevated levels of TP, TSS and NO$_2$-N were also measured within the impaired section of the Malše River (Figure 3). A high TP concentration can result in a period of high algal productivity and reduction in oxygen levels, but it is not directly toxic to FPM (BSI, 2017). Suspended solid concentrations may influence the viability of juveniles: directly, causing mussels to clam up, leading to stress and death; or indirectly, clogging the river bed and preventing oxygen change with surface water (BSI, 2017). In the case of caged mussels, regular maintenance can prevent device clogging (Bílý et al., 2018).

Little work has been performed to characterize the effects of nitrite on freshwater mussels (Soucek & Dickinson, 2012). The EPA criteria for nitrite (USEPA, 1986) recommend a maximum NO$_2$-N of 0.06 mg L^{-1}; a guide threshold for Czech surface waters is three times higher (Government of the Czech Republic, 2006) (all values for salmonid waters). The former value was exceeded downstream of the plant outfall (up to 0.083 mg L^{-1}; see Supporting Information 3), and it could contribute to effluent-induced mortality (Eybe et al., 2013; Boon et al., 2019). Elevated levels of ammonia and nitrite as well as declining DO levels have been associated with the extirpation of mussels downstream of the treatment facilities (Gillis et al., 2017).

Other contaminants may have also affected mussel performance (e.g. potassium and chloride ions; Wang et al., 2017; Wang et al., 2018); however, no data were collected in this study.

4.6 | Effluent-induced effects on freshwater mussels

Although this study did not focus on the detection of the primary source of pollution, based on the performed measurements the potential origin of increased or decreased values of some water quality parameters during sampling campaigns could be identified.

A positive relationship was indicated between lower survival/ growth (when site-specific data were investigated) and a sudden event causing elevated total ammonia concentrations together with decreased DO levels (see Supporting Information 3, 4). Combined sewer overflow at the sewage treatment plant and effluent-induced effects on freshwater mussels were recorded in the Malše River. Municipal wastewater treatment plants are known to be significant sources of anthropogenic nutrient enrichment in aquatic environments (Augspurger et al., 2003; Haag, 2012; USEPA, 2013). Effluent-induced effects on field-deployed mussels have been described by many authors (Farcy et al., 2011; Nobles & Zhang, 2015; Černá et al., 2018). Levels of total ammonia were expected to be lower further downstream of the plant outfall because of dispersal and dilution of the effluent (Mummert et al., 2003; Patnode et al., 2015). Surprisingly, the juvenile FPM response did not correspond to the pollution gradient. This could be a sign of the combined effects of more environmental factors; however, the

understanding of interactions between contaminants and other environmental stressors is limited (Kienzler et al., 2016; Lopes-Lima et al., 2017; Belamy et al., 2020).

There is a polluted tributary of the Malše River upstream of the urban area with a treatment facility. Nutrient-rich water may come from human settlements and/or grazing in the basin; however, the source of pollution remains unknown. It has reduced river water quality to a lesser extent than effluent discharge from sewage treatment plants (see Supporting Information 2). TAN concentrations below the confluence with the stream reached 0.364 mg L^{-1} (approximately one-third of that recorded downstream of the plant outfall), and DO levels were not markedly affected (see Supporting Information 3). Although the tributary seemed not to have a clearly adverse effect on deployed FPM immediately downstream of the confluence (see Supporting Information 4), it may have partially contributed to exacerbated mussel performance further downstream.

4.7 | Implications for conservation

The assessment of the ecotoxicological responses of freshwater mussels to water quality degradation is considered one of the priorities for conservation research (Ferreira-Rodríguez et al., 2019) because of the need to protect declining populations of an increasing number of threatened or endangered mussel species (Armstead & Yeager, 2007). In situ testing using freshwater mussels has been used in numerous studies but is generally limited to upstream/downstream comparisons with discrepancies between data from reference sites and contaminated sites (Armstead & Yeager, 2007; Salazar & Salazar, 2007). The caged bivalve model can provide insights into the sensitivity of mussels to environmental stressors (Salazar & Salazar, 2007); however, there is a lack of information relevant to freshwater mussel ecotoxicology outside North America (Beggel et al., 2017; Kleinhenz et al., 2019; Nakamura et al., 2021).

Based on the results of this and other recent studies (Patnode et al., 2015; Bílý et al., 2018; Rogers et al., 2018), three main recommendations can be proposed for the use of the caged bivalve model in streams and rivers: (i) covering longer longitudinal profiles extending outside the impaired sections; (ii) the use of multiple sites in each section (including reference ones), and replicated cages and samples/measurements to supplement water quality monitoring; and (iii) even a short-term study (1-month duration) focused on the critical season (high water temperature, low flow) can be sufficient for reaching the study goals. Much effort has gone into developing and testing the robust design and the statistical analyses used were able to determine the significant role of stream section (for both mussels and their environment). A simple method for removing the effect of temperature was included when the effects of other water quality indices were investigated. Only if these approaches were applied was it possible to provide key data for conservation practitioners and stakeholders in the Malše River.

In situ testing in the Malše River demonstrates the adverse development of young mussels exposed to multiple-stress effects of water pollution. The effects of multiple point-source pollution have been investigated in a few recent studies (Patnode et al., 2015; Rogers et al., 2018). In the present study, persistent water pollution and episodic changes in organic loads were recorded within areas designed for endangered species protection and intended to restrict the intensity of sewage water management (NCA CR, 2013). Poor control and the failure of sewage treatment plants can lead to contamination of surface waters and groundwaters (Akaishi et al., 2007; Cuttelod, Seddon & Neubert, 2011). If pollution prevention controls become more effective, point-source pollution is likely to have a decreasing influence on water and sediment quality (Newton, 2003) and vice versa.

These findings could result in the designation of a pollution control programme for mussel conservation (Nakamura et al., 2021), e.g. setting unique ecological thresholds for translation into enforceable water quality regulations (Ferreira-Rodríguez et al., 2019). However, this can be complicated because water quality attributes depend on changing environmental parameters and often act synergistically, making the delineation of single threshold values inappropriate (Boon et al., 2019). In addition, specific levels found in guidance standards only help in target setting (BSI, 2017). Regarding the setting of local protective criteria, the present study indicates that target values for the long-term occurrence of mussel populations should be differentiated based on site-specific conditions (Simon & Dort, 2014). Nevertheless, rigid threshold values are not sufficient for predicting mussel development (Wagner et al., 2018).

The new experimental approach used may provide a useful tool for assessing the effect of multiple point-source (episodic) pollution on the receiving environment and improving water quality management for FPM streams. This may also be applicable for other mussel species with broad potential uses in freshwater mussel action plans, information campaigns and stream biotope conservation. Future studies should focus on long-term exposure to assess the full impact of contamination (Belamy et al., 2020), and ex situ and in situ exposure can be combined to improve water quality testing for the conservation of aquatic species (ASTM, 2013a; Patnode et al., 2015; Pollard et al., 2017; Wagner et al., 2018). Caged experiments could be performed simultaneously with bivalve surveys (Nobles & Zhang, 2015; Patnode et al., 2015) to compare field-deployed mussel results with wild populations. These data will be increasingly important for the protection of existing populations and for the success of active conservation measures such as planning the release of captive-bred mussels.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Vojtěch Barák: conceptualization (equal); data curation (lead); formal analysis (supporting); funding acquisition (lead); investigation (equal); methodology (supporting); project administration (lead); resources (equal); visualization (equal); writing – original draft preparation (supporting); writing – review and editing (equal). *Michal Bílý*: methodology (supporting); resources (equal); writing – review and editing (supporting). *Karel Douda*: conceptualization (equal); formal analysis (lead); methodology (lead); supervision (lead); validation (lead); visualization (equal); writing – original draft preparation (lead); writing – review and editing (equal). *Václava Jahelková*: funding acquisition (supporting); investigation (equal); resources (equal); writing – review and editing (supporting). *Ondřej Simon*: funding acquisition (supporting); methodology (supporting); resources (equal); writing – review and editing (supporting).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are partially available in the supplementary material of this article and fully available from the corresponding author upon reasonable request.

ORCID

Karel Douda https://orcid.org/0000-0002-7778-5147

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

ČLÁNEK 4: Hodnocení přímého antropogenního vlivu na jedince perlorodky říční (Barák et al., 2022) – „přijatý k uveřejnění" (J_{imp})

Visitor pressure in protected areas: Interactions between river tourists and freshwater pearl mussels in the Central European national park Šumava

Vojtěch Barák, Christoph Hauer, Ondřej Simon, Peter Flödl

Podíl autora: Koncept článku jsem dával dohromady s pomocí kolegů z BOKU Vídeň (Ch. Hauer, P. Flödl). Na metodické části článku jsem spolupracoval s Ondřejem Simonem. Provedl jsem sběr dat a také se významně podílel na jejich zpracování a vizualizaci, stejně jako na psaní rukopisu včetně revizí.

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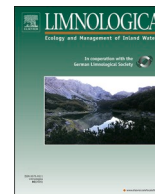
Vojtěch Barák [a,*], Christoph Hauer [b], Ondřej Simon [c], Peter Flödl [b]

[a] *Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Praha Suchdol, Czech Republic*
[b] *CD-Laboratory for Sediment Research and Management, Institute of Hydraulic Engineering and River Research, Department of Water, Atmosphere and Environment, University of Natural Resources and Life Sciences Vienna, Muthgasse 107, 1190 Vienna, Austria*
[c] *T.G.Masaryk Water Research Institute, Podbabská 30, 160 00 Praha 6, Czech Republic*

ABSTRACT

The freshwater pearl mussel *Margaritifera margaritifera* (FPM) is an endangered bivalve species occurring in oligotrophic rivers. FPMs can be found in the Upper Vltava River in the Šumava National Park (Czech Republic, Central Europe), where suitable conditions exist for both adult and juvenile mussels. Non-consumptive human leisure activities are known to negatively affect wildlife in fragile aquatic ecosystems, and in the Vltava River boating tourism in particular may be a threat to local mussel populations. This study focused on an assessment of interactions between river tourists and the FPM, using "pseudo-mussels" in both field and lab experiments. In the field, fake concrete mussels were exposed at three rest sites for river tourists, and in the lab experiment glued shells were placed at the water-sediment interface in an aquarium tested for mechanical impacts of paddles (dislodgement and crushing). Interactions of river tourists with fake mussels were most frequent at low water levels (10–20 cm), and within 2 m from the nearest river bank. Mussel visibility and the presence/absence of a guide played an important role in people's reactions, but site-specific effects were also found. Unintentional interactions (60–69%) were mostly observed at less-attractive sites (with a limited area of shallow water where people spent most time outside the river channel), whereas visual and manipulative interactions (76%) dominated at a more-attractive site (the confluence of two rivers where people move across and inspect the river channel). Crash tests revealed that 8.03 ± 1.37 (mean$\pm$SD) and 7.88 ± 1.13 (mean$\pm$SD) hits by paddles were needed for dislodgment and crushing, respectively. Those findings indicate that the direct effects of recreational boating might be less detrimental than those of accompanying activities (such as wading, bathing, and swimming). The role of trampling (and handling) disturbances should be investigated in more detail to help conserve FPM populations in protected areas.

1. Introduction

Aquatic ecosystems are one of the most endangered ecosystems worldwide (Malmqvist and Rundle, 2002). The freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) (FPM) is particularly affected by anthropogenic negative influences due to its complex life cycle (including host fish species) and habitat requirements (Bauer, 1988; Clements et al., 2018; Geist, 2010). FPMs require slightly acidic river waters, which are often found in catchments where granite and gneiss are predominant (Bauer, 1988; Geist, 2010; Hauer, 2015). In Central Europe, these geological preconditions are mainly present in the Bohemian Massif, which extends across the Czech Republic, Germany

and Austria (Flödl and Hauer, 2019; Hauer, 2015). The greatest problems facing the FPM and its habitat include eutrophication, excessive sediment input, and the absence of fish hosts (Bauer, 1988; Denic and Geist, 2015; Flödl and Hauer, 2019; Geist, 2010; Geist and Auerswald, 2007; Hauer, 2015; Hoess and Geist, 2020). One issue that has received less research attention is recreational inland navigation and boating. Industrialized inland navigation poses a serious threat to aquatic ecosystems due to the spread of invasive species (Boltovskoy et al., 2006; Drake et al., 2007), the action of waves (Fleit et al., 2021; Gabel et al., 2017), and chemical pollution (Dafforn et al., 2011; French McCay et al., 2004). However, boating tourism has also been associated with environmental problems in water bodies and their surroundings (Graham

and Cooke, 2008; Křenová, 2018; Polat et al., 2016).

Outdoor leisure activities are common and widespread, and participation in those activities has been growing (Knight and Gutzwiller, 1995; Miller et al., 2001). Non-consumptive types of recreation are rapidly increasing, and hiking, mountain biking and recreational boating have all become popular forms of outdoor activities (Blanc et al., 2006; Graham and Cooke, 2008; Taylor and Knight, 2003). Although such "quiet" recreation can be considered an essential element of local economies (Blanc et al., 2006) and even a benign use of natural areas (Reed and Merenlender, 2008), interference with wildlife makes such recreation a potential source of disturbance (Blanc et al., 2006).

Reserve networks around the world attempt to provide recreational use while conserving wildlife (Hardiman and Burgin, 2011; Reed and Merenlender, 2008). Most studies have focused on interactions between non-consumptive leisure activities and both aquatic and terrestrial birds or mammals (for recent review, see Dertien et al., 2021). Visitors of protected areas with high-profile sites (such as national parks, which are typically extremely popular; McGinlay et al., 2020) are unaware of the majority of species and consequently of their current status of endangerment (Behrens et al., 2009), and difficult to observe and/or unknown species may be much more threatened by visitors. Examples include the endangered rock partridge *Alectoris graeca saxatilis* in the Alpine Hohe Tauern National Park (Bednar-Friedl et al., 2012; Behrens et al., 2009), and the capercaillie *Tetrao urogallus* in Central European national parks (Šumava and Bavarian Forest National Parks; Rösner et al., 2014).

Several authors have been interested in the impacts of leisure activities on aquatic ecosystems, as assessing their components in protected areas is critical for sustaining both ecosystem health and recreation (Cao et al., 2016; Hardiman and Burgin, 2011; Sordello et al., 2020). For instance, bathers in Peñalara Lake (Natural Park of Peñalara, Spain) were found to cause resuspension of the sediment, changing (micro-)habitat conditions (Toro and Granados, 2002). Deposited eggs of fairy shrimps (*Chirocephalus marchesonii* and *C. sibyllae*) endemic to the Pilato and Palazzo Borghese Lakes (Sibillini Mountains National Park, Italy) are exposed to the pressure of trampling, pushing them deeper into the sediments (Carosi et al., 2022, 2021). Endangered benthic diatomic species have also been recorded in those high-elevation aquatic habitats (Padula et al., 2021). In comparison, running waters have been relatively neglected and only a few studies have focused on recreational activities within river environments; e.g., the effects of boating on the filtration activity of mussels (Lorenz and Pusch, 2012; Lorenz et al., 2013).

The Šumava National Park (Czech Republic, Central Europe) (ŠNP) was established in 1991 to protect a forested mountain range with various aquatic ecosystems (rivers, streams, springs, peatlands and glacial lakes) over an area of 68.500 ha (Křenová, 2018; Křenová and Kiener, 2012; Křenová and Kindlmann, 2015). The ŠNP together with the adjacent Bavarian Forest National Park (24.300 ha) in Germany form the largest wilderness area in the Central European cultural landscape (Křenová and Vrba, 2014). Public access in the core zone of the ŠNP is restricted in order to promote species conservation, mainly those sensitive to human-induced disturbance; e.g., the capercaillie *Tetrao urogallus*, the Eurasian lynx *Lynx lynx* and the FPM (Křenová, 2018; Křenová and Kiener, 2012; Křenová and Kindlmann, 2015). Although the previously numerous FPM population in the Upper Vltava River has been largely reduced due to water pollution and water reservoir construction (Simon et al., 2015), suitable conditions for adult and juvenile FPMs (e.g., water chemistry, oxygen saturation and food supply (detritus from water macrophytes)) have been found there (Bílý et al., 2021; Černá et al., 2018; Matasová et al., 2013; NCA CR, 2013).

Despite being situated within a restricted protected area, the ŠNP mussel population is exposed to recreational boating tourism (Křenová, 2018; Křenová and Kindlmann, 2015; NCA CR, 2013). Boating in the Upper Vltava River has been permitted since 1993, and it is the only way to enter into the most protected areas of the ŠNP (Nykles, 2014). The intensity of boating rapidly increased and resulted in damage to water macrophytes during low-flow conditions (Zelenková, 2008). While the risk of direct contact between humans and mussels was also likely high (Lorenz et al., 2013; NCA CR, 2013), this was not investigated. However, starting in 2004 boating became more regulated (allowed when a minimum water level is exceeded; Simon and Kladivová, 2006), and in 2009 additional rules were established; e.g., restrictions in the number of boats and registration with a user fee required (Diviš, 2009). Since 2012, almost all boats must be accompanied by a guide. The level of boating has thus gradually been reduced: whereas more than 12.000 boats were recorded in 2005 and 2006, this was down to 4.000 boats in 2009. Since then, it has ranged from 2.000 to 4.000 boats per year, mainly canoes with two passengers (Křenová, 2018). While such severe reductions in visitor numbers can lead to higher-quality tourism (Bednar-Friedl et al., 2012), there is still a lack of knowledge on the direct effects of regulated recreational boating on the FPM. Boating is assumed to be accompanied by other activities, such as wading and river bed disturbance (Cole and Landres, 1995). Studies have demonstrated the negative effects of "trampling" on mussel beds (Calcagno et al., 2012) as well as handling of individual mussels (Ohlman and Pegg, 2020), and FPM shells can be susceptible to damage from mechanical impact (Bílek, 2013). However, the responses of freshwater mussel populations to these anthropogenic disturbances have been poorly studied (Ziuganov et al., 2000).

The present study was therefore focused on the following research questions: (i) could interactions between river tourists and FPMs be related to the abiotic conditions; e.g., water depth, substrate and flow conditions; (ii) what is the typical behavior (and reasons) of tourists as a result of interactions; and (iii) how many hits by paddles are needed for a FPM to be mechanically disturbed/damaged? Our aim was to provide data that could be used to improve management related to conservation of the FPM in the ŠNP.

2. Study area

The Upper Vltava River flows through the ŠNP, and is a unique river floodplain system in Central Europe (see Fig. 1). It is a mountain meandering river with high water quality, a natural bed with dense macrophyte cover and regular floods, and is surrounded by numerous peatbogs, oxbow lakes, wet meadows and forest communities (Bílý et al., 2021; Černá et al., 2018; Křenová, 2018; NCA CR, 2013). The Upper Vltava River is a part of several protected areas: the UNESCO Biosphere Reserve "Šumava", Ramsar Convention Site "Šumava peatlands", and European Union's Natura 2000 Site "Šumava" (for the protection of endangered FPM and habitat type 3260 (Water courses of plain to montane levels with the *Ranunculion fluitantis* and *Callitricho-Batrachion* vegetation); Křenová, 2018; Křenová and Kindlmann, 2015). The floor of the river valley slopes gently from 760 to 720 m a. s. l. over about 15 km distance, but with a much longer meandering river channel (about 30 km), and the floodplain is relatively wide (about 1.5 km) (Černá et al., 2018; Křenová, 2018).

The oligotrophic river with high-level water chemistry, oxygen-rich river-bed substrate and presence of water macrophytes as a food source (Bílý et al., 2021; Černá et al., 2018; NCA CR, 2013) provides suitable habitats for sensitive mussel species, and FPMs have been confirmed within a 30-km long river stretch (Lenora-Nová Pec) (Černá et al., 2018; NCA CR, 2013) although the population is sparse (Matasová et al., 2013; NCA CR, 2013). The population size has been estimated at up to several hundreds of adults and several tens of subadults (captive-bred individuals that were released in 1998) (Matasová et al., 2013; NCA CR, 2013; Simon et al., 2015), along with other single individuals that have been recently observed (J. Horáčková, pers. communication).

The studied stretch of the Vltava River is situated between S. Most (48°54'27"N, 13°49'38"E; river km 389.8) and Pěkná (48°51'7"N, 13°55'14"E; river km 373.5). The width of the river channel ranges from 6 to 15 m (in river meanders up to 30 m) during mean flow conditions (Bílý et al., 2021; Simon and Kladivová, 2006). Mean annual values of water depth and flow at the gauging station "Chlum" (river km 377.6)
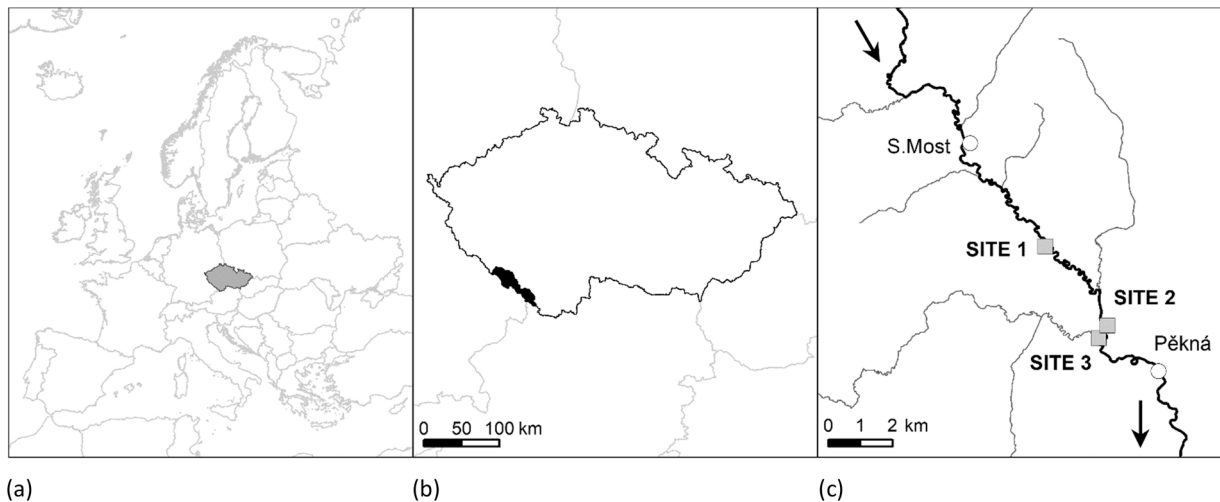
Fig. 1. Location of the study area: (a) Czech Republic; (b) Šumava National Park; (c) Upper Vltava River (the river stretch with the most strict rules for boating; entrance/exit site – white circle, rest site – grey square).

are 72 cm and 5.8 m^3.s^{-1}, respectively (CHMI, 2022). The river bed composition varies from sand, gravel to stones (Bílý et al., 2021). This river stretch covers the main area with the occurrence of FPMs, and therefore boating has been permitted only under very strict conditions. Rules for boating include allowed days, times and types of boats (canoes and kayaks), a minimum water level, a limited number of boats per hour, and a guide mandatory for groups. A registration charge is also required (Bílek, 2013) (500–600 CZK (20–24 EUR) per boat in 2022), with boat registrations on the river controlled by park rangers. Rafts and larger boats are no longer allowed (Křenová, 2018). According to the international scale of river difficulty in boating, the river stretch can be classified as "Easy" (Class I) to "Novice" (Class II). There are three rest sites where people are allowed to get out of their boats: "Dobrá" (Site 1, river km 383.2), "Chlum" (Site 2, river km 377.6), "Soutok" (Site 3, river km 376.7) (see Fig. 1c; Bílek, 2013).

3. Material and methods

3.1. Field study

The interactions and impacts of river tourists on FPMs were investigated at the rest sites described above. Due to the fact that the FPM is a critically endangered species in the Czech Republic (Simon et al., 2015), fake mussels had to be used for that testing. These were made from fine-grained grey concrete to mimic the actual shape of FPM shells, and placed along the river bank, mostly in five groups (with 5–6 individuals per group; see Fig. 2) to represent variable abiotic conditions (depth, river-bed substrate, distance from the river bank and local flow

conditions). It was assumed that mussels occurring in colonies would be exposed to an increased susceptibility to disturbance (Blanc et al., 2006). Based on pilot testing in June 2015, several parameters were distinguished for groups of fake concrete mussels during the main experiment period (June-September 2016) (see Table 1), adjusted to site-specific conditions. Placement of the fake mussels simulated the real conditions for FPMs based on mussel requirements (cf. Degerman et al., 2009; Geist, 2010; Skinner et al., 2003) as well as local knowledge from the Upper Vltava River.

Exposures of fake mussels were performed at each site from the morning to the early evening on 19 separate days (3 in 2015, 16 in 2016). Two researchers were positioned on the river bank during testing and recorded the behavior of passing people ("reactions"). No distinct effect of the researchers' presence on tourists' behavior was observed in 2015, thus the study conditions did not change in 2016. Three types of reactions were distinguished: (1) *unintentional* (people stepped on mussels and/or hit them by boats or paddles), (2) *intentional visual* (people observed mussels and discussed them with each other or with the guide), (3) *intentional manipulative* (people took mussels out of the water and put them back, threw them away, brought them to the researchers or even stole them). All reactions were noted for each particular group of fake mussels (see Table 1).

3.2. Lab experiment

During pilot phase of the field study, it was confirmed that people did hit the fake concrete mussels with paddles. Therefore, a supplementary lab experiment was designed to isolate the effects of this human-induced disturbance (see Ohlman and Pegg, 2020) and quantify that effect

Fig. 2. A group of fake mussels during a field exposure.

Table 1
Basic characteristics for groups of fake mussels during a field exposure. (Depth category: A 10–20 cm, B 20–50 cm, C 50–80 cm. The visibility assessment was based on the effects of water depth, substrate, local flow conditions and sunshine.).

	Group 1	Group 2	Group 3	Group 4	Group 5
Depth	Low (A)	Low (A)	Middle (B)	Middle (B)	High (C)
Substrate	Sand	Sand	Gravel (Sand)	Gravel	Stones (Gravel)
Visibility	Very good	Good	Very good	Good	Bad
Distance from the bank (m)	0–2	0–2	2–4	2–4	4–6

("crash tests"). In August 2015, eight models were made from empty FPM shells and cotton stuffing to simulate mussel tissue. Previous studies have used similar "sham" mussels (shells filled with sand or agar and bonded together with glue or a non-toxic silicone) to examine the effects of freshwater mussels (specimens and their shells) on U.S. and Australian stream benthic and fish communities (e.g., Hopper et al., 2019; McCasker and Humphries, 2021; Spooner and Vaughn, 2006; Spooner et al., 2013). In the present study, most cracks on the shell surface were fixed with glue, and then all parts of the models joined together (with some gaps left for the entry of water into the model body to simulate live mussels) (see Fig. 3a). For the experiments, a 425-liter aquarium with a substrate layer (wet-sieved sand with gravel, grain size >0.2 mm, thickness ca 20 cm) and water column (tap water, height ca 40 cm) was used. The risk of contact between a paddle and the river-bed substrate had been observed in the field to increase with lower water columns, with the highest level of disturbance at depths of 35–45 cm (boating in shallower sites is almost impossible). Mussel models were deployed at the water-sediment interface according to photos of real mussels living within the Upper Vltava River (see Fig. 3b). The paddle motion used for crash-tests was simulated from video records of real tourists during boating along the river (in July 2015).

The main direct effects of human disturbances on aquatic organisms in heavily visited areas include the dislodgement and crushing of individuals (Brosnan and Crumrine, 1994). Therefore, the first part of experiment was focused on knocking a FPM model out of the substrate. Each FPM model was tested alone in the aquarium (with four replicate series of paddling attempts). After that, actual crash tests with the same models were performed. After each hit, it was necessary to return the FPM model to the initial position (paddle hits were repeatedly aimed at the upper part of the "emerged mussel", but its orientation in the soft substrate changed during the experiment). The experiment was ended when at least one shell of each model was partially broken.

3.3. Statistical analysis

Numbers of interactions between people and fake concrete mussels (yes-no) among categories of abiotic conditions (depth, substrate and visibility) were compared using Pearson's chi-squared test after checking the test requirements (i.e., expected frequencies higher than five). The same test was used for comparisons of numbers of unintentional and intentional reactions among sites (with visual and manipulative types joined together to meet the aforementioned requirements). Mean numbers of hits by paddles for dislodgement and crushing of each mussel model (crash-tests) were compared using a paired t-test after checking the test requirements (i.e., normality of data (by Shapiro-Wilk test)). All analyses were performed in R version 3.6.0 (R Core Team, 2020).

4. Results

4.1. Field study

During 19 observation days, 1079 people getting out of boats and 165 reactions to exposed fake concrete mussels were recorded at three rest sites between S. Most and Pěkná in the Upper Vltava River. Most people stopped at Site 1 (the first rest site in the studied river stretch), but almost 60% of all reactions were found at Site 3 (a more-attractive site at the confluence of the Studená and Teplá Vltava Rivers; see Table 2).

4.1.1. Abiotic conditions

Interactions of tourists with fake mussels were most frequent for those mussels exposed at the shallowest depth (A; 11.6% of potential interactions). For groups installed at the intermediate depth, the interaction rate decreased (B: 6.5%), and was lowest for groups at the highest depth (C: 0.6 %; see Fig. 4a). Differences in interactions among depth categories were found to be significant ($p < 0.001$).

Based on the prevalent river-bed grain size at experimental sites (according to the local investigation), tourists had the highest level of interaction with fake mussels placed in sandy and stony bottoms (both 6.1 % of potential interactions). When mussels were placed in gravel, the interaction rate dropped by half (3.3 %; see Fig. 4b). However, differences in interactions among substrate types were non-significant ($p > 0.05$).

According to the visibility of fake mussels, interactions of tourists were most numerous when mussel visibility was very good (13.7 % of potential interactions). Frequencies of interactions when visibility was poorer decreased, but was similar for both "good" and "bad" visibility (6.2 % and 5.8 %, respectively; see Fig. 4c). Differences in interactions among categories of visibility were found to be significant ($p < 0.001$).

4.1.2. Site-specific reaction types

The most frequent tourist reactions to fake mussels were unintentional (60 %) at Site 1, where people mostly stepped into mussel groups. Intentional visual reactions were less numerous (33 %; people mostly observed mussels and discussed them with each other). Manipulative reactions were the least frequent (7 %; see Fig. 5a); however, two

Table 2
Number of observation days, people getting out of boats and their reactions to fake mussels at three rest sites in the Upper Vltava River (river stretch S. Most-Pěkná).

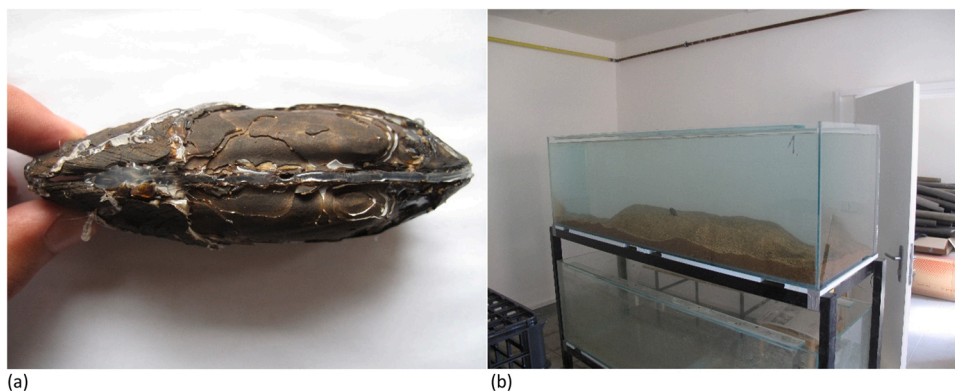|	Site 1	Site 2	Site 3	SUM
Days	8 (42.1 %)	5 (26.3 %)	6 (31.6 %)	19
People	481 (44.6 %)	161 (14.9 %)	437 (40.5 %)	1079
Reactions	57 (34.5 %)	13 (7.9 %)	95 (57.6 %)	165

Fig. 3. (a) Mussel model (close-up), and (b) deployment in the aquarium for the lab experiment.
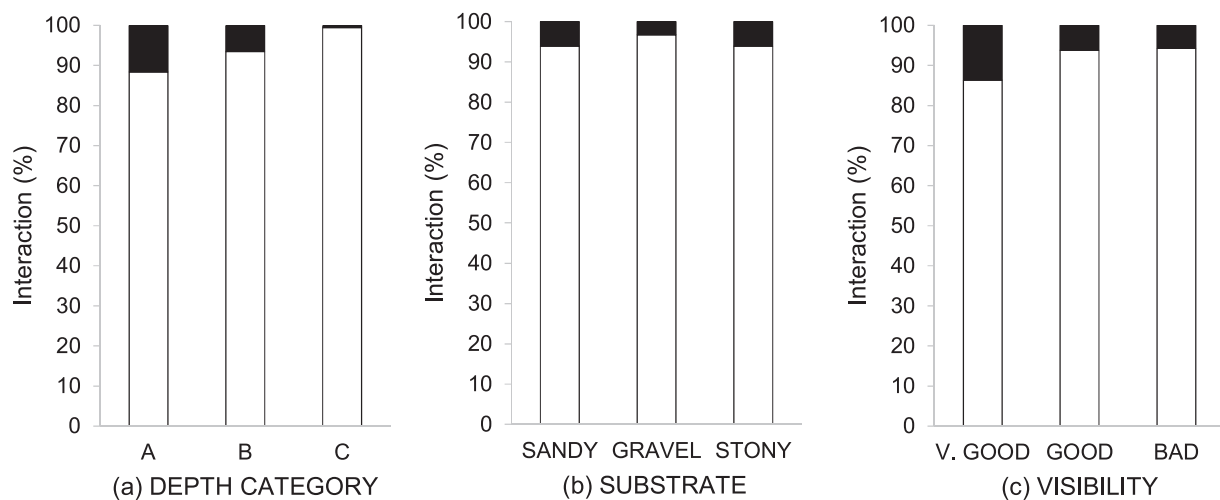
Fig. 4. Interactions of river tourists and FPMs (yes – black, no – white): (a) Effect of water depth ("A": N = 862, "B": N = 949, "C": N = 530), (b) river-bed substrate (sandy: N = 587, gravel: N = 544, stony: N = 621), and (c) visibility (very good: N = 445, good: N = 801, bad: N = 928) during the field study with fake mussels.
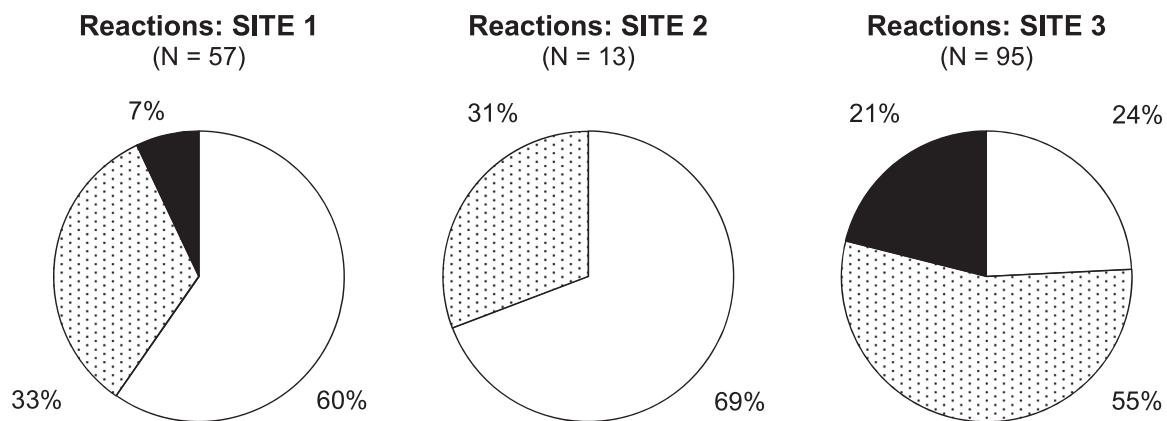

Fig. 5. Site-specific types of reactions (*unintentional* – white, *intentional visual* – dotted, *intentional manipulative* – black) during the field study with fake mussels.

tourists stole a few fake mussels despite a guide being present. Although not many reactions were observed at Site 2, their distribution was similar to at Site 1, being dominantly unintentional (almost 70 %; people impacted fake mussels by trampling or with paddles) and visual reactions (people only observed mussels) reaching 30 % frequency (see Fig. 5b). No manipulative interactions were recorded. The most frequent tourist reactions were observed at Site 3. Visual reactions represent the most common type (55 %), and people mostly spoke about their findings with each other or with a guide. Unintentional reactions were less frequent (24 %, with the same characteristics as at Site 2). As for manipulative reactions (21 %; see Fig. 5c), people usually took mussels out of the water and then put them back. But one unique event was recorded in July 2016 when a large guided group of tourists found the fake concrete mussels. Assuming that the mussels were alive, people brought them to the researchers and wanted to open them for pearls! Differences in reactions among the experimental sites were found to be significant (p < 0.001).

4.2. Lab experiment

The lab experiment using FPM models revealed that mussels were knocked out of the sediment after 8.03 ± 1.37 (mean±SD) paddle hits. Actual crash tests showed that mussel shell damage (see Fig. 6a, b) was caused by 7.88 ± 1.13 (mean±SD) hits (see Fig. 6c). Differences in hit frequencies for mussel disturbance among both tests were found to be non-significant (p > 0.05).

5. Discussion

Within the ŠNP area in the Czech Republic, navigating the Upper Vltava River in a small boat (canoe and kayak) is an important and popular tourist attraction. However, recreational boating can have negative impacts on the aquatic ecosystem. This study focused on a field assessment of interactions between river tourists and fake FPMs under variable abiotic and site-specific conditions. Moreover, the effects of mechanical impacts on FPMs were investigated in a lab experiment using mussel shells.

5.1. Visitor pressure and the role of abiotic conditions

The effects of recreational boating on FPMs in the ŠNP area were characterized by the placement of fake mussels in both shallow and deep sites along the river banks. River tourists mostly affected fake mussels in very shallow locations with depths of 10–20 cm. Several authors have stated that the FPM shows a habitat preference for "shallow" running waters (e.g., Degerman et al., 2009; Moorkens and Killeen, 2014; Outeiro et al., 2008); however, the term *shallow* is not precisely defined. An average depth of 15–18 cm was found in an Irish river with sustainable FPM recruitment (Moorkens and Killeen, 2014). River tourists in the Upper Vltava River were observed to move across shallow locations (<0.5 m) at rest sites to get out of their boats and spend a short period of time (a few minutes) in the river channel. Thus, both adult and younger specimens may be threatened by recreational boating and
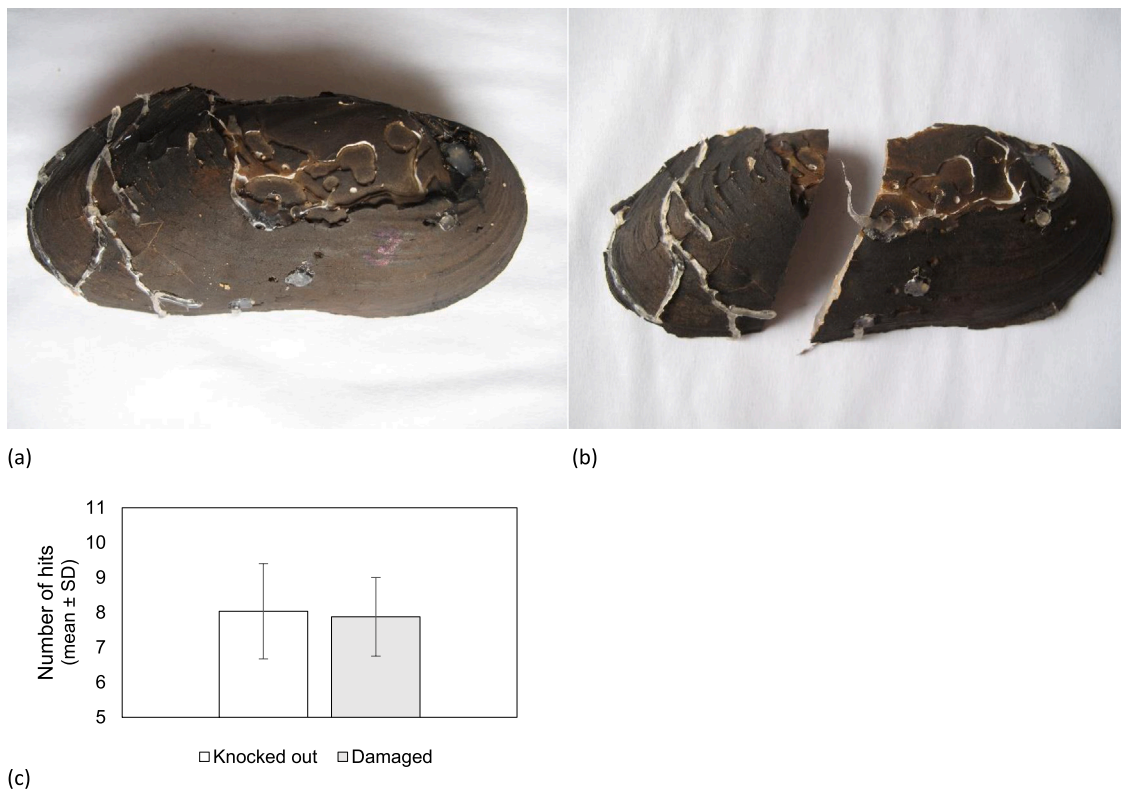
(a)

(b)

(c)

Fig. 6. A mussel model (a) before and (b) after a crash test; (c) number of paddle hits needed for a mechanical effect to be seen on mussel models (knocked out of the sediment/shell damaged).

accompanying activities. Nevertheless, FPMs have also been found in deeper locations across Europe (e.g., Hastie et al., 2000; Ostrovsky and Popov, 2011; Varandas et al., 2013), and Degerman et al. (2009) stated that Scandinavian mussel populations occurred at various depths up to 5–6 m. Visitors often use only areas for bathing where the depth does not exceed 2 m (Escarpinati et al., 2011), but wading is more widespread in the ŠNP area. In the present study, the interaction rate between river tourists and FPMs decreased with increasing water level, therefore mussels occurring at deeper sites (>0.5 m) should be less likely to be disturbed.

At the rest sites along the Upper Vltava River investigated here, the water depth increased with distance from the river banks. The most affected fake mussels were located in the 2-m wide strip close to the edge of the river where people stopped and their boats accumulated. Thus, locations with the most numerous interactions between river tourists and FPMs could be described not only by shallow water but also by closeness to the riparian zone. FPMs are generally found within 4 m of the nearest bank, as confirmed by several mussel surveys (e.g., Hastie et al., 2000; Outeiro et al., 2008; Sousa et al., 2015), which makes mussels vulnerable to visitor disturbance. Few FPMs were observed in the middle of the river channels in Portugal (Sousa et al., 2015, 2013), although those in large populations can spread out across the river bed and colonize open areas (Varandas et al., 2013). Interactions of wading tourists with mussels in small rivers and streams with narrow channels could be much more frequent; however, it can be assumed that such watercourses are not generally used for boating. Nevertheless, habitat quality for the FPM depends strongly on river bottom characteristics (Geist and Auerswald, 2007).

Results from the field study indicate that river tourists mostly affected fake mussels located in both sandy and stony substrates. The FPM requires a stable river-bed substrate, usually made from sand, gravel and small stones (Degerman et al., 2009; Moorkens and Killeen, 2014; Ostrovsky and Popov, 2011; Outeiro et al., 2008) where adults and juveniles can burrow. Mussels were found to be lacking in large

areas of sandy sediments in the Waldaist River (Austria) (Jung et al., 2013), but large FPM populations in Russia can inhabit those habitats due to the slow current velocity and specific river morphology reducing flood forces (Ostrovsky and Popov, 2011). In the ŠNP area, sand was the preferred type of river-bed substrate for people to get out of their boats, explaining why most interactions of visitors with fake mussels were recorded at such sites. Surprisingly, FPMs located among larger stones were also exposed to visitor disturbances, but mussel visibility and visitors' reactions played important roles in this case (see Section 5.2). Larger sized material can improve the stability of sand and gravel in suitable FPM locations (Geist and Auerswald, 2007; Hastie et al., 2000; Hauer, 2015; Jung et al., 2013). Sandy (or gravel) patches among stones were also recorded at the rest sites in the Upper Vltava River. On the contrary, unstable silted substrates are a poor habitat for the FPM (Boon et al., 2019). Rare silty locations in the Upper Vltava River were found to be not suitable for placing fake mussels, and were excluded from the field study.

5.2. Visitor pressure and the behavior of people

Despite being regulated, boating in the ŠNP area can still potentially have negative impacts on the aquatic environment including mussels, fish, and their habitats (Křenová and Kindlmann, 2015; Simon and Kladivová, 2006). The consequences of underwater noise pollution have already been documented for freshwater fish (e.g., changes in behavior and physiology) (Butler and Maruska, 2020; Graham and Cooke, 2008; Wysocki et al., 2006), but other animal groups have received far less attention (Sordello et al., 2020). No studies exist on the effects of paddle noise on mussels, but it may be expected that both acoustic and mechanical visitor-induced disturbances affect aquatic biota (see below and Section 5.3) in the Upper Vltava River. However, assessing the response of endangered species is difficult because in situ disturbance should be avoided, and the capture of individuals for ex situ testing is often restricted (Thiel et al., 2008). Thus, instead of directly

investigating the responses of the FPM to visitor pressure, the reactions of people to endangered mussel species were assessed using fake FPMs. Interactions between river tourists and FPMs in the Upper Vltava River were found to be dependent on the visibility of fake mussel groups. Very good visibility, related with a shallow water depth and light-colored substrate (contrasting with dark mussels), induced higher numbers of visitor reactions. However, the type of reaction played an important role. Whereas visual and related manipulative reactions (i.e., handling) were more frequent for well-visible mussels, the numbers of unintentional reactions (mainly trampling within mussel groups) increased with worsening visibility.

Traditional pearl hunting including the process of handling was a major problem for the FPM populations. Although currently the mussels are protected and pearl hunting prohibited (Degerman et al., 2009; NCA CR, 2013; Skinner et al., 2003), and manipulating FPMs during any phase of their life cycle is prohibited by law (Simon et al., 2015), illegal pearl hunters still examine mussels and specimens can be killed using destructive methods (Bauer, 1988). In the present study, some of the river tourists, who were apparently not able to differentiate the fake animals from real FPMs, acted like illegal pearl hunters when attempted to open mussel "shells".

Visitors of shallow river areas generally come into contact with the river bed: trampling can cause the death of some organisms (Escarpinati et al., 2014, 2011), and should be minimized in rivers and streams carrying FPM populations (Skinner et al., 2003). Visitor movement across the river channel is forbidden in the core zone of the ŠNP, but wading, bathing, and swimming tourists were observed at the rest sites, despite a guide being present. Such activities may lead to a prolonged time spent in the river channel, and consequently an increased risk of contact (both trampling and handling) between humans and FPMs. Juvenile mussels, which have more fragile shells, may be more protected because they live within the river bed, but some individuals move up to the water-sediment interface as a result of adverse oxygen conditions (Bílý et al., 2021). In such cases, young mussels would be potentially exposed to trampling by tourists. Trampling can also have an indirect negative consequences for FPM populations; e.g., through river-bed compaction resulting in low exchange between the water column and the bottom substrate (Boon et al., 2019; Geist and Auerswald, 2007), or the resuspension of fine material with adverse effects on mussel filter-feeding and oxygenation (Moorkens and Killeen, 2014).

Site-specific investigation in the Upper Vltava River divided the rest sites into "less-attractive" (Site 1 and Site 2) and "more-attractive" groups (Site 3). At less-attractive sites, people stop and get out of their boats within a limited area of shallow water, resulting in disturbance of the river-bed environment. They also spend most time outside the river channel, so mostly unintentional reactions with fake FPMs were recorded at those sites. On the contrary, at the more-attractive site at the confluence of the Studená and Teplá Vltava Rivers, visitors have larger areas for getting out of boats, and the water depths are very low. People often move across and inspect the river channel, so visual reactions were more frequent at that site. Overall, the typical interactions of visitors with mussels observed in this study can be characterized as non-manipulative.

5.3. Visitor pressure and mechanical impacts

River tourists can also affect mussels while boating along the river, mainly by the action of their paddles. The lab experiments performed here to quantify human mechanical impacts showed that mussel models were knocked out of the sediment after mean number of eight paddle hits. It must be stated that the relatively unstable substrate used during lab testing may have contributed to a higher risk of dislodgement. Behavioral responses of four freshwater mussel species to their removal from the substrate were investigated by Waller et al. (1999), who described three locomotor behaviors: righting (realignment to a vertical position), horizontal movement, and burrowing into the substrate.

While FPMs can also re-bury themselves after dislodgement (Skinner et al., 2003), this process can induce stress, especially if there are repeated hits by paddles and changes in mussel positions.

In addition to the dislodgment, mussels can be also damaged by the paddles of river tourists. The mean number of paddle hits needed to damage FPM models was 7.88 (similar to mussels being knocked out of the substrate). In the lab experiment, the models were replaced to the vertical position after each hit. The movement of real FPMs after such a disturbance has not been documented in greater detail, though in the experiment mentioned above Waller et al. (1999) found that many mussels did not right themselves during 168 h. Ziuganov et al. (2000) investigated mutilations including shell damage (a cracked right shell) and their effects on individual FPM survival using experimental animals placed in the River Varzuga (Russia) and checked each third day. Half of 30 mussels died during the first 9 days, and the remaining specimens were dead between "day 9″ and "day 15″. Thus, having only a cracked shell and posterior adductor damage led to 100 % mortality, though handling and aerial exposure during check days may have contributed to this mortality. The same authors evaluated FPM regeneration experiments in the Thorma River (Russia) and found that three adults with shells previously damaged had completely repaired shells after two years. The FPM population in the Upper Vltava River is characterized by a decrease in longevity and increase in growth compared to northern populations (NCA CR, 2013), and may suffer from insufficient shell regeneration (Ziuganov et al., 2000). Moreover, the mechanical impacts caused by river tourists and their paddles may be repeated and also intensified by trampling during wading (and bathing).

The effects of recreational boating on river environments can also be more detrimental due to the altered flow conditions that have become more extreme as a result of climate change (Degerman et al., 2009; Santos et al., 2015; Sousa et al., 2012). Negative interactions between river tourists and FPMs might be higher when mussels are exposed to low flow and decreased water depth, as noticed for other bivalves by Lorenz et al. (2013). Animals would be more vulnerable to hits by paddles, trampling and (potential) handling. However, in the Upper Vltava River low-flow periods could also have positive effects on the FPM population, as the minimum water level used by the ŠNP Administration for permitting boating could help protect local mussel population from visitor-induced disturbances during low flows. Nevertheless, water levels are only checked at the entrance site of the study river stretch, and even under conditions permitting boating many very shallow areas were observed along the river. Moreover, prolonged low-flow conditions may lead to illegal boating without any check on tourists´ behavior. On the contrary, high-flow periods can also lower visitor impacts on the FPM population, as no interactions of river tourists with fake mussels were recorded during highly increased water levels. Extreme floods may greatly affect mussel populations, as FPMs can be damaged/crushed by moving substrates and/or large sediment deposits, or washed out onto riverbanks where they desiccate and die (Álvarez-Claudio et al., 2000; Hastie et al., 2001; Sousa et al., 2012). However, the Upper Vltava River has a relatively wide floodplain that can mitigate the negative impacts of high-flow conditions on mussel populations by reducing flow velocities (also see Hauer et al., 2022, this issue).

5.4. Implications for management

River tourism and regulation can be complicated due to the competing interests of nature conservation authorities, the tourism industry, and the general public. This was also the case in the ŠNP area, with discussions of a ban on recreational boating in the Upper Vltava River contrasting with an "open river" policy without any restrictions (Křenová and Kindlmann, 2015). Long-term negotiations by the ŠNP Administration led to compromise rules acceptable for the Czech Canoe Union, the major boating industry representatives, fishermen, scientists, NGO groups and local authorities. Interactions of river tourists with

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mussels were not studied during this process, though assessing and monitoring visitor behavior is a great challenge for managers of protected areas (McGinlay et al., 2020).

Based on the results of the present study, the effects of recreational boating along the river might be less detrimental than those of accompanying activities, such as wading, bathing, and swimming. Tourists in protected areas should be educated on how their activities affect wildlife and how they can modify their behavior to mitigate pressure on endangered species (Miller et al., 2001). The ŠNP Administration has used a set of tools for visitor education (i.e., guide presentations, an information point at the entrance site, a virtual natural trail (http://vltava.perlorodkaricni.cz/), an official boating webpage (https://splouvani.npsumava.cz/en/) and leaflets). However, our discussions with river tourists revealed them to be in doubt regarding the rules for regulated boating and FPM protection in the Upper Vltava River, especially if they had never had contact with FPMs before.

Simple and harmless method of using fake mussels can provide insights into the interactions of people and endangered mussel species and improve the level of knowledge on visitor behavior. Surprisingly, in the present study in the ŠNP area the reactions of river tourists to fake mussels were less frequent for non-guided groups. However, mostly visual reactions were recorded when guides were present, whereas non-guided visitors more often manipulated mussels. Two rest sites are located close to each other in the Upper Vltava River, and tourists with no guide were observed to stop at Site 2 (a less-attractive site), whereas guided visitors generally stopped downstream at Site 3 (a more-attractive site). This finding could be useful for the management of protected areas, and the establishment of a "buffer site" upstream of the most visited sites would mitigate visitor impacts. Nevertheless, the movement of visitors within the river channel should be strictly checked, as demonstrated by the presence of recently dead adult FPM with broken shells found in the Upper Vltava River in 2015 (Zelenková et al., 2015). If a guide is not present, information and training before recreational activities would likely help to minimize negative impacts (Escarpinati et al., 2014).

A minimum number of interactions between river tourists and mussels was found in locations deeper than 0.5 m, so the negative impacts of visitor behavior might be lower if mussels were moved to deeper sites. However, artificial relocation of adult FPMs has not yet been shown to be effective (Cosgrove and Hastie, 2001; Hastie et al., 2003). Álvarez-Claudio et al. (2000) marked and relocated 124 specimens to conserve a FPM population in the River Narcea (Spain), but none could be found in the next year during re-sampling. Mussel watchers, photographs and scientists using manipulative monitoring methods might also harm some small mussel populations, but the level of such effects remains unknown and needs to be investigated in more extended studies. In any case, manipulating FPMs should be limited to severe threat situations (Skinner et al., 2003), such as moving mussels subject to desiccation (Sousa et al., 2018). No FPMs were found at or close to the present rest sites in the Upper Vltava River, so it is currently not necessary to relocate animals to adjacent deeper river areas. If FPM monitoring in the future would confirm the presence of the species near rest sites, the conflict between river tourism and nature conservation could be resolved by e.g., changes in the locations of rest sites. Protecting adult mussels and the maintenance of juvenile habitat should be key conservation measures for the FPM (Cosgrove and Hastie, 2001), and the non-invasive methods used here could be applied to assess visitor-induced pressure in other protected (and non-protected) areas.

6. Conclusion

The present study clearly showed that fake FPM specimens were mostly affected by river tourists in shallow running waters near the edge of the river, with both fine and coarse river-bed substrates. Based on previous surveys and studies, such habitat could be generally described as "ideal" for the FPM. Therefore, this sensitive bivalve species could be

threatened by river tourism. Fake mussels that were well visible were also frequently disturbed, but the type of reaction (unintentional, visual, and manipulative) varied under site-specific conditions. Lab experiment indicated that several repeated hits by paddles are needed to both dislodge mussels and to damage their shells. Thus, the effects of recreational boating might be less detrimental than those of accompanying activities, such as wading, bathing, and swimming. Human-induced FPM disturbances related with trampling and handling should be investigated in greater detail to support mussel conservation. Relocation actions have not been very effective, so conservation measurements should be focused on the protection and maintenance of mussels and their habitats. Although effective regulation of the daily numbers of river tourists was implemented in the ŠNP area to minimalize disturbances to the FPM population, understanding visitor behavior is critical for keeping the core zone of the national park open to tourist use.

CRediT authorship contribution statement

Vojtěch Barák: Data curation (lead), Formal analysis (lead), Investigation (lead), Methodology (equal), Project Administration (lead), Validation (lead), Visualization (lead), Writing – original draft preparation (equal), Writing – review & editing (equal). **Christoph Hauer:** Conceptualization (equal), Resources (supporting), Supervision (equal), Writing – review & editing (equal). **Ondřej Simon:** Funding acquisition (lead), Investigation (supporting), Methodology (equal), Resources (lead), Supervision (equal), Writing – review & editing (supporting). **Peter Flödl:** Conceptualization (equal), Writing – original draft preparation (equal), Writing – review & editing (supporting).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Authors' contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by VB and OS (analysis by VB). The first draft of the manuscript was written by VB and PF. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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4. Diskuze

ČLÁNEK 1: Hloubková preference juvenilních jedinců perlorodky říční

Pro determinaci hloubkové preference mladých perlorodek byly využity síťové trubičky neboli sondičky, vyplněné říčním pískem o velikosti zrna 1–2 mm. Uvedená frakce již byla zjištěna jako vhodná pro vývoj juvenilů v laboratorních podmínkách (Lavictoire et al., 2016) a využita při sestavení zařízení (pískových klícek) pro terénní pokusy (viz **Bílý et al., 2018 – článek 2**). Při pilotním in situ experimentu v prostředí Teplé Vltavy (**Bílý et al., 2021 – článek 1**) bylo zjištěno, že juvenilové mají tendenci v sondičkách setrvávat vzhledem k nalezitelnosti přesahující 50 % při dlouhodobé (několikaměsíční) expozici a blížící se 70% hranici při krátkodobé (několikatýdenní) expozici. Svoji roli při hodnocení preferované vrstvy substrátu však hrají jak sezónní podmínky prostředí, tak efekt mikrohabitatu.

V letním období se juvenilní perlorodky vyskytovaly převážně v mělké hloubce 2 až 3 cm. Jelikož Teplá Vltava je charakteristická svým dobře prokysličeným dnem (dle dlouhodobých měření na toku) a mladí mlži se nezahrabali hlouběji než 8 cm, lze předpokládat, že na jiných perlorodkových tocích by tento limit nebyl výrazně hlubší. Větší i menší jedinci zástupců velkých mlžů se obvykle zahrabávají v prvních 10 cm hloubky sedimentu (Neves and Widlak, 1987; Balfour and Smock, 1995; Schwalb and Pusch, 2007; Negishi et al., 2011) a juvenilní mlži se většinou nacházejí ve svrchní vrstvě dna (tj. 0–3 cm hluboko) (Yeager et al., 1994; Negishi et al., 2011; Archambault et al., 2014). Význam horních vrstev zóny hyporeálu jako životního prostoru mladých mlžů zmiňují již Buddensiek et al. (1993), ačkoli vycházeli z hodnocení stanovišť na základě statutu populací několika druhů velkých mlžů včetně perlorodky říční. Mělká zóna hyporeálu musí umožnit výměnu vody mezi vodním sloupcem a prostředím říčního dna. Na stanovištích s výskytem mladých perlorodek byla doložena vyšší koncentrace O_2 v prvních 2 až 3 cm hloubky dna oproti hlubším vrstvám, kdy do hloubky 2 cm úroveň prokysličení neklesla pod 8 mg l^{-1} (Buddensiek et al., 1993). Byť Quinlan et al. (2015) upozorňují, že v uvedené práci jsou zmíněny pouze průměrné hodnoty, na Teplé Vltavě byly zjištěny obdobné výsledky, přičemž daná úroveň prokysličení platila ve většině měření dokonce i v hloubce 10 cm!

Při prvním a druhém přezimování byly juvenilní perlorodky nalézány převážně v hloubce 3,0 až 4,5 cm, tedy hlouběji než v letním období. Podobné sezónní chování mladých mlžů zaznamenali již Negishi et al. (2011), zkoumající vertikální/horizontální

migraci jedinců druhu *Pronodularia japanensis* (Lea, 1859), a lze jej vysvětlit teplotou vody, stabilnější a vzdálenější od nuly právě ve větší hloubce. Nízká míra přežití mlžů během první zimy byla patrně způsobena poklesem obsahu O_2 během expozice, jak naznačuje hloubkový gradient teploty mezi mělkou a hlubší vrstvou hyporeálu (tzn. omezení výměny vody) a teplotní oscilace (zejména na konci experimentu). Vzhledem k dobře zachovalému stavu schránek jedinců lze předpokládat úhyn jedinců v období právě před koncem experimentu. Oproti tomu během druhé zimy většina jedinců přežila, a to hlavně díky příznivým a stabilním podmínkám na zkoumaném stanovišti.

Proč však mladé perlorodky preferovaly velice mělkou zónu hyporeálu (oproti předpokladu 10 cm hloubky; Geist and Auerswald, 2007) a přitom se ve větší míře nevyskytovaly při povrchu dna? Jde patrně o příklad kompromisu („trade-off"), kdy při povrchu dna hrozí vyplavení ze substrátu (Yeager et al., 1994), zatímco ve větších hloubkách bývají chudší podmínky prostředí, zejména zhoršené O_2 poměry kvůli vlivu podzemní vody (Hauer and Hill, 2007; Quinlan et al., 2015). Prostředí velmi mělkého říčního dna tedy umožňuje únik jedinců v případě změny abiotických podmínek (viz dále). Takřka identické hloubkové rozložení po 26- a 55-denní letní expozici navíc ukazuje, že když juvenilní perlorodky zaujmou vhodnou pozici v dnovém substrátu, tak v ní setrvávají tak dlouho, dokud to podmínky prostředí dovolují.

Při zohlednění efektu mikrohabitatu lze definovat různé typy prostředí. Stanoviště A se stabilními podmínkami bylo charakteristické vysokou mírou přežití juvenilů a jejich schopností přezimování. Podobný popis platí taktéž pro stanoviště C. Mladé perlorodky prospívaly i na stanovišti D vzhledem k dobrým kyslíkovým poměrům, zprostředkovaným díky výraznému downwellingu (s pohybem vody ve směru seshora dolů; Dahm et al., 2007), jak dokládají minimální rozdíly teploty vody mezi vodním sloupcem a zónou hyporeálu. Navzdory tomu juvenilové vykazovali nižší nalezitelnost s velkou variabilitou mezi sondičkami a větší akumulaci v nejsvrchnější vrstvě dna oproti ostatním stanovištím. Hloubka vody na stanovišti D se pohybovala pouze mezi 0,10 a 0,15 m. Přestože Moorkens and Killeen (2014) uvádějí průměrnou hloubku 0,15–0,18 m na irském toku s výskytem rozmnožující se populace perlorodky říční, nejvyšší hodnoty dosahovaly až 0,4 m. Proto mohlo být stanoviště D příliš mělké na to, aby tam mladí mlži setrvávali a raději jej opouštěli nebo se k úniku chystali. Již dříve bylo zjištěno, že v případě nepříznivých podmínek pro juvenilní vývojová stádia se mlži mohou přesouvat blíže k povrchu dna, vykazovat známky stresového chování nebo dokonce uhynout (Polhill and Dimock, 1996; Sparks and Strayer, 1998; Hruška,

1999). Výrazný podíl jedinců vyskytujících se při povrchu dna byl zaznamenán též na posledním stanovišti (B), avšak zde došlo k absolutnímu úhynu juvenilů. Kvůli mírnému poklesu hladiny během expozice došlo k obnažení dna nad stanovištěm a změně charakteru tamního prostředí na stojatou vodu, typickou svými chudými kyslíkovými poměry (ASTM, 2013). V důsledku toho poklesl obsah O_2 ve dně na 4–6 mg l^{-1} ve 3 cm, resp. 2–3 mg l^{-1} v 10 cm hloubky. (Podobně na tom byl i teplotní režim, kdy rozdíly mezi volnou a vodou a zónou hyporeálu byly největší právě na stanovišti B.) Schopnost rozlišení (ne)vhodných podmínek prostředí u juvenilních perlorodek a jejich vynoření na povrchu nedávno popsali v laboratorním prostředí také Hyvärinen et al. (2021), přičemž jako možnou příčinu vynoření uvádějí jemnozrnný sediment (se sníženým obsahem kyslíku). První podrobnější data o toleranci mladých perlorodek vůči hypoxii přináší studie Hyvärinen et al. (2022): během 10-denního laboratorního experimentu s koncentrací O_2 <1,3 mg l^{-1} nepřežil žádný z testovaných juvenilů. Konstantní teplotní podmínky stejně jako absence dalších stresorů i vrstvy substrátu neumožňují provést podrobnější srovnání s terénními výsledky z Teplé Vltavy, kde měli navíc juvenilové možnost úniku za nepříznivých podmínek, nicméně lze konstatovat, že delší epizody se zhoršenými kyslíkovými poměry (Hyvärinen et al. (2022): 10 dní, **Bílý et al. (2021) – článek 1**: ~20 (možná i více?) dní) mají negativní vliv z hlediska vývoje juvenilní fáze perlorodky říční.

ČLÁNEK 2: Provádění bioindikačních in situ experimentů s juvenilními jedinci perlorodky říční

Pro provádění in situ bioindikačních experimentů byl vytvořen podrobný protokol pro dva typy zařízení: modifikovanou perforovanou destičku s individuálním držením (Buddensiek, 1995) a pískovou klícku s hromadným držením mladých mlžů (Hruška, 1999) (**Bílý et al., 2018 – článek 2**). Destičky a klícky lze instalovat v prostředí volné vody a/nebo v zóně hyporeálu. Postup prací při využití dalšího typu experimentálního zařízení (tj. síťových trubiček umístěných v říčním dně) byl popsán již v práci **Bílý et al. (2021) – článek 1**.

Všechny výše uvedené varianty (destička volná voda, klícka volná voda, destička dno a klícka dno) byly využity pro zkoumání vhodného prostředí pro perlorodku říční na Teplé Vltavě ve dvou odlišných prostorových měřítkách: podélného profilu toku a mikrohabitatu dna (více viz Černá et al., 2018).

Bioindikační destičky umístěné ve volné vodě jsou běžně využívanou metodou při terénních experimentech s juvenilními perlorodkami (Schmidt and Vandré, 2010; Scheder et al., 2014; Denic et al., 2015; Černá et al., 2018). Při měsíční expozici v prostředí Teplé Vltavy v roce 2014 byla zjištěna velice nízká míra růstu (většinou <10 %) bez zjevného podélného gradientu. Oproti tomu v roce 2015 byly přírůstky mlžů již vyšší (28–42 %), kdy směrem po proudu docházelo k významnému zvyšování míry růstu. Příznivější podmínky pro růst lze vysvětlit vyšší průměrnou měsíční teplotou vody ve druhém roce, což potvrzuje teplotní závislost růstu juvenilních i adultních perlorodek (Hruška, 1992; Buddensiek, 1995; Lange and Selheim, 2011; Denic et al., 2015). Míra přežití byla obecně na vysoké úrovni (>83 %). Jelikož jedinci v destičkách ve volné vodě obvykle nejsou vystaveni deficitu O_2, jenž bývá nejpravděpodobnější příčinou úhynu juvenilů (**Bílý et al., 2021 – článek 1**), dobré podmínky pro vývoj mlžů na Teplé Vltavě byly zjištěny i na těch stanovištích, kde perlorodky prospívaly hůře v klíckách (ve volné vodě i hyporeálu). Nicméně měsíční expozice je dostatečná pro rozlišení rozdílu přírůstků mezi stanovišti, a tak lze s využitím destiček celkem rychle detekovat charakteristiky jednotlivých stanovišť.

Odlišné výsledky ve vývoji juvenilních perlorodek byly získány při hodnocení expozice klícek v prostředí volné vody. Míra růstu mlžů s významným podélným gradientem se v roce 2014 nejprve zvyšovala směrem po proudu a poté opět klesala, což bylo potvrzeno také o rok později. Průměrná teplota vody pro tříměsíční expozici v obou letech se nelišila: zatímco i míra růstu byla obdobná, míra přežití byla vyšší v roce 2015 (max. 72 % vs. 29 % v roce 2014). I přes odlišnou délku expozice platí, že v hromadných systémech typu sedimentačních boxů přežívá méně jedinců, patrně v důsledku sníženého obsahu O_2 uvnitř zařízení (Denic, 2018). Totéž lze předpokládat v pískových klíckách, kde se juvenilové mohou pohybovat a uniknout z mikrozón s chudými O_2 poměry, nicméně nemohou takové zařízení zcela opustit (srovnej **Bílý et al., 2021 – článek 1**). Doplňkové hodnocení odhalilo absolutní úhyn jedinců na jednom z přítoků Teplé Vltavy, zasaženém havárií ČOV, jež způsobila krátkodobý, ale silný nárůst koncentrace amoniaku (Černá et al., 2018). Svoji negativní roli však mohl sehrát i deficit O_2 v důsledku aktivity mikroorganismů kolonizujících písková zrna v klíckách.

A co platí pro vývoj jedinců v podmínkách říčního dna? Zde jsou simulovány reálnější podmínky života juvenilů, avšak kvůli značné časoprostorové variabilitě podmínek (viz Dahm et al., 2007; Braun et al., 2012; Quinlan et al., 2014; **Bílý et al.,**

2021 – článek 1) může být interpretace výsledků ztížena a často je třeba využít vyšší počet zařízení. Informativní hodnota experimentů může být zvýšena, pokud se hodnotí vývoj nejlépe rostoucích jedinců (3 MAX pro destičku, 10 MAX pro klícku), jež jsou zásadní pro obnovu populací perlorodky říční. Míra růstu juvenilů byla každopádně vždy nižší ve srovnání se stejnými typy zařízení na daném stanovišti, umístěnými v prostředí volné vody, a podíl přeživších jedinců se vyznačoval velkou variabilitou. Nicméně v případě mikrohabitatu bylo zjištěno, že podmínky pro růst a přežití mlžů umístěných v destičkách ve dně se zhoršují s jemnozrnnějším substrátem a chudšími kyslíkovými poměry (přičemž souvislost s ucpáváním prostorů mezi zrny a zhoršením přístupu O_2 zmiňují již Geist and Auerswald (2007) a nově také Stoeckl et al. (2020)). Významné rozdíly (či rozdíly na hranici významnosti) byly odhaleny zejména pro kamenité a písčité dno: zatímco v prokysličeném dně s kameny (>96% nasycení O_2) dosahovala míra přežití až 100 %, nejméně jedinci prospívali v písku s nízkým a velmi proměnlivým množstvím kyslíku (40–80% nasycení O_2 → přežití 0–83 %). Význam kyslíku pro vývoj (zejména přežívání) juvenilů byl potvrzen stejně jako v práci **Bílý et al. (2021) – článek 1**.

ČLÁNEK 3: Hodnocení vlivu vícenásobného bodového znečištění na juvenilní jedince perlorodky říční

Nový experimentální přístup založený na principu CBM byl využit pro hodnocení účinků komunálního znečištění na mladé perlorodky v jemném prostorovém měřítku podélného profilu horní Malše (**Barák et al., 2022 – článek 3**). Tradiční aplikace CBM je spojena s designem „above-below" (tzn. hodnocení podmínek na jednom stanovišti nad a pod zdrojem znečištění) (např. Martel et al., 2003) či s rozšířenou gradientovou variantou (využívající jedno/dvě stanoviště nad a tři/čtyři stanoviště pod zdrojem znečištění; Salazar and Salazar, 2007) (např. Nobles and Zhang, 2015; Patnode et al., 2015). Armstead and Yeager (2007) stejně jako Salazar and Salazar (2007) upozorňují na možný nesoulad mezi daty z kontaminovaných (dolních) a referenčních (horních) stanovišť, jelikož i kontrolní stanoviště mohou být ovlivněna např. výtoky z ČOV a/ nebo městskou zástavbou (Gillis et al., 2014). Malšská studie založená na kombinaci vyššího počtu stanovišť a experimentálních (bioindikačních) zařízení pro spolehlivější detekci vlivu znečištění odpovídá přístupu v práci Rogers et al. (2018), avšak navíc umožňuje detailní charakteristiku patternů vývoje mlže a/nebo abiotických faktorů

prostředí ve třech rovnocenných úsecích (úsek se znečištěním a horní/dolní kontrolní úsek) s možností hodnocení gradientu referenčních podmínek v kontrolních úsecích.

Bioindikační experimenty s juvenilními perlorodkami umístěnými v destičkách ve volné vodě byly provedeny v souladu s protokolem viz **Bílý et al. (2018) – článek 2**. Na základě hodnocení 33-denní expozice v toku Malše dosahovala míra růstu mlžů 30,8 % v poškozeném, resp. 30,7 % v dolním kontrolním úseku. V horním kontrolním úseku byla významně nižší, kdy činila 21,2 %. Větší přírůstky byly doprovázeny vyšší teplotou vody stejně jako v případě Teplé Vltavy (**Bílý et al., 2018 – článek 2**), přičemž míra růstu pro podobné teplotní podmínky byla srovnatelná na obou řekách (24–44 % vs. 28–42 %).

Přírůstky juvenilů po odstranění vlivu teploty (pomocí regresního vztahu) odhalily, že překvapivě významně lépe perlorodky prospívaly v úseku se znečištěním zatíženém živinami (zejména amoniakem). Autoři již v minulosti dávali do souvislosti zvýšené množství živin s lepší dostupností potravy (Buddensiek, 1995; Strayer, 2008; Gagné et al., 2011; Strayer, 2014). Bartsch et al. (2003) ve své terénní studii zjistili pozitivní vztah růstu juvenilních mlžů (*Lampsilis cardium* (Rafinesque, 1820)) a koncentrace amoniaku (po 4 a 28 dnech, nikoli však po 10 dnech experimentu). Zatímco při nízkých koncentracích může amoniak působit jako živina, při vyšším množství naopak jako kontaminant (Salazar and Salazar, 2007; Strayer, 2014). Nicméně obecně je eutrofizace hodnocena jako proces s nepříznivým vlivem na vývoj perlorodky říční (Buddensiek, 1995; Degerman et al., 2009; Eybe et al., 2013).

Přežívání juvenilů bylo dle předpokladu významně nižší v úseku se znečištěním (73–95 %) oproti kontrolním úsekům (91–100 %). Zhoršené podmínky prostředí lze charakterizovat zejména zvýšeným množstvím amoniaku a sníženým obsahem O_2.

Mladí mlži jsou velice citliví na přítomnost amoniaku (Nakamura et al., 2021), avšak vztah mezi úhynem jedinců a množstvím látky nemusí být vždy zcela jasný (Bartsch et al., 2003). Na Malši byla zjištěna 73% míra přežití juvenilů při max. koncentraci 1,01 mg NH_4^+ l^{-1}, zatímco na přítoku Teplé Vltavy všichni jedinci uhynuli v prostředí s koncentrací max. 1,22 mg NH_4^+ l^{-1} (**Bílý et al., 2018 – článek 2**; Černá et al., 2018). V obou případech došlo k zachycení epizodické události znečištění pocházející z ČOV (odlehčení kanalizace, resp. havárie čistícího zařízení). Nicméně hodnoty toxické formy amoniaku NH_3 (Mummert et al., 2003; Haag, 2012; Strayer, 2014) byly srovnatelně nízké a k úhynu mlžů v povodí Teplé Vltavy spíše vedl odlišný typ experimentálního zařízení (tj. klícka s výplní písku) s rizikem deficitu O_2 (viz **Bílý**

et al., 2018 – článek 2). Akutní ekotoxikologické testy (t = 96 h.) s juvenilními jedinci příbuzných druhů odhalily hodnoty EC_{50} (subletální koncentrace pro 50 % jedinců) = 7,7–8,4 mg TAN l^{-1} (*Margaritifera falcata* (Gould, 1850); Wang et al., 2017) a LC_{50} (letální koncentrace pro 50 % jedinců) = 11,5–16,7 mg TAN l^{-1} (*P. auricularius*; Nakamura et al., 2021). Na Malši byly zaznamenány koncentrace 0,020–1,037 mg TAN l^{-1} a úhyn juvenilů 5–27 % během 33-denního experimentu, přičemž max. koncentrace celkového amoniakálního N byla velmi blízká bezpečné úrovni 1,15 mg l^{-1} (= 10% LC_{50}; Mummert et al., 2003). Maximální hodnoty jak pro akutní, tak chronickou expozici sladkovodních organismů včetně velkých mlžů v USA (USEPA, 2013) nebyly překročeny. To již neplatí v případě limitních hodnot pro perlorodkové toky (viz tab. 1, str. 17), nicméně autoři uvádějí jako kritérium NH_4^+ a nikoli TAN či NH_3. Nejen eutrofní prostředí, ale také s ním související snížený obsah kyslíku mohly přispět k nepříznivému vývoji mlžů (Cooper et al., 2005; Geist and Auerswald, 2007; Lopes-Lima et al., 2017).

Bartsch et al. (2003) prokázali pozitivní vztah mezi úhynem *L. cardium* a sníženou úrovní O_2. Ze srovnání výsledků bioindikačních testů s juvenilními perlorodkami na Malši (**Barák et al., 2022 – článek 3**) a Teplé Vltavě (**Bílý et al., 2018 – článek 2**; Černá et al., 2018) vyplývají obdobná zjištění. Při poklesu množství O_2 na úroveň blížící se 75 % došlo ke snížení míry přežití na 73 % jedinců v destičkách ve volné vodě (Malše), přičemž v prostředí říčního dna (Vltava) byly zachyceny dva extrémy: na jedné straně úroveň O_2 >85 % a 100% přežití, a na druhé straně úroveň O_2 <65 % a 0% přežití mladých perlorodek. V obou případech byl prokázán význam prokysličení vodního prostředí pro život juvenilního vývojového stádia perlorodky ve shodě s prací **Bílý et al. (2021) – článek 1**. Environmentální limity denních a týdenních minimálních hodnot O_2 pro lososové vody v USA (USEPA, 1986) byly na Malši překročeny nebo se úroveň prokysličení vody těmto limitům velice přiblížila (pozn. autora: losos jako jeden z možných hostitelů glochidií perlorodky; viz kap. 1.4.3). To opět neplatí pro kritéria perlorodkových toků, kdy se běžně uvádí hodnoty O_2 blízko 100% nasycení (BSI, 2017), ovšem tolerance juvenilních perlorodek vůči sníženému množství kyslíku nebyla donedávna podrobněji zkoumána (Quinlan et al., 2015). Hyvärinen et al. (2022) zjistili, že dokonce i při déletrvající situaci se sníženým obsahem O_2 až k 70% úrovni mohou mladé perlorodky přežívat. Ve srovnání s jejich výsledky z laboratorních testů však lze předpokládat, že v přirozených podmínkách toku Malše sehrála negativní roli kombinace většího počtu stresorů (tj. vyšší hodnoty amoniaku a pokles množství O_2

spolu se zvýšenou koncentrací dusitanů; viz níže). Zde je ještě třeba připomenout, že množství O_2 ve vodě může klesat v důsledku zvýšené teploty (Hauer and Hill, 2007), přičemž laboratorní experimenty jsou zpravidla prováděny za konstantních teplotních podmínek (viz např. Hyvärinen et al., 2022). Na Malši byly vyšší teploty detekovány pouze v dolním kontrolním úseku, tudíž snížení obsahu kyslíku ve vodě lze opravdu spojovat se zvýšenou koncentrací amoniaku (Černá et al., 2018).

V úseku se znečištěním odhalily laboratorní analýzy chemismu vody mimo vyšší koncentrace amoniakálního dusíku též zvýšené hodnoty fosforu (TP), nerozpuštěných látek a dusitanů. Fosfor je známkou eutrofizace vodního prostředí, nicméně se uvádí, že pro perlorodku není přímo toxický (BSI, 2017). Nerozpuštěné látky mohou přímo i nepřímo ovlivnit životaschopnost juvenilů. Mlži (zejména ti umístěni v destičkách) mohou zavírat své schránky v reakci na přítomnost suspendovaných látek ve vodě, a tak trpět stresem kvůli absenci příjmu potravy a kyslíku s možným následkem úhynu. Navíc jemný materiál po sedimentaci může ucpávat dno a jedinci obývající zónu hyporeálu by i v tomto případě byli vystaveni zhoršeným podmínkám prostředí (BSI, 2017). Na druhou stranu bioindikační zařízení v toku se mohou pravidelně čistit, jak ostatně doporučují **Bílý et al. (2018) – článek 2**, což snižuje riziko zanášení prostor obývaných juvenilními perlorodkami. Podrobnosti o citlivosti perlorodky říční (a mlžů obecně; Soucek and Dickinson, 2012) vůči dusitanům bohužel dosud chybí. Přitom jak environmentální limity pro lososové vody v USA (USEPA, 1986), tak pro německé perlorodkové toky (Jungbluth, 2011) byly překročeny při epizodické události na Malši, kdy dosahovaly úrovně až 0,083 mg NO_2-N l^{-1}. Dusitany tudíž mohly přispět k úhynu juvenilů (Eybe et al., 2013; Boon et al., 2019), přičemž již Gillis et al. (2017) uvádí, že v důsledku vyšších koncentrací amoniakálního a dusitanového N a poklesu O_2 jsou tekoucí vody v úsecích pod výtoky ČOV charakteristické absencí výskytu mlžů.

Ačkoli primárním cílem malšské studie nebyla identifikace zdrojů znečištění, v úseku se znečištěním byl zjištěn pozitivní vztah mezi nižší mírou růstu/přežívání juvenilních mlžů a epizodickou událostí (odlehčením kanalizace na ČOV) způsobující zvýšené koncentrace TAN a NO_2-N a snížení úrovně O_2. Nesoulad mezi hodnocením přírůstků jedinců na úrovni úseku a jednotlivých stanovišť vyplývá z podmínek na konkrétních stanovištích. Pod výtokem z ČOV, obecně považovanými za významné zdroje obohacení vod živinami (Augspurger et al., 2003; Haag, 2012; USEPA, 2013), byly zjištěny zvýšené přírůstky mlžů (stanoviště IMP-2), zatímco dále po proudu se již snižovaly (IMP-3 a IMP-4). Nejvyšší koncentrace TAN byly zaznamenány přímo

pod výtokem a jejich pokles směrem po proudu byl očekáván vzhledem k ředění v toku (Mummert et al., 2003; Patnode et al., 2015). Přesto juvenilové s rostoucí vzdáleností od výtoku hůře prospívali, což lze vysvětlit současným vlivem více abiotických faktorů prostředí (amoniaku postupně ubývá, přičemž na jeho odbourání se postupně spotřebovává kyslík a v důsledku oxidace amoniaku přibývají ve vodě dusitany). Na základě monitoringu jakosti vody je možno konstatovat, že ČOV trvale znečišťuje tok Malše a tento nepříznivý stav byl v průběhu experimentu ještě umocněn již popsanou epizodickou událostí. Její závažnost přitom dokumentovaly dočasně zvýšené hodnoty dusitanů prakticky v celém dolním kontrolním úseku. (Výše proti proudu se ještě do Malše vlévá malý znečištěný přítok, přinášející živiny ze sídel a/nebo pastevních ploch v povodí. Tento přítok s mnohem menším dopadem na jakost vody oproti ČOV neměl bezprostřední negativní vliv na jedince pod soutokem (IMP-1), avšak mohl částečně přispět ke zhoršenému vývoji mlžů dále po proudu).

ČLÁNEK 4: Hodnocení přímého antropogenního vlivu na jedince perlorodky říční

Terénní studie pro zkoumání interakcí mezi vodáky a mlži byla provedena na Teplé Vltavě, kde je umožněno rekreační splouvání a vyskytuje se zde populace perlorodky říční (**Barák et al., 2022 – článek 4**) s příznivými stanovištními podmínkami také pro mladé jedince (**Bílý et al., 2021 – článek 1**; **Bílý et al., 2018 – článek 2**). Bylo přitom využito „falešných" mlžů (betonových maket), umístěných na třech odpočinkových místech podél břehu v různých abiotických podmínkách dle habitatových požadavků perlorodky říční (viz Skinner et al., 2003; Degerman et al., 2009; Geist, 2010).

Významný rozdíl míry interakcí byl zjištěn při srovnání kategorií hloubky vody: zatímco v nejmělčích místech (hl. 0,1–0,2 m) dosahoval jejich podíl 11,6 %, v hlubších vodách se již snižoval (0,2–0,5 m: 6,5 %, resp. 0,5–0,8 m: 0,6 %). Perlorodkové toky bývají zpravidla hlubší než uvedených 0,2 m (viz Hastie et al., 2000; Ostrovsky and Popov, 2011; Jung et al., 2013; Varandas et al., 2013), nicméně není výjimkou, že dokonce i rozmnožující se populace druhu se vyskytují ve velmi mělkých úsecích toků (Moorkens and Killeen, 2014). Vodáci na Teplé Vltavě se většinou pohybují v místech s hloubkou do 0,5 m, kdy vystupují z lodí a několik málo minut tráví v korytě toku. V takových „mělkých" habitatech mohou prospívat i juvenilní perlorodky, jak uvádí **Bílý et al. (2021) – článek 1**. Z toho plyne, že splouváním a doprovodnými aktivitami,

jako jsou brodění, koupání a plavání, mohou být ohrožena různá vývojová stádia mlžů. Perlorodka se však vyskytuje i v hlubších částech toků (viz např. Degerman et al., 2009) a také lidé při koupání vstupují do míst, kde může hloubka dosahovat až 2 m (Escarpinati et al., 2011). Na druhou stranu na území NP Šumava bylo zaznamenáno častější brodění než koupání: jelikož míra interakcí klesá s hloubkou, mlži ve větších hloubkách (nad 0,5 m) by měli být méně vystaveni disturbancím.

Na odpočinkových místech na Teplé Vltavě bylo nejvíce ovlivněných maket mlžů zjištěno ve zhruba dvoumetrovém pásmu poblíž říčního břehu. Tedy nejen v mělkých, ale zároveň i příbřežních vodách lze sledovat nejčastější interakce mezi vodáky a mlži. Perlorodka se většinou vyskytuje ve vzdálenosti do 4 m od břehu (viz Hastie et al., 2000; Morales et al., 2004; Outeiro et al., 2008; Sousa et al., 2015), kde příbřežní vegetace poskytuje stín, snižuje výkyvy teploty vody a omezuje nadměrný vstup živin z okolních ploch (Degerman et al., 2009). Z toho plyne, že mlži nacházející se v blízkosti příbřežní zóny mohou být zranitelní vůči tlaku návštěvníků daného území. Interakce vodáků a mlžů v malých tocích s úzkými koryty by tedy mohly být velice četné, nicméně takové toky patrně nejsou obecně využívány k rekreačnímu splouvání.

Při zohlednění typu substrátu bylo zaznamenáno větší zastoupení interakcí ve dně písčitého a kamenitého charakteru (vždy 6,1 %) než ve štěrkovitém substrátu (3,3 %), ovšem bez významného vlivu na interakce. Velké plochy písku jsou obvykle nestabilní a mlži se zde nevyskytují (Jung et al., 2013), ovšem populace perlorodky říční v Rusku obývají právě tyto habitaty (kvůli příznivým proudovým a morfologickým poměrům; Ostrovsky and Popov, 2011). Písek byl preferovaným substrátem pro zastavení vodáků na Teplé Vltavě, proto nepřekvapí jejich četné interakce s mlži ve zdejším prostředí. Ač příhodné pro zahrabávání živočichů, písečné dno se zejména kvůli zhoršeným kyslíkovým poměrům nejeví jako vhodné pro vývoj mladých perlorodek (viz **Bílý et al., 2018 – článek 2**). Nicméně i perlorodky vyskytující se mezi kameny, jež přispívají ke stabilitě dnového substrátu (viz Hastie et al., 2000; Geist and Auerswald, 2007; Jung et al., 2013; Hauer, 2015), mohou být vystaveny tlaku návštěvníků, důležitou roli však hraje zejména viditelnost jedinců a s ní související chování vodáků (viz níže).

Při hodnocení reakcí ohrožených druhů na antropogenní disturbanci je doporučeno vyhnout se in situ disturbanci, přičemž ex situ testování pro změnu ztěžují omezení sběru jedinců vzhledem ke statutu ohrožení (Thiel et al., 2008). Proto bylo přistoupeno k problému z druhé strany, kdy bylo hodnoceno chování lidí při setkání s ohroženým druhem mlže s využitím maket jedinců. Doplňující hodnocení viditelnosti ukázalo, že

velmi dobře viditelní mlži by byli vystaveni významně většímu tlaku návštěvníků (13,7 % interakcí) oproti hůře či dokonce špatně viditelným jedincům (6,2, resp. 5,8 % interakcí). Dobrá viditelnost v mělkých vodách s kontrastem světlého substrátu a tmavých maket vedla k vyšší míře interakcí lidí a mlžů. Je však třeba rozlišit vizuální a manipulativní reakce vodáků vůči dobře viditelným jedincům, a naopak neúmyslné reakce vůči jedincům s horší viditelností pod hladinou toku. Lov perel zahrnující manipulaci s mlži byl významným problémem pro populace perlorodky říční, dnes je však zakázán (Skinner et al., 2003; Degerman et al., 2009; Švanyga et al., 2013) stejně jako „pouhá" manipulace s jedinci (Simon et al., 2015). Nicméně někteří lidé stále ilegálně hledají perlorodky v jejich přirozeném prostředí a jedince mohou zabíjet za použití destruktivních metod (Bauer, 1988; Cosgrove and Hastie, 2001; Stoeckl et al., 2020). Někteří vodáci na Teplé Vltavě nedokázali rozlišit makety od živých jedinců a chovali se jako lovci perel, když chtěli domnělé mlže otevřít. Chování při pohybu návštěvníků v mělkých vodách, kdy přicházejí do kontaktu s prostředím dna, bylo často spojeno s jeho neúmyslným sešlapem, jež může zapříčinit úhyn některých sladkovodních organismů (Escarpinati et al., 2011, 2014). Proto by i v případě perlorodkových toků měl být sešlap minimalizován (Skinner et al., 2003), což však neplatí pro vodáky v NP Šumava. Ačkoli mají zákaz pohybovat se v korytě toku, obecně je brodění (někdy též koupání a plavání) tolerováno i v přítomnosti odborného průvodce. Doprovodné aktivity vedou k delší době strávené v toku a zvyšuje se tak riziko kontaktu člověka a perlorodky říční. Juvenilové mající křehké schránky mohou být vzhledem k životu v hyporeálu více chráněni, nicméně někteří jedinci se dostávají na povrch dna (**Bílý et al., 2021 – článek 1**) a mohou být vystaveni sešlapu nohou vodáků. Sešlap dna může mít také nepřímý vliv, kdy hrozí riziko stlačení dna a snížení výměny vody s vodním sloupcem (Geist and Auerswald, 2007; Boon et al., 2019) či resuspendace jemného materiálu a jeho možné nepříznivé účinky na filtraci a příjem kyslíku u mlžů (Moorkens and Killeen, 2014).

Svůj význam mají také stanovištně-specifické podmínky, kdy se typy reakcí napříč stanovišti významně lišily. Na prvních dvou odpočinkových místech totiž převažovaly neúmyslné zásahy do dna, kdežto na posledním z nich byly většinou zaznamenány vizuální či manipulativní reakce. To lze vysvětlit rozdílnou atraktivitou obou skupin stanovišť. Zatímco na méně atraktivních místech lidé zastavují a vystupují z lodí na omezeném mělkém prostoru (což ústí v disturbanci dna) a většinu času pak tráví mimo

koryto toku, na atraktivnějších lokacích mají mnohem více prostoru a pohybují se v mělkých oblastech toku (což vede k nárůstu zejména vizuálních reakcí).

Mimo interakce vodáků a mlžů je také třeba se zabývat splouváním podél toku, kdy lidé ovlivňují prostředí říčního dna zejména zásahy svých pádel. Při laboratorních pokusech (**Barák et al., 2022 – článek 4**) byly vytvořeny modely mlžů z reálných schránek perlorodek, a následně umístěny v akváriu na rozhraní vody (výška sloupce 40 cm odpovídala experimentálně zjištěné nejvyšší míře disturbance při splouvání) a vrstvy štěrkopískového substrátu. Zkoumány přitom byly dva typy disturbance, tj. vyražení z polohy a destrukce (Brosnan and Crumrine, 1994). Pro vyražení z polohy bylo potřeba v průměru 8,03 úderů pádlem, přičemž tento výsledek mohl být ovlivněn relativně nestabilním dnovým substrátem použitým při pokusech. Ačkoli perlorodka se dokáže znovu zahrabat do sedimentu (Skinner et al., 2003), tento proces může vyvolat stres u mlžů, zejména pokud by byly údery opakovány a jedinci často měnili svou pozici. Destrukce modelu vyžadovala v průměru 7,88 úderů pádlem (v tomto případě zase bylo nutné po každém úderu vrátit model do původní vztyčené polohy), přičemž rozdíly v počtu zásahů pro vyražení z polohy a poškození schránky nebyly zjištěny jako významné. Vliv prasknutí schránky perlorodek na jejich přežití zkoumali Ziuganov et al. (2000), kdy během dvou týdnů od poškození došlo k úhynu všech zkoumaných jedinců. Na druhou stranu stejní autoři hodnotili v dalším terénním experimentu dlouhodobou regeneraci schránek mlžů a u třech jedinců odhalili zcela opravené schránky. Perlorodky na Teplé Vltavě nicméně mohou trpět nedostatečnou regenerační schopností oproti severským populacím (Ziuganov et al., 2000; Švanyga et al., 2013) a je třeba upozornit, že mohou být vystaveni opakovanému mechanickému poškození, umocněnému sešlapem nohou návštěvníků vodních toků (**Barák et al., 2022 – článek 4**).

5. Závěry a doporučení

V rámci bližšího zkoumání prostorových nároků juvenilních jedinců perlorodky říční v přirozeném prostředí říčního dna (**Bílý et al., 2021 – článek 1**) bylo zjištěno, že mladí mlži mají tendenci setrvávat v experimentálních sondičkách, což umožnilo vyhodnocení jejich hloubkové preference. Ukázalo se, že se většinou vyskytují ve vrstvě několika málo cm pode dnem (nepronikají hlouběji než 8 cm), přičemž se zdá, že juvenilové preferují pro svůj výskyt a vývoj nejmělčí možnou vrstvu dnového substrátu, na druhou stranu však dostatečně hlubokou pro zajištění stabilní polohy jedinců. Velice mělký hyporeál představuje příhodné životní prostředí pro juvenilní perlorodky, přičemž kvůli stabilnějším teplotním podmínkám přezimovávají o něco hlouběji ve srovnání s letním obdobím. Významnou roli pro přežití mlžů pak hrají především kyslíkové poměry.

Obsah kyslíku ve vodě pro úspěšný vývoj mladých perlorodek se objevuje též při hodnocení stanovištních podmínek s využitím in situ bioindikačních experimentů. Protokol pro provádění terénních pokusů byl přednostně vypracován pro juvenilní perlorodky (**Bílý et al., 2018 – článek 2**), nicméně jej lze v modifikované podobě využít i pro raná vývojová stádia ostatních druhů mlžů (zejména s přihlédnutím k jejich velikosti; Bauer and Wächtler, 2001). Využití různých typů bioindikačních zařízení ve volné vodě a prostředí říčního dna odhalilo rozdíly ve vývoji juvenilních mlžů: zejména vhodné podmínky pro jedince v klíckách/destičkách ve střední/dolní části toku a zhoršené (nicméně reálné) podmínky pro jedince v zóně hyporeálu. Svou roli přitom hrají také podmínky konkrétního stanoviště v podélném profilu vodního toku (s výrazným pozitivním vlivem teploty vody na růst jedinců) a mikrohabitatu dna (s výrazným pozitivním vlivem hrubozrnnosti substrátu a jeho prokyslíčení, zejména na přežití mlžů).

Výše uvedený protokol byl využit při hodnocení vlivu bodového znečištění na juvenilní perlorodky, kdy pro aplikaci CBM v tekoucích vodách platí tato doporučení: (1) sledování podélného profilu s rozšířením mimo úsek se znečištěním, (2) využití většího množství stanovišť, experimentálních zařízení a měření, resp. vzorkování při monitoringu jakosti vody, (3) i krátkodobá expozice za kritických podmínek (nízký vodní stav, vysoká teplota vody) může být dostačující pro detekci rozdílů ve vývoji mlžů napříč rovnocennými úseky toku s/bez znečištění (**Barák et al., 2022 – článek 3**). Představený robustní design umožnil determinaci významného efektu úseku toku,

přičemž pomocí jednoduché metody došlo k odstranění vlivu teploty vody na juvenilní perlorodky (zmíněného již v práci **Bílý et al. (2018) – článek 2**), což usnadnilo další zkoumání vlivu parametrů jakosti vody. Nepříznivý vývoj juvenilních mlžů na úrovni stanovišť v důsledku trvalého znečištění vody umocněného epizodickou událostí na ČOV (efekt havárie takového zařízení viz **Bílý et al., 2018 – článek 2**; Černá et al., 2018) byl s největší pravděpodobností způsoben zvýšenou koncentrací amoniaku a dusitanů a též sníženým obsahem kyslíku (a jejich synergistickým působením). Tato skutečnost potvrzující citlivost mladých mlžů vůči (zejména náhlým) změnám jakosti vody byla doložena v chráněném území s výskytem ohrožených druhů a omezením nakládání s odpadními vodami (Švanyga et al., 2013), tudíž je zde nutná efektivní prevence před znečištěním (Newton, 2003). Stejně jako v případě protokolu viz **Bílý et al. (2018) – článek 2**, také metodické postupy použité v práci **Barák et al. (2022) – článek 3** lze aplikovat na jiné druhy velkých mlžů.

V chráněném území byl také hodnocen přímý vliv člověka na perlorodku říční, kdy nejvýraznější interakce vodáků a (adultních) mlžů byly zjištěny v mělkých vodách při okraji příbřežní zóny (s jemným i hrubým typem substrátu), kde se mlži přirozeně vyskytují. Svou roli přitom hraje i viditelnost mlžů a reakce lidí se liší dle stanovištně-specifických podmínek (**Barák et al., 2022 – článek 4**). Návštěvníci chráněných území by měli být vzděláváni o tom, jak mohou svými aktivitami ovlivnit volně žijící druhy a jak mohou změnit své chování pro zmírnění tlaku na ohrožené druhy (Miller et al., 2001). Nejde přitom jen o přímý kontakt s jedinci (typický pro atraktivnější místa), ale také o neúmyslný zásah do habitatu druhů v adultní i juvenilní fázi života (na méně atraktivnějších místech). Jednoduchá a neškodná metoda s maketami mlžů dovoluje porozumět interakcím lidí a jedinců ohrožených druhů, a zlepšit tak úroveň znalostí o chování návštěvníků (to je velká výzva pro správce chráněných území; McGinlay et al., 2020). Laboratorní experiment (**Barák et al., 2022 – článek 4**) odhalil opakovaný počet (osmi) úderů pádlem pro vyražení (modelu) mlže z polohy či jeho destrukci, tzn. že splouvání může mít méně nepříznivé účinky než doprovodné aktivity při pohybu v korytě toku. Jelikož přesun mlžů není efektivním řešením (Álvarez-Claudio et al., 2000; Cosgrove and Hastie, 2001; Hastie et al., 2003b), je třeba se zaměřit na ochranu jedinců a udržování jejich habitatu (Cosgrove and Hastie, 2001). Navzdory efektivní regulaci počtu vodáků pro minimální disturbanci perlorodky říční hraje zásadní roli pochopení chování lidí pro zachování jejich přístupu do chráněného území.

Souhrn

Na základě výše uvedených článků tvořících jádro předkládané disertační práce lze konstatovat, že pomocí modifikovaných či zcela nových metodických postupů byly determinovány tyto klíčové abiotické faktory pro výskyt a vývoj ohrožených druhů mlžů na příkladu perlorodky říční: **obsah O$_2$ ve vodě**, substrátové poměry a jakost (+ teplota) vody. Největší význam má prokyslичení vody, kdy snížené množství kyslíku v zóně hyporeálu i volné vodě vede k poklesu růstu a přežívání juvenilních jedinců, přičemž chudé kyslíkové poměry také nutí mladé mlže opouštět říční dno a hledat vhodnější habitaty pro život. V tomto smyslu je třeba zdůraznit význam výměny vody na rozhraní voda-sediment.

Získané poznatky o ekologii perlorodky říční, kvalitě prostředí a vhodnosti biotopů pro juvenilní jedince lze uplatnit v rámci záchranného programu v ČR (Švanyga et al., 2013) i v Evropě (Araujo and Ramos, 2001): ať už jde o ochranu stávajících populací druhu včetně jeho habitatu, nebo o provádění propopulačních opatření (Lopes-Lima et al., 2017). Tato práce také přináší nové údaje o vlivu stresorů v prostředí na velké mlže mimo prostředí Severní Ameriky. Kromě toho byly představeny metodické postupy s možným využitím v obecné rovině pro hodnocení biotopu organismů pomocí izolátů („caged model") či antropogenního tlaku na volně žijící druhy („falešní" jedinci). Ty by mohly najít uplatnění při zlepšení managementu jakosti vody, resp. ochraně biotopů tekoucích vod.

Jelikož zde uvedené výsledky představují pouze střípek v celém obraze života sladkovodních organismů a zejména fascinující skupiny velkých mlžů, je třeba se v budoucím výzkumu zaměřit na následující aspekty:

- hodnocení in situ experimentů a současné provádění průzkumů přirozených populací (mlžů) na daném toku/tocích (Nobles and Zhang, 2015; Patnode et al., 2015),

- hodnocení (sub)letálních účinků stresorů a jejich synergistického působení (na mlže) (Kienzler et al., 2016; Lopes-Lima et al., 2017; Belamy et al., 2020),

- kombinace ex/in situ experimentů pro provádění testů jakosti vody a ochranu sladkovodních organismů (ASTM, 2013; Patnode et al., 2015; Pollard et al., 2017),

- disturbance sladkovodních organismů obývajících mělké vody sešlapem dna a manipulací s jedinci.

6. Přehled použitých zdrojů

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7. Seznam příloh

Příloha 1. Odborný životopis

Příloha 2. Publikační činnost