PROMJENE U SASTAVU MIKROBNIH ZAJEDNICA I ULOGA IMUNOSNOG ODGOVORA U ŠIRENJU AREALA INVAZIVNE STRANE VRSTE SIGNALNOGA RAKA Pacifastacus leniusculus (Dana, 1852)

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PRIRODOSLOVNO-MATEMATIČKI FAKULTET

BIOLOŠKI ODSJEK

Paula Dragičević

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DOKTORSKI RAD

Mentor:

doc. dr. sc. Sandra Hudina

Zagreb, 2022.

University of Zagreb

FACULTY OF SCIENCE

DEPARTMENT OF BIOLOGY

Paula Dragičević

CHANGES IN MICROBIAL COMMUNITIES AND THE ROLE OF IMMUNE RESPONSE IN THE RANGE EXPANSION OF THE INVASIVE SIGNAL CRAYFISH *Pacifastacus leniusculus* (Dana, 1852)

DOCTORAL THESIS

Supervisor:

Dr. Sandra Hudina, Asst. Prof.

Zagreb, 2022

Ovaj je doktorski rad izrađen na Zoologijskom zavodu Biološkog odsjeka Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, pod vodstvom doc. dr. sc. Sandre Hudine, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Doktorski rad izrađen je u sklopu projekta „Promjene sastava patogena i imunološkog odgovora tijekom širenja areala uspješnih invazivnih vrsta slatkovodnih rakova“ (HRZZ UIP-2017-05-1720).

Informacije o mentoru

Sandra Hudina (MBZ: 291626) je docent na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta (PMF-a) Sveučilišta u Zagrebu. Po završetku studija *Biologije* na PMF-u 2002. godine, radila je na nekoliko stručnih pozicija u različitim tvrtkama u javnom i privatnom sektoru te 2006. godine završava jednogodišnji magistarski studij *Biodiversity, conservation and management* na Sveučilištu u Oxfordu, Velika Britanija. Na Biološkom odsjeku započinje raditi 2007. godine u području istraživanja vezanom uz ekologiju invazivnih stranih vrsta, s fokusom na slatkovodnim ekosustavima i beskralješnjacima. Doktorirala je 2012. godine na tematici invazivnog uspjeha slatkovodnih deseteronožnih rakova. Objavila je preko 30 znanstvenih radova u časopisima s međunarodnom recenzijom te je bila suradnik na više od deset i voditelj pet znanstvenih i stručnih projekata, uključujući i projekt „Promjene sastava patogena i imunološkog odgovora tijekom širenja areala uspješnih invazivnih vrsta slatkovodnih rakova“ (HRZZ UIP-2017-05-1720) u sklopu kojeg je izrađen doktorski rad Paule Dragičević. Do danas je bila recenzent više od 20 znanstvenih radova u međunarodnim časopisima. Nositelj je dva te je bila suradnik na dodatnih osam nastavnih kolegija u sklopu studijskih programa na Biološkom odsjeku. Bila je mentor više od 20 završnih i diplomskih radova te tri rada studenata kojima je dodijeljena Rektorova nagrada. Trenutno obavlja funkciju pomoćnice pročelnice za međunarodnu suradnju i provedbu bolonjskog procesa. Tijekom karijere, bila je dobitnica nekoliko nagrada uključujući nagradu BRDO Studentskog zbora PMF-a za najboljeg asistenta Biološkog odsjeka (2016. godine) i Priznanje Prirodoslovno-matematičkog fakulteta za postignute rezultate u znanstvenom i stručnom radu (2012. godine). Kroz cijelu karijeru kontinuirano se bavi popularizacijom znanosti i struke te je provela brojne edukativne radionice i objavila nekolicinu edukativnih letaka, priručnika i knjiga za razne ciljne skupine.

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Invazivne strane vrste jedna su od najvećih prijetnji vrlo osjetljivim slatkovodnim ekosustavima, a predviđanje uspjeha invazije predstavlja globalni izazov. Invazivni uspjeh određen je, između ostaloga, i imunosnim sustavom, kojeg oblikuju patogeni i nepatogeni mikrobi domaćina te ekološki procesi koji proizlaze iz širenja areala. Ovo istraživanje ispituje promjene u sastavu mikrobnih zajednica i ulogu imunosnog odgovora tijekom širenja areala jedne od najuspješnijih invazivnih stranih vrsta slatkovodnih beskralježnjaka u Europi, signalnoga raka. Mikrobi u različitim tkivima jedinki duž invazivnog areala detektirani su analizom metagenoma (sekvenciranjem nove generacije), dok su razlike u imunosnom odgovoru jedinki ispitane analizom nekoliko standardnih imunosnih parametara. Navedene analize pokazale su da se mikrobne zajednice i imunosni odgovor signalnoga raka značajno mijenjaju tijekom širenja invazivnog areala te da su oblikovani, u različitoj mjeri, okolišnim čimbenicima i karakteristikama populacije. Ovo istraživanje također ukazuje na vrlo slabu istraženost patogena slatkovodnih deseteronožnih rakova te ističe potrebu za standardizacijom eksperimentalnih metoda prilikom ispitivanja patogenosti.

(202 stranice, 3 slike, 212 literaturnih navoda, jezik izvornika: hrvatski)

Ključne riječi: signalni rak, invazivni uspjeh, mikrobiom, imunosni odgovor, patogeni

Mentor: doc. dr. sc. Sandra Hudina

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Doctoral thesis

CHANGES IN MICROBIAL COMMUNITIES AND THE ROLE OF IMMUNE RESPONSE IN THE RANGE EXPANSION OF THE INVASIVE SIGNAL CRAYFISH *Pacifastacus leniusculus* (Dana, 1852)

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Invasive alien species are among the largest threats to sensitive freshwater ecosystems, and predicting the success of an invasion represents a global challenge. Invasive success is determined, among other things, by the immune system, which is shaped by pathogenic and non-pathogenic host microbes and ecological processes arising from range expansion. This study examines changes in microbial communities and the role of immune response during range expansion of one of the most successful invasive alien species of freshwater invertebrates in Europe, the signal crayfish. Microbes in different tissues of individuals along the invasion range were detected by metagenome analysis (next generation sequencing), while differences in the immune response of individuals were examined by analysis of several standard immune parameters. These analyses have shown that microbial communities and the immune response of signal crayfish change significantly during range expansion and that they are shaped, to varying degrees, by environmental factors and population characteristics. This study also highlights a gap of knowledge regarding crayfish pathogens and underlines the need for standardization of experimental methods when testing pathogenicity.

(202 pages, 3 figures, 212 references, original in: Croatian)

Keywords: signal crayfish, invasive success, microbiome, immune response, pathogens

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Sadržaj

1. UVOD

1.1. Biološke invazije

Invazivna strana vrsta je vrsta slučajno ili namjerno unesena u novi okoliš izvan svog prirodnog područja rasprostranjenosti, za koju je utvrđeno da njeno unošenje ili širenje ugrožava ili štetno utječe na bioraznolikost i povezane usluge ekosustava (uredba EU 1143/2014). Proces širenja invazivne strane vrste naziva se biološka invazija i predstavlja jedan od glavnih uzroka smanjenja bioraznolikosti diljem svijeta (Simberloff i sur., 2013). Invazivna strana vrsta u novom okolišu mijenja strukturu i narušava funkcije ekosustava (Pyšek i Richardson, 2010) te negativno utječe na gospodarstvo i/ili ljudsko zdravlje (Bradshaw i sur., 2016; Jones, 2017; Ogden i sur., 2019). K tome, nabrojene negativne učinke dodatno pogoršavaju globalizacija i klimatske promjene koje podupiru širenje invazivnih stranih vrsta: globalizacija omogućava (namjerno i nenamjerno) širenje vrsta diljem svijeta različitim prijevoznim sredstvima, dok klimatske promjene i globalno zatopljenje prisiljavaju vrste na migraciju izvan njihovog prirodnog područja rasprostranjenosti (Essl i sur., 2020; Seebens i sur., 2020). Dolaskom u novi okoliš, unesena strana vrsta u njemu pokušava opstati i uspostaviti samoodrživu populaciju. Uspješno uspostavljena populacija počinje se širiti, a u slučaju da njeno širenje ima štetne učinke u novom okolišu, unesena strana vrsta smatra se invazivnom. Iako samo mali dio unesenih stranih vrsta postane invazivan u novom okolišu (Pyšek i Richardson, 2010), upravo zbog izrazito negativnih učinaka relativno malog broja vrsta, identifikacija i predviđanje uspješnih invazivnih stranih vrsta predstavljaju globalni izazov (Marchetti i sur., 2004).

Mnoge invazivne strane vrste pokazuju sposobnost prilagodbe novom okolišu preusmjeravanjem energetskih resursa u procese koji potiču rast, razmnožavanje i širenje jedinki (Lagos i sur., 2017). U novom okolišu, širenje populacije podrazumijeva povećanje broja jedinki u populaciji i prostorno širenje jedinki u novi, nekolonizirani teritorij (Phillips, 2016). Izvorna, polazišna uspostavljena populacija iz koje se jedinke šire naziva se invazijsko središte. S rastom gustoće populacije u invazijskom središtu, raste i kompeticija za ograničene resurse, zbog čega se jedinke udaljavaju, populacija raspršuje i dolazi do povećanja invazivnog areala. Zbog toga je, od invazijskog središta prema rubovima invazivnog areala, vidljivo smanjenje gustoće jedinki. Jedinke na rubovima areala, koje karakterizira manja gustoća populacije, čine invazijsku frontu (Phillips, 2016).

Brzina širenja populacije u novom okolišu, odnosno invazivni uspjeh, može znatno varirati među različitim invazivnim stranim vrstama (Pyšek i Richardson, 2010). Invazivni uspjeh ovisi

o mnogim čimbenicima koje je moguće razvrstati u tri primarne skupine: biološke karakteristike invazivne strane vrste, karakteristike novog okoliša i sličnost novog okoliša s prirodnim okolišem invazivne strane vrste (Marchetti i sur., 2004). Važnu ulogu u invazivnom uspjehu ima imunosni sustav, što potvrđuju brojna istraživanja (npr. Lee i Klasing, 2004; Dunn i sur., 2012; White i Perkins, 2012; Vogel i sur., 2017). Imunosni sustav u stalnoj je interakciji s mikrobnim zajednicama domaćina i njegovog okoliša (Zheng i sur., 2020). Mikrobne zajednice s kojima invazivna strana vrsta dolazi u kontakt, kao i one koje donosi sa sobom u novi okoliš, utječu na niz interakcija između domaćina i okoliša te na ekologiju samog domaćina (Diagne i sur., 2016). Međusobno oblikovanje i interakcija imunosnog sustava invazivne strane vrste s mikrobnim zajednicama (Bahrndorff i sur., 2016) jedna je od odrednica invazivnog uspjeha.

1.1.1. Mikrobiom u biološkim invazijama

Mikrobiom čini zajednica komenzalnih, simbiotskih i patogenih mikroba (npr. virusa = virom; bakterija = bakteriom; mikrogljiva = mikobiom) koji žive unutar nekog staništa s određenim fizikalno-kemijskim svojstvima (npr. unutar tijela domaćina ili drugog okoliša kao, primjerice, vodenog stupca ili tla; Lederberg i McCray, 2001). Mikrobiom je dinamičan i interaktivan mikroekosustav: podložan je promjenama tijekom vremena, pri čemu se mijenja njegov sastav i veličina (Berg i sur., 2020). Sastav i veličinu mikrobioma određuju mnogi čimbenici, kao primjerice: prehrana, dob ili životni stadij domaćina, izloženost mikrobima drugih jedinki, stres, okolišni uvjeti i njihove promjene (npr. promjene temperature ili pH vrijednosti) i izloženost različitim zagađivalima u okolišu (npr., farmaceuticima ili toksičnim kemijskim spojevima; Karl i sur., 2018; Davila Aleman i Valenzano, 2019; Chiu i sur., 2020; Sze i sur., 2020; Weersma i sur., 2020). Budući da je mikrobiom integriran u makroekosustave (npr. eukariotske domaćine) te s njima koevoluira, dolazi do stvaranja međuovisnosti između domaćina i njegovog mikrobioma. Mikrobiom ima vrlo važnu ulogu u biologiji i funkcioniranju domaćina, kao i u očuvanju njegovog zdravlja, otpornosti ili podložnosti bolestima (Hauffe i Barelli, 2019; Antwis i sur., 2020). Primjerice, mikrobiom jedinke koja se širi u novi okoliš može doprinijeti zaštiti domaćina od infekcije mikrobnim patogenima (Chiu i sur., 2017).

Tijekom procesa invazije, invazivna strana vrsta i njen mikrobiom dolaze u kontakt s mikrobnim zajednicama u novom okolišu, a interakcije između ovih mikrobioma mogu utjecati na fiziologiju, imunosni status i kondiciju domaćina (Braga i sur., 2016; Gould i sur., 2018; Hauffe i Barelli, 2019). Nekoliko hipoteza opisuje moguće interakcije invazivne strane vrste s mikrobima pri širenju u novi okoliš:

- Hipoteza o oslobađanju od neprijatelja (*engl.* enemy release hypothesis)

  Tijekom brzog širenja populacije u novom okolišu, jedinke invazivne strane vrste mogu izgubiti svoje prirodne neprijatelje (npr. patogene mikrobe, ali i parazite, predatore, kompetitore) koji zaostaju za njima. Oslobađanje od prirodnih neprijatelja može dovesti do smanjenja učestalosti određenih (patogenih) mikroba u invazivnoj populaciji u novom okolišu. U konačnici, gubitak prirodnih neprijatelja može rezultirati boljom kondicijom jedinki na invazijskoj fronti u odnosu na invazijsko središte, budući da neprijatelji ostaju u invazijskom središtu (Colautti i sur., 2004; Phillips i sur., 2010a; Llewellyn i sur., 2012; Brown i Shine, 2014; Cornet i sur., 2016).

- Hipoteza prelijevanja (*engl.* spillover hypothesis) / Hipoteza o novom oružju (*engl.* novel weapon hypothesis)

  Jedinke koje se šire potencijalno su domaćini nekim mikrobima koji nisu prisutni u novom okolišu. Dolaskom u novi okoliš, invazivna strana vrsta unijet će u njega nove mikrobe koji će kolonizirati zavičajne vrste (proces opisan hipotezom prelijevanja). Nadalje, navedeni novi mikrobi mogli bi se pokazati patogenima te uzrokovati pojavu bolesti u populacijama zavičajnih vrsta, što je opisano hipotezom o novom oružju. Ovaj proces u konačnici može dovesti do selektivne prednosti invazivne strane vrste u kompeticiji sa zavičajnim vrstama (Strauss i sur., 2012; Vilcinskas, 2015).

- Hipoteza izlijevanja (*engl.* spillback hypothesis)

  Širenjem u novom okolišu, jedinke dolaze u kontakt s novim mikrobima, koji ih pritom mogu kolonizirati. U nekim slučajevima, jedinke invazivne strane vrste djelovat će kao rezervoar u kojem se novostečeni mikrobi uspješno umnažaju. Brzo umnažanje patogenih mikroba u novouspostavljenoj invazivnoj populaciji dovodi do višestrukog povećanja njihovog negativnog utjecaja na zavičajne vrste, budući da se patogeni mikrobi u velikom broju oslobađaju i „izlijevaju“ iz invazivnih jedinki. Međutim, ova pojava također može smanjiti kondiciju i imati negativne posljedice za invazivne jedinke (Kelly i sur., 2009).

Dakle, proces invazije i sve mikrobne interakcije koje iz njega proizlaze, u kombinaciji s karakteristikama novog okoliša (tj. okolišnim mikrobiomom, fizikalno-kemijskim parametrima, zavičajnim vrstama itd.), utječu na sastav mikrobioma jedinki invazivne strane

vrste, što može izravno i neizravno utjecati na njihovo zdravlje, kondiciju i u konačnici, invazivni uspjeh vrste.

Tema mikrobioma u biološkim invazijama do sad je slabo istražena, stoga je trenutno dostupan relativno mali broj empirijskih dokaza i zaključaka o ulogama mikrobioma u invazivnom uspjehu. Međutim, nedvojbeno je da biološke invazije uzrokuju negativne posljedice za zavičajne mikrobne zajednice u novom okolišu. Primjerice, biološke invazije mijenjaju funkcije ekosustava, što može uzrokovati promjene u bioraznolikosti ekosustava te u sastavu mikrobnih zajednica okoliša, tj. može dovesti do gubitka raznolikosti mikrobnih zajednica (Hauffe i Barelli, 2019; Malacrinò i sur., 2020). Nadalje, invazivne strane vrste unose mikrobne patogene u novi okoliš te tako nadjačavaju zavičajne vrste i potencijalne kompetitore koji nisu koevoluirali s novim patogenom i nemaju spreman brzi imunosni odgovor (Crowl i sur., 2008.; Ogden i sur., 2019). Usto, invazivne strane vrste predstavljaju vektor za širenje potencijalno invazivnih mikroba (Litchman, 2010).

1.1.1.1. Definicija i identifikacija patogena

Patogen je (mikro)organizam koji može prouzročiti štetu u podložnom domaćinu i uzrokovati negativne učinke na kondiciju domaćina. Pritom, navedena šteta u organizmu može biti vidljiva na bilo kojoj razni biološke organizacije (tj. na razini jedinke, organskih sustava, organa, tkiva i stanica) i u bilo kojem intenzitetu (tzv. virulentnost ili stupanj patogenosti; Casadevall i Pirofski, 2003). Iako je patogen teoretski jasno definiran, klasifikacija mikroba na patogene i nepatogene u praksi vrlo je zahtjevan izazov iz dva glavna razloga:

i. Virulentnost patogena je promjenjiva i njena kvantifikacija je zahtjevna

Interakcija između patogena i domaćina rezultira nastankom štete u organizmu domaćina. Navedena šteta, koja nastaje zbog djelovanja patogena i zbog imunosnog odgovora domaćina, vidljiva je u različitim oblicima (npr. poremećaji u unutarstaničnim procesima, lezije na tkivima, promjene u funkciji organa, promjene u ponašanju, smanjenje kondicije domaćina) i naziva se virulentnost (Pirofski i Casadevall, 2012; Méthot i Alizon, 2014). Virulentnost određenog patogena je promjenjiva varijabla koja ovisi o karakteristikama patogena, ali i o svojstvima zaraženog domaćina, npr. o njegovom imunosnom sustavu (Casadevall i Pirofski, 2001). Patogeni iste vrste, ali različitih sojeva i/ili izolata, mogu značajno varirati u virulentnosti budući da je virulentnost patogena često uvjetovana njegovim genetskim materijalom (Nathanson i González-Scarano, 2016). Virulentnost patogena može se kvantificirati mjerenjem

brojnih faktora virulentnosti (Nathanson i González-Scarano, 2016), no teško je definirati granicu između "niske" i "visoke" virulentnosti i odrediti koja je minimalna virulentnost potrebna da se mikrob proglasi patogenom (Méthot i Alizon, 2014). Usto, virulentnost je jednostavnije mjeriti na višim razinama biološke organizacije (npr. na razini jedinke, organskih sustava i organa) nego na nižim (npr. na razini tkiva, stanica i staničnih procesa).

ii. Podložnost domaćina često je određena vanjskim čimbenicima i stohastičkim događajima

Interakcija patogena i domaćina vrlo je dinamičan proces podložan brojnim promjenama. Određeni broj patogena spada u generaliste, tj. imaju sposobnost zaraze širokog spektra domaćina različitih taksonomskih pripadnosti. Primjerice, virus ptičje gripe H5N1 patogen je ptica i brojnih sisavaca (Poovorawan i sur., 2013), dok bakterija *Vibrio harveyi* uzrokuje bolest u morskim kralježnjacima (ribama) i beskralježnjacima (Zhang i sur., 2020a). S druge strane, mnogi patogeni specijalisti su za određenu manju taksonomsku skupinu, vrstu ili rod (npr. baciliformni virus vrste *Austropotamobius pallipes*; Edgerton, 2003). U odgovarajućim uvjetima, patogeni specijalizirani za određene taksonomske skupine mogu prijeći na jedinke druge taksonomske skupine u kojima pokazuju značajno višu virulentnost (npr. virus ebole u šišmišima je asimptomatski, dok u ljudima i primatima može uzrokovati broje simptome i mortalitet; Longdon i sur., 2015). Nadalje, ako domaćin živi u određenim (stresnim) uvjetima i/ili mu je imunosni sustav oslabljen, prethodno nepatogeni mikrob u takvom domaćinu može izazvati bolest (tzv. oportunistički patogen; Casadevall i Pirofski, 1999). U suprotnome slučaju, patogen i njegov domaćin mogu dugoročno koevoluirati dok se među njima ne uspostavi ravnoteža, pri čemu patogen postaje avirulentan i u domaćinu uzrokuje latentnu infekciju koja ne narušava zdravlje ili kondiciju domaćina (Alizon i sur., 2009), kao u slučaju uzročnika bolesti račje kuge *Aphanomyces astaci* Schikora, 1906 i sjevernoameričkih vrsta rakova (Svoboda i sur., 2017).

S obzirom na to da mnogi čimbenici (npr. okoliš, imunosni sustav domaćina, interakcije s drugim mikrobima itd.; Pirofski i Casadevall, 2012) utječu na pojavu ili nestanak patogenosti, nije uvijek moguće odrediti jasnu granicu između nepatogena i patogena. Usto, mikrob definiran kao patogen ne mora u svakom kontekstu pokazati patogenost.

1.1.1.2. Mikrobiom u zdravlju i bolesti

Bolest ili poremećaj zdravstvenog stanja često su uzrokovani jednim određenim patogenom (tj. uzročnikom bolesti). Međutim, promjene u odnosu i/ili sastavu mikroba unutar

mikrobioma domaćina također mogu negativno utjecati na zdravlje i stanje domaćina. Takav neuravnoteženi mikrobiom, koji izaziva bolest ili narušava zdravlje domaćina, naziva se patobiom (Bass i sur., 2019). Patobiom domaćina posljedica je narušenih interakcija mikroba unutar mikrobioma. Vanjski stresori, oslabljeni imunosni sustav ili infekcija patogenima vode do promjena u sastavu mikrobioma, kao npr. pretjerane proliferacije ili nestanka nekih mikrobnih skupina, kolonizacije novih mikrobnih skupina iz okoliša itd. (Vayssier-Taussat i sur., 2014). U konačnici, mijenja se odnos (promijenjenog) mikrobioma i imunosnog sustava domaćina, zbog čega dolazi do pojave bolesti (Zheng i sur., 2020).

Održavanje uravnoteženog, tj. „zdravog“ mikrobioma, od iznimne je važnosti za funkcioniranje svake jedinke, budući da zdravi mikrobiom pozitivno utječe na brojne procese u organizmu (npr. Hoffmann i sur., 2015; Lloyd-Price i sur., 2016; Graystock i sur., 2017; Rajeev i sur., 2021). U teoriji, zdravi mikrobiom je funkcionalna zajednica sastavljena od određenih genetski raznolikih mikroba, njihovih metaboličkih procesa i regulacijskih sustava, koja je otporna na promjene izazvane vanjskim ili unutarnjim čimbenicima, ili se, unatoč promjenama, može vratiti u prvobitno funkcionalno stanje kojim se održava stabilna i uravnotežena ekologija domaćina (Bäckhed i sur., 2012; Rajeev i sur., 2021). Međutim, iako mnoga istraživanja ukazuju na postojanje razlika između mikrobioma zdravih i bolesnih jedinki (npr. McKenna i sur., 2008; Ganz i sur., 2017; McKenney i sur., 2017; Holt i sur., 2021) u praksi nije jednostavno odrediti što čini zdravi mikrobiom, tj. od kojih je mikrobnih taksona sastavljen. Zbog brojnih čimbenika koji oblikuju mikrobiom – prehrana, stanište, način života, dob itd. – zdravi mikrobiom određene jedinke ne mora biti zdravi mikrobiom u nekom drugom kontekstu, npr. okolišu ili jedinki, čak ni ako se radi o pripadnicima iste vrste (Eisenstein, 2020). Stoga, ne postoji jedan univerzalni zdravi mikrobiom, nego više varijacija zdravog mikrobioma koje imaju pozitivan učinak na funkcioniranje i zdravlje domaćina (Zaneveld i sur., 2017).

1.1.2. Imunosni sustav u biološkim invazijama

Različiti čimbenici, kao što su geni, spol, prehrana, dob ili životni stadij, fizička aktivnost i okoliš, sudjeluju u oblikovanju imunosnog sustava (Gomez i sur., 2008; Edwards i sur., 2018; Hagai i sur., 2018; Strandin i sur., 2018; Weyh i sur., 2020). Kao što je već prethodno spomenuto, vrlo važnu ulogu u oblikovanju imunosnog sustava imaju i mikrobne zajednice, odnosno mikrobiomi domaćina i njegovog okoliša, što je prepoznato u mnogim istraživanjima (npr. Broom i Kogut, 2018; Tizard i Jones, 2018; van Veelen i sur., 2020; Zhang i sur., 2020b; Zitvogel i Kroemer, 2021). Visoko funkcionalni imunosni sustav od neosporive je važnosti za

svaku jedinku koja se suočava s napadom patogena. Imunosuprimirane jedinke imaju povećani rizik od zaraze, što često rezultira smanjenjem njihove kondicije (Marie i sur., 2001). S obzirom na potrebu za brzim i učinkovitim imunosnim odgovorom na napad patogena, lako je pretpostaviti da će jedinke snažnog imunosnog odgovora imati selektivnu prednost nad jedinkama slabijeg imunosnog odgovora. Međutim, snažan imunosni odgovor nije nužno bolja opcija. Osim što će onemogućiti napredovanje patogena u tijelu, presnažan imunosni odgovor može izazvati sintezu prekomjernog broja reaktivnih obrambenih molekula koje će naštetiti fiziološkom stanju jedinke (tzv. imunopatologija; Graham i sur., 2005), što je dokazano kod kralježnjaka (Bertrand i sur., 2006), ali i kod beskralježnjaka koji ne posjeduju stečenu imunost, kao npr. člankonošci (Sadd i Siva-Jothy, 2006). Dakle, primjereni imunosni odgovor na imunosni izazov odlika je funkcionalnog imunosnog sustava (Viney i sur., 2005).

Imunosni odgovor složena je fiziološka funkcija koja, iako nije izravno vezana za širenje populacije, ima važnu ulogu u invazivnom uspjehu vrste (Lee i Klasing, 2004; Dunn i sur., 2012; White i Perkins, 2012; Vogel i sur., 2017). Imunosni odgovor može varirati među jedinkama invazivne strane vrste unutar invazivnog areala. Ove varijacije dijelom proizlaze iz različitih interakcija imunosnog sustava svake jedinke s patogenim, komenzalističkim i mutualističkim mikrobima, a njihov uzrok mogu biti i uvjeti lokalnog okoliša, kao i ekološki procesi koji proizlaze iz širenja areala (Galloway i Depledge, 2001; Phillips i sur., 2010b; Brown i Shine, 2014; Brown i sur., 2015). Na primjer, tijekom širenja areala, invazivna strana vrsta može doći u područje s visokim pritiskom patogena, i/ili visokim kompetitivnim pritiskom, i/ili velikim brojem predatora, i/ili visokom razinom okolišnog stresa. U tom slučaju, jedinke invazivne strane vrste mogu preusmjeriti energetske resurse u imunosni odgovor, umjesto ulaganja u procese koji potiču rast, spolno sazrijevanje, razmnožavanje i širenje populacije (Keane, 2002; Zuk i Stoehr, 2002; Lee i Klasing, 2004; Phillips i sur., 2010b). Međutim, ulaganje u imunosni odgovor je energetski skupo (Klasing, 2004; Viney i sur., 2005), stoga postojeća ekološka istraživanja predlažu dvije suprotstavljene hipoteze o ulaganju u imunosni odgovor tijekom širenja invazivnog areala. Dolaskom u novi okoliš (na invazijsku frontu), jedinke invazivne strane vrste mogle bi pokazati:

a) Smanjeno ulaganje u imunosni odgovor

   Brzo širenje jedinki na invazijskoj fronti dovest će do smanjenja učestalosti patogena koji ostaju u starom okolišu (prema hipotezi o oslobađanju od neprijatelja, opisanoj u poglavlju 1.1.1.), posebice ako su jedinke koje prve dolaze u novi okoliš zdravije, tj.

manje opterećene patogenima (Phillips i sur., 2010a). Ovaj scenarij omogućio bi nižu zastupljenost patogena na invazijskoj fronti, a usto i potencijalno manju brzinu njihovog prijenosa jer je gustoća raspoloživih invazivnih domaćina na invazijskoj fronti niska. Niski pritisak patogena u okolišu rezultira manjim brojem imunosnih izazova za domaćine te smanjuje potrebu za snažnim imunosnim odgovorom (Torchin i sur., 2003; Phillips i sur., 2010a; White i Perkins, 2012). U konačnici, opisani procesi omogućili bi smanjeno ulaganje u imunosni odgovor, a povećano ulaganje energije u rast, reprodukciju i preživljavanje (tj. procese koji promiču širenje populacije), čime raste invazivni potencijal vrste (Blossey i Nötzold, 1995; Colautti i sur., 2004).

b) Povećano ulaganje u imunosni odgovor

Tijekom širenja areala, jedinke invazivne strane vrste vjerojatno će doći u kontakt s novim patogenima koji će ih kolonizirati (Kelly i sur., 2009), zbog čega bi jedinke na invazijskoj fronti trebale povećati ulaganje u imunosni odgovor (Therry i sur., 2014; Cornet i sur., 2016; Diagne i sur., 2016; Kołodziej-Sobocińska i sur., 2017). Nadalje, pretpostavlja se da širenje jedinki nije nasumično i da će se manje zaražene jedinke u populaciji širiti prve (Ochocki i Miller, 2017), a bolje fiziološko stanje ovih jedinki moglo bi ukazivati na njihov veći imunosni potencijal (Norris i Evans, 2000; Therry i sur., 2014). Stoga bi se imunosni odgovor jačeg intenziteta u jedinkama na invazijskoj fronti mogao pojaviti i kao neizravna posljedica selekcije jedinki bolje kondicije prilikom širenja areala (Therry i sur., 2014).

Jedinke na invazijskoj fronti mogle bi imati koristi i od smanjenog i od povećanog ulaganja u imunosni odgovor, stoga nije odmah očito koja će od ovih suprotstavljenih hipoteza prevladati tijekom širenja areala.

Dosadašnja istraživanja na temu kompromisa između ulaganja u imunosni odgovor i ulaganja u procese koji promiču širenje populacije kod invazivnih vrsta su malobrojna i uglavnom se odnose na kopnene kralježnjake (npr. Brown i sur., 2007; Brown i Shine, 2014; Poirier, 2019). Ova tema slabo je istražena kod beskralježnjaka, posebice vodenih, poput deseteronožnih rakova koji su ključni organizmi u slatkovodnim ekosustavima (Weinländer i Füreder, 2016).

1.2. Slatkovodni deseteronožni rakovi

Slatkovodni deseteronožni rakovi pripadaju u taksonomski nadred Astacidea, koji uključuje pet porodica s ukupno 38 rodova (Crandall i De Grave, 2017). Slatkovodni

deseteronožni rakovi vrlo su raznolika i evolucijski uspješna skupina: broje više od 700 vrsta rasprostranjenih diljem svijeta, iako je njihova rasprostranjenost u nekim dijelovima svijeta posljedica ljudskog djelovanja (Kawai i sur., 2016; Crandall i De Grave, 2017). Ova skupina uključuje najveće pokretne slatkovodne beskralježnjake prilagodljive fiziologije i ponašanja (Holdich, 2002), što im je omogućilo nastanjivanje vrlo širokog spektra staništa s različitim okolišnim uvjetima (Reynolds i sur., 2013). Slatkovodne deseteronožne rakove karakterizira dugi životni vijek, velika veličina tijela i omnivorni način prehrane, zbog čega imaju značajnu ulogu u hranidbenoj mreži slatkovodnih ekosustava (Momot, 1995; Holdich, 2002; Reynolds i sur., 2013). Zbog istovremenog utjecaja na više trofičkih razina, oni predstavljaju ključne vrste u slatkovodnim ekosustavima: u interakciji su s velikim brojem drugih vrsta u ekosustavu, upravljaju protokom energije između trofičkih razina i utječu na strukturu ili funkcije ekosustava (Momot, 1995; Reynolds i sur., 2013). Nadalje, slatkovodni deseteronožni rakovi nazivaju se i inženjerima ekosustava: svojim kretanjem i prehranom utječu na akumulaciju i prijenos sedimenta u slatkovodnim ekosustavima, čime mijenjaju karakteristike staništa i dostupnost resursa za ostale vrste u staništu (Johnson i sur., 2011; Reynolds i sur., 2013). K tome, neke vrste slatkovodnih deseteronožnih rakova ukopavaju se u sediment čime mijenjaju izgled obale vodnih tijela i/ili uzrokuju eroziju obale (Holdich, 2002; Reynolds i sur., 2013). Stoga su slatkovodni deseteronožni rakovi vrlo važna karika u slatkovodnim ekosustavima.

1.2.1. Mikrobiomi slatkovodnih deseteronožnih rakova

Iako su tijekom posljednjih godina istraživanja mikrobioma sve brojnija, ona su najčešće fokusirana na ljudski mikrobiom (i općenito, na mikrobiom kralježnjaka) zbog primjene u medicini, dok su mikrobiomi beskralježnjaka slabije istraženi (Petersen i Osvatic, 2018). Svega nekoliko radova bavi se mikrobiomima slatkovodnih deseteronožnih rakova. Najčešće je istraživan mikrobiom crijeva rakova, pogotovo u vrsta od ekonomskog značaja, s ciljem boljeg razumijevanja što čini zdravi mikrobiom uzgojnih vrsta (npr. Foysal i sur., 2020; Zhang i sur., 2020c; Liu i sur., 2020a; Shui i sur., 2020; Wu i sur., 2021), dok su istraživanja koja se bave drugim tkivima slatkovodnih deseteronožnih rakova i/ili interakcijama račjih mikrobnih zajednica i okoliša malobrojna (npr. Skelton i sur., 2017; Orlić i sur., 2021).

Metode sekvenciranja nove generacije tek su prije nekoliko godina postale cjenovno pristupačne i dostupne široj znanstvenoj zajednici (Kumar i sur., 2019), što djelomično može objasniti ovaj niski broj znanstvenih radova o račjim mikrobiomima. Međutim, čak je i znanje o patogenim mikrobima, koji su već desetljećima tema istraživanja, relativno skromno.

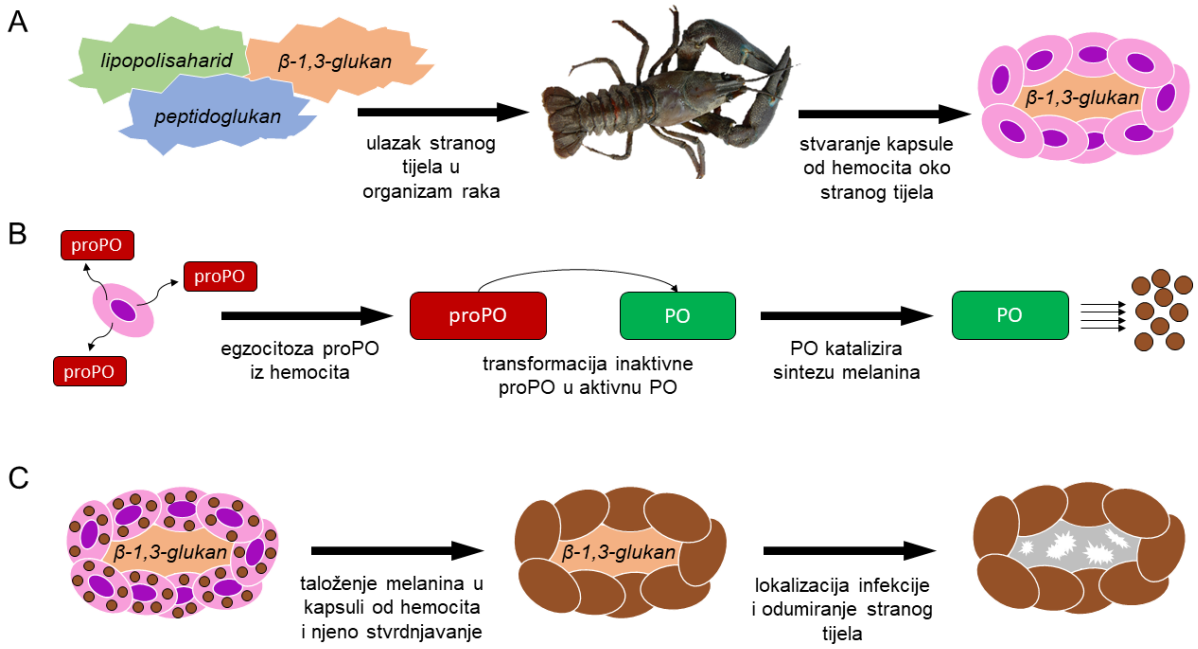
Patogeni mikrobi slatkovodnih deseteronožnih rakova istraživani su uglavnom u populacijama u akvakulturi (npr. Edgerton i Owens, 1993; Jones i Lawrence, 2001), dok su mikropatogeni u divljim populacijama često zapostavljeni u istraživanjima, osim u slučajevima kad su uzrokovali značajni mortalitet (npr. Grandjean i sur., 2019). Slatkovodni deseteronožni rakovi vrlo su osjetljivi na zarazu mikrogljivama i gljivama sličnim organizmima (npr. vodenim plijesnima; Bower i sur., 1994), od kojih je najpoznatiji patogen *A. astaci*, uzročnik bolesti račje kuge. Brojni gubici populacija slatkovodnih deseteronožnih rakova u divljini i akvakulturi uzrokovani račjom kugom rezultirali su izrazito velikom zastupljenošću patogena *A. astaci* u znanstvenim istraživanjima. Posljedično, to je dovelo do zanemarivanja ostalih mikrobnih patogena rakova: virusa, bakterija i drugih mikrogljiva i gljivama sličnih organizama, kojih je u posljednjih nekoliko desetljeća identificirano relativno malo (Edgerton i sur., 2004).

1.2.2. Imunosni sustav slatkovodnih deseteronožnih rakova

Kao i svi beskralježnjaci, slatkovodni deseteronožni rakovi nemaju stečenu imunost, tj. nemaju mogućnost proizvodnje specifičnih protutijela za određeni antigen (Söderhäll, 1999). Pri ulasku stranog tijela u organizam, rakovi se oslanjaju samo na mehanizme urođene imunosti: sintezu pigmenta melanina (Sritunyalucksana i Söderhäll, 2000), aktivaciju sustava koagulacije (Theopold i sur., 2004) i proizvodnju antimikrobnih peptida (Ferrandon i sur., 2007). Melanin sintetiziran tijekom imunosnog odgovora raka vrlo je važan mehanizam obrane budući da sudjeluje u procesu inkapsulacije stranog tijela, tj. „zatvaranja“ i izoliranja stranog tijela u melaninsku čahuru, čime mu onemogućuje daljnje širenje kroz organizam (Sritunyalucksana i Söderhäll, 2000).

Po ulasku u organizam raka, strano tijelo (npr. lipopolisaharide, peptidoglukane, beta-1,3-glukane, tj. dijelove bakterija, gljivica itd.) prepoznaju hemociti, što dovodi do nakupljanja dodatnih hemocita koji okružuju strano tijelo i stvaraju kapsulu (tzv. inkapsulacija) (Slika 1A; Gillespie i sur., 1997; Sritunyalucksana i Söderhäll, 2000). Istovremeno dolazi do aktivacije sustava profenoloksidaze. Profenoloksidaza (proPO), neaktivni prekursor enzima fenoloksidaze (PO), egzocitozom izlazi iz hemocita (specifično, granulocita i semigranulocita) u hemolimfu, gdje se pomoću serinske proteaze transformira u svoj aktivni oblik (tj. PO) (Johansson i Söderhäll, 1985; Söderhäll i Cerenius, 1998). Zatim PO katalizira sintezu melanina (Slika 1B), koji se taloži u kapsuli od hemocita, što rezultira stvrdnjavanjem kapsule i lokalizacijom infekcije te u konačnici, odumiranjem i/ili eliminacijom stranog tijela (Slika 1C; Nappi i sur., 1995; Gillespie i sur., 1997; Söderhäll i Cerenius, 1998). K tome, melanin sudjeluje

u procesima zacjeljivanja i (ponovnog) otvrdnjavanja kutikule nakon oštećenja (Sritunyalucksana i Söderhäll, 2000; Moreno-García i sur., 2013).



Slika 1. Aktivacija sustava profenoloksidaze i melanizacija stranog tijela. (A) Strano tijelo (lipopolisaharid, peptidoglukan, beta-1,3-glukan i sl.) ulazi u tijelo raka. Po ulasku, prepoznaju ga hemociti koji se zatim nakupljaju oko njega i zatvaraju ga u kapsulu. (B) Istovremeno dolazi do aktivacije sustava profenoloksidaze. Profenoloksidaza (proPO) egzocitozom izlazi iz hemocita u hemolimfu, gdje se pomoću serinske proteaze transformira u svoj aktivni oblik fenoloksidazu (PO). PO zatim katalizira sintezu melanina. (C) Melanin se taloži u kapsuli od hemocita, što vodi do stvrdnjavanja kapsule i lokalizacije infekcije te odumiranja stranog tijela. Autor slike: Paula Dragičević.

Dakle, mehanizam sinteze melanina uključuje brojne složene procese koji u konačnici rezultiraju odgovorom na imunosni izazov. Intenzitet imunosnog odgovora rakova moguće je odrediti mjerenjem nekoliko standardnih imunosnih parametara koji sudjeluju u ovom mehanizmu: ukupne koncentracije proPO u hemolimfi, enzimske aktivnosti PO, ukupnog broja hemocita u hemolimfi i snage reakcije inkapsulacije (tj. razine melanizacije). S obzirom na to da su navedeni imunosni parametri međusobno ovisni, intenzitet imunosnog odgovora oblikovan je njihovim zajedničkim djelovanjem.

1.2.3. Slatkovodni deseteronožni rakovi u ulozi invazivnih stranih vrsta

Zbog karakteristika i uloga slatkovodnih deseteronožnih rakova u slatkovodnim ekosustavima (navedenih u poglavlju 1.2.), nestanak ili pojava nove račje vrste u nekom vodnom tijelu može dovesti do značajnih promjena i posljedica za ostale vrste u ekosustavu. Mnoge račje vrste cijenjene su u ljudskoj prehrani, što je dovelo do njihovog prekomjernog izlova u prirodnom staništu i znatnog smanjenja njihovih populacija (Reynolds i sur., 2013). Osim toga, smanjenje i/ili nestanak prirodnih populacija zavičajnih vrsta rakova uzrokovani su i nizom drugih čimbenika, kao što su zagađenje, klimatske promjene i modifikacije staništa (Kawai i sur., 2016). S druge strane, neke su račje vrste, za potrebe prehrambene industrije i uzgoja u akvakulturi, unesene u nova područja gdje nisu prirodno rasprostranjene (Holdich, 2002). Određeni postotak jedinki ovih unesenih vrsta pobjegao je iz akvakulture u novi okoliš, gdje su uspostavile alohtone populacije. Usto, neke su račje vrste slučajno unesene u novi okoliš: kao neiskorišteni ili „odbjegli" riblji mamci, ili kao neželjeni akvarijski ljubimci (Souty-Grosset i sur., 2016). Slučajne i namjerne translokacije račjih vrsta koje pokazuju izrazitu fenotipsku plastičnost i eurivalentnost, nemali broj puta rezultirale su njihovim uspješnim uspostavljanjem i širenjem populacije u novom okolišu i brojnim negativnim posljedicama za zavičajne vrste i ekosustav, tj. biološkim invazijama.

Tijekom prošlih nekoliko desetljeća, zabilježeni su brojni negativni učinci invazivnih stranih vrsta slatkovodnih deseteronožnih rakova u novom okolišu: smanjenje biomase i raznolikosti makrobeskralježnjaka, makrofita i perifitona (npr. Lodge i Lorman, 1987), smanjenje i nestanak populacija zavičajnih vrsta slatkovodnih deseteronožnih rakova (npr. Light i sur., 1995), unos novih patogena (npr. Diéguez-Uribeondo i Söderhäll, 1993), modifikacija staništa (npr. Rodríguez i sur., 2003), degradacija staništa (npr. Rodríguez i sur., 2005) te ekonomski gubici uzrokovani štetnim djelovanjem ovih vrsta (npr. Anastácio i sur., 2005), ali i visokim troškovima upravljanja i eradikacije (npr. Williams i sur., 2010). Stoga slatkovodni deseteronožni rakovi u ulozi invazivnih stranih vrsta predstavljaju jednu od najvećih prijetnji bioraznolikosti, strukturi i funkcioniranju slatkovodnih ekosustava.

1.2.3.1. Signalni rak *Pacifastacus leniusculus* (Dana, 1852)

Jedna od najuspješnijih invazivnih stranih vrsta slatkovodnih deseteronožnih rakova u Europi je signalni rak *Pacifastacus leniusculus* (Dana, 1852) (Slika 2). Ova vrsta prirodno je rasprostranjena na zapadu sjevernoameričkoga kontinenta (Lewis, 2002), ali ljudskim djelovanjem proširena je u novi okoliš: u Japan (Hiruta, 1996) i diljem Europe (Kouba i sur.,

2014). U Europu je prvi put unesena tijekom 1960-ih godina, u skandinavske zemlje, gdje su zavičajne europske vrste rakova (posebice vrste od akvakulturnog značaja) bile pogođene bolešću račje kuge, koja je uzrokovala značajno smanjenje broja i veličina njihovih populacija te ekonomske gubitke. Stoga je namjera bila zamijeniti uzgojne (zavičajne) vrste osjetljive na račju kugu, vrstama otpornima na račju kugu koje imaju otprilike podjednaku konzumnu vrijednost, tj. signalnim rakom (Lowery i Holdich, 1988). Međutim, signalni rak se, nakon unošenja u Europu, počeo nekontrolirano širiti europskim slatkovodnim ekosustavima.

Slika 2. Signalni rak *Pacifastacus leniuculus* (Dana, 1852). Autor fotografije: Paula Dragičević.

Važnu ulogu u invazivnom uspjehu signalnoga raka imaju njegove biološke osobine kao što su brzi rast, spolno sazrijevanje u ranoj dobi i visoki fekunditet, ali i visoka razina agresivnosti prilikom kompetitivnih interakcija s drugim jedinkama rakova (Söderbäck, 1991; Usio i sur., 2001; Gherardi, 2006; Pintor i sur., 2008). Ove osobine omogućuju signalnom raku brzo širenje

populacije te istiskivanje populacija zavičajnih vrsta rakova u područjima njihovog kontakta (Hudina i sur., 2016). Uz nabrojene osobine, negativnom učinku ove vrste doprinosi i činjenica da je signalni rak prijenosnik račje kuge, bolesti koja ima poguban utjecaj na populacije zavičajnih europskih rakova (Bohman i sur., 2006). Invazivne američke vrste rakova, kao što je signalni rak, otporne su na bolest račje kuge budući da su koevoluirale s njenim uzročnikom, patogenom *A. astaci*. Također, signalni rak pokazuje kontinuirano povišenu razinu ekspresije gena za proPO, koja je temelj za sintezu PO i melanizaciju, tj. lokalizaciju, infekcije (opisano u poglavlju 1.2.2.), što ovoj vrsti omogućuje brz imunosni odgovor (Cerenius i sur., 2003) te se bolest račje kuge ne razvija, ali su jedinke latentno zaražene i vektori su širenja patogena. Nasuprot tome, imunosni sustav zavičajnih vrsta rakova reagira tek po kontaktu s patogenom, zbog čega im je imunosni odgovor sporiji. Širenje signalnoga raka Europom rezultiralo je gubitkom populacija zavičajnih vrsta rakova u mnogim europskim državama tijekom posljednjih nekoliko desetljeća (npr. Söderbäck, 1995; Usio i sur., 2001; Westman i sur., 2002; Bohman i sur., 2006). Stoga, zbog izrazito negativnih učinaka na bioraznolikost i ekosustave u Europi, signalni rak je uvršten na popis invazivnih stranih vrsta koje izazivaju zabrinutost u Uniji, za koje države članice trebaju razviti učinkovite mjere upravljanja (uredba EU 1143/2014).

Signalni rak rasprostranjen je u kontinentalnom dijelu Hrvatske (Maguire i sur., 2018). Prvi put je u Hrvatskoj zabilježen 2008. godine u rijeci Muri (Maguire i sur., 2008), u koju je došao iz slovenskoga dijela Mure nizvodnim širenjem (Bertok i sur., 2003; Govedič 2006; Govedič i sur., 2007). Daljnjim širenjem niz rijeku Muru, ušao je u rijeku Dravu gdje nastavlja širenje populacije jednom od najbržih stopa u Europi (18 – 24,4 kilometara godišnje; Hudina i sur., 2009). U 2011. godini zabilježen je i u krškoj rijeci Korani (Slika 3), gdje se smatra da je ilegalno unesen (Hudina i sur., 2013). U Korani signalni rak uspješno širi svoj invazivni areal uzvodno (prema jugu, tj. Plitvičkim jezerima) i nizvodno (prema sjeveru, tj. gradu Karlovcu; Hudina i sur., 2017). Izvor rijeke Korane nalazi se u Nacionalnom parku Plitvička jezera (Roglić, 1974), a samu rijeku i njezine pritoke nastanjuju populacije tri zavičajne vrste slatkovodnih deseteronožnih rakova: riječni rak *Astacus astacus* (Linnaeus, 1758), potočni rak *Austropotamobius torrentium* (von Paula Schrank, 1803) i uskoškari rak *Pontastacus leptodactylus* (Eschscholtz, 1823) (Maguire i Gottstein-Matočec, 2004; Maguire i sur., 2011; Hudina i sur., 2013). Pritom su riječni i potočni rak u nepovoljnom statusu koji se pogoršava u većini europskih biogeografskih regija (https://nature-art17.eionet.europa.eu/article17/species/summary/?period=5&group=Arthropods&subject=Austropotamobius+torrentium&region=),

zbog čega su zaštićeni na nacionalnoj (Zakon o zaštiti prirode, NN 80/2013) i međunarodnoj razini (Zakon o potvrđivanju konvencije o zaštiti europskih divljih vrsta i prirodnih staništa (Bernska konvencija), Dodatak III, NN 6/2000). Stoga, širenje signalnoga raka duž rijeke Korane predstavlja prijetnju zavičajnim vrstama slatkovodnih deseteronožnih rakova u Hrvatskoj, a njegov negativni utjecaj vidljiv je na primjeru uskoškaroga raka čije populacije signalni rak postupno smanjuje i s vremenom potpuno zamjenjuje (Hudina i sur., 2013; Rebrina i sur., 2015).

Slika 3. Rijeka Korana, u koju je signalni rak ilegalno unesen. Autor fotografije: doc. dr. sc. Sandra Hudina.

Navedena populacija u rijeci Korani izabrana je za provedbu ovog istraživanja jer je unesena relativno nedavno i njezino se širenje redovito prati, stoga je moguće odrediti invazijsko središte i invazijsku frontu (uzvodnu i nizvodnu) te usporediti karakteristike (mikrobiom i imunosni odgovor) jedinki na invazijskim frontama s jedinkama u invazijskom središtu. Nadalje, na temelju rastućeg znanja o odrednicama invazivnog uspjeha signalnoga raka u Europi, javlja se

potreba za razvojem novih i proširenih planova upravljanja populacijom signalnoga raka u Korani s ciljem zaštite populacija zavičajnih vrsta slatkovodnih deseteronožnih rakova. Kako bi se razvile učinkovite mjere upravljanja invazivnim stranim vrstama, nužno je ispitati ulogu mikrobnih zajednica i imunosnog sustava u uspješnom širenju areala, odnosno invazivnom uspjehu, te identificirati patogene u njihovim populacijama.

1.3. Ciljevi istraživanja

Smatra se da jedinke koje se šire na rubovima invazivnog areala nisu nasumično odabrane unutar populacije, odnosno, jedinke koje prve dolaze na invazijsku frontu imaju određene karakteristike te njihova nenasumična akumulacija na invazijskoj fronti posljedično dovodi do značajnih razlika u osobinama naspram jedinki koje ostaju u invazijskom središtu (engl. *spatial sorting theory;* Shine i sur., 2011; Phillips, 2016). Prethodnim istraživanjima zabilježene su razlike u karakteristikama jedinki signalnoga raka duž invazivnog areala u rijeci Korani: na invazijskim frontama pronađeno je više mužjaka nego ženki, ženke su pokazivale bolji energetski status hepatopankreasa i gonada te su, općenito, jedinke na invazijskim frontama bile manje agresivne, boljeg tjelesnog i fiziološkog stanja u odnosu na jedinke iz invazijskog središta (Hudina i sur., 2015; Rebrina i sur., 2015). S obzirom na ove, prethodno zabilježene razlike među jedinkama, za očekivati je da će postojati i razlike u mikrobnim zajednicama i imunosnom odgovoru jedinki duž invazivnog areala. Usto, budući da se mikrobne zajednice i imunosni sustav međusobno određuju, za očekivati je da će se oboje mijenjati duž invazivnog areala, tj. procesa invazije.

Cilj istraživanja je istražiti promjene u sastavu mikrobnih zajednica tijekom širenja invazivnog areala signalnoga raka te ulogu imunosnog odgovora u tom procesu.

Specifični ciljevi su:

1. Ispitati postoje li razlike u sastavu mikrobnih zajednica u različitim tkivima i duž invazivnog areala.

2. Temeljem pretraživanja dostupne literature, klasificirati identificirane mikroorganizme rakova na potvrđene patogene, potencijalne patogene, nepatogene i one za koje ne postoje podaci o patogenosti, te ustanoviti postoji li razlika duž invazivnog areala u njihovoj zastupljenosti.

3. Ispitati postoje li razlike u imunosnom odgovoru jedinki duž invazivnog areala.

Radi bolje preglednosti, specifični ciljevi su u raspravi spojeni u cjeline koje opisuju: identifikaciju mikrobnih patogena signalnoga raka u rijeci Korani (poglavlje 3.1.) i razlike u mikrobiomu i imunosnom odgovoru signalnoga raka duž invazivnog areala u rijeci Korani (poglavlje 3.2.) te su na temelju dobivenih rezultata dane smjernice za daljnja istraživanja (poglavlje 3.3.).

2. ZNANSTVENI RADOVI

Znanstveni rad br. 1 **Dragičević P**, Faller M, Kutleša P, Hudina S (2020) Update on the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) range expansion in Croatia: a 10-year report. BioInvasions Records 9: 793-807.

Znanstveni rad br. 2 **Dragičević P**, Bielen A, Petrić I, Hudina S (2021) Microbial pathogens of freshwater crayfish: A critical review and systematization of the existing data with directions for future research. Journal of Fish Diseases 44: 221-247.

Znanstveni rad br. 3 **Dragičević P**, Bielen A, Petrić I, Vuk M, Žučko J, Hudina S (2021) Microbiome of the Successful Freshwater Invader, the Signal Crayfish, and Its Changes along the Invasion Range. Microbiology Spectrum 9: e00389-21.

Znanstveni rad br. 4 **Dragičević P**, Grbin D, Maguire I, Blažević SA, Abramović L, Tarandek A, Hudina S (2021) Immune Response in Crayfish Is Species-Specific and Exhibits Changes along Invasion Range of a Successful Invader. Biology 10: 1102.

Znanstveni rad br. 1

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Research Article

Update on the signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) range expansion in Croatia: a 10-year report

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Abstract

The signal crayfish (*Pacifastacus leniusculus*) is considered to be the most successful crayfish invader in Europe. It is present across the continental part of Croatia, with first records from the Mura-Drava river basin in 2008 and from the karstic Korana River in 2011. In both rivers, *P. leniusculus* presents an imminent threat to indigenous crayfish species (ICS). The aim of the study was to explore the range expansion of *P. leniusculus* 10 years after its first record in Croatia. Based on the data collected via an extensive literature search and our own fieldwork performed in 2018, we here demonstrated that *P. leniusculus* is the most successful non-indigenous crayfish species (NICS) in Croatia, with the invasion range covering the largest extent of Croatia compared to other NICS. Its dispersal rates in the rivers of the continental part of Croatia have remained among the highest in Europe. In the Drava River, recorded dispersal rate (21.3 km/yr) corresponded to the predictions made by the earlier study that estimated *P. leniusculus* dispersal over 10 years. However, due to the low number of caught individuals along the 128 km of the Drava River course, dispersal rates as well as invasion pathways (natural *vs.* human-mediated) need to be further explored. The predicted encounter of *P. leniusculus* with the invasive *Faxonius limosus* was not observed. In the Korana River, we observed a continued expansion of *P. leniusculus* invasion range in both upstream and downstream direction. Dispersal rates were higher in the upstream direction, potentially due to differences in competitive pressure by the native crayfish, *Pontastacus leptodactylus*, which abundances were 6 times higher at the downstream invasion front. In both rivers, a displacement of the native *P. leptodactylus* has been observed. Even though *P. leptodactylus* has been considered to be the most robust ICS, that successfully expands its range in water bodies of Croatia and Europe, it is also the species the most adversely affected by the range expansion of the *P. leniusculus* in the continental part of Croatia. Thus, the status of *P. leptodactylus* in Croatia should be monitored and re-examined.

Key words: non-indigenous crayfish species, dispersal, distribution, competitive pressure

Introduction

The introduction of non-indigenous crayfish species (NICS) is among the major causes of decline of indigenous crayfish species (ICS) in freshwater ecosystems in Europe (Holdich et al. 2009) and worldwide (Taylor 2002).

During the last two decades, three NICS have been recorded in Croatian freshwater ecosystems: the spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), and the marbled crayfish *Procambarus virginalis* Lyko, 2017 (Samardžić et al. 2014; Maguire et al. 2018). These species possess advantageous life history traits such as fast growth, early maturation, and high fecundity, as well as higher aggression in competitive interactions (Söderbäck 1991; Usio et al. 2001; Gherardi 2006; Pintor et al. 2008) and can outcompete ICS. More importantly, they are chronic carriers of the crayfish plague pathogen *Aphanomyces astaci* Schikora, 1906 (Holdich et al. 2009; Keller et al. 2014; Svoboda et al. 2017), which is listed among the 100 World's Worst Invasive Alien Species (Lowe et al. 2000). Crayfish plague is considered to be a major driver of the decline of the European ICS, although recent records suggest that some ICS populations can exhibit resistance to crayfish plague (e.g. *Astacus astacus* (Linnaeus, 1758): Jussila et al. 2011; Makkonen et al. 2012; *Austropotamobius pallipes* (Lereboullet, 1858): Martín-Torrijos et al. 2017; *Austropotamobius torrentium* (Schrank, 1803): Kušar et al. 2013; *Pontastacus leptodactylus* (Eschscholtz, 1823): Kokko et al. 2018), even to its most virulent genotypes (Martín-Torrijos et al. 2017; Kokko et al. 2018).

Pacifastacus leniusculus is currently the most widespread NICS in Europe and, according to the EU Regulation on invasive alien species No. 1143/ 2014, is on the list of species of EU Concern. It has been recorded in 29 European countries (Kouba et al. 2014) with the most recent records reported from EU candidate countries bordering with Croatia (Bosnia and Herzegovina; Trožić-Borovac et al. 2019). In Croatia, it is distributed across the continental part of the country, in the drainage system of the Sava and the Drava Rivers (Maguire et al. 2018). *Pacifastacus leniusculus* was first recorded in Croatia in 2008, in the Mura River (Maguire et al. 2008), where it spread as a result of the natural downstream dispersal of Slovenian and Austrian populations (Bertok et al. 2003; Govedič 2006; Govedič et al. 2007). It spread further downstream through the Mura to the Drava River at dispersal rate of 18–24.4 km/yr, which is among the highest recorded rates in Europe (Hudina et al. 2009). In 2011, it was recorded in the Korana River in the continental part of Croatia, where it was illegally introduced (Hudina et al. 2013). Here, it presents an immense threat to ICS diversity, since three out of four Croatian ICS inhabit Korana River and its tributaries (Hudina et al. 2017). In the Korana River *P. leniusculus* is expanding its range both upstream and downstream with the dispersal rate of 2.23–2.84 km/yr (Hudina et al. 2017) and gradually displaces the indigenous narrow-clawed crayfish *P. leptodactylus* (Hudina et al. 2013; Rebrina et al. 2015).

The aim of this study was to estimate *P. leniusculus* distribution and dispersal rates in Croatia, a decade after its first record (Maguire et al. 2008). In a study by Hudina et al. (2009) it was estimated that at the recorded

dispersal rate (18–24.4 km/yr), in 10 years *P. leniusculus* would come in contact with *F. limosus* in the lower section of the Drava River, 50 km upstream from the confluence with the Danube. Therefore, the additional aim was to examine whether predicted range expansion rates in the Drava River were accurate and whether contact with *F. limosus* has occurred as predicted by the study by Hudina et al. (2009). Finally, while there were recent studies of *F. limosus* range expansion in the Danube and Drava Rivers and its tributaries in Croatia (Maguire et al. 2018), such data for *P. leniusculus* downstream dispersal in the Drava River are lacking. The collected data provide an insight into the extent of the invasion, current distribution of *P. leniusculus* populations in Croatia and their distance from the native crayfish populations, as well as into potential management implications for both the invasive *P. leniusculus* and the native crayfish in the region.

Materials and methods

The field study was performed in the continental part of Croatia, in the Drava and the Korana Rivers. Both rivers belong to the Danube River basin.

The Korana River is a 134 km long karstic river belonging to the Sava River basin, with numerous natural and man-made cascades along the whole course of its length. Its springs are located at the end of a chain of sixteen barrage lakes in the Plitvice Lakes National Park. The river flows northward (Roglić 1974) to its confluence with the Kupa River in the town of Karlovac. Due to its karstic character and the man-made cascades, the discharge averages at approximately 29 m^3/s (cf. Rebrina et al. 2015). Korana River and its tributaries are inhabited by populations of three ICS: *A. astacus*, *P. leptodactylus* and *A. torrentium* (Maguire and Gottstein-Matočec 2004; Maguire et al. 2011; Hudina et al. 2013). *Astacus astacus* (recorded in the Drava and Korana River and tributaries) and *A. torrentium* (recorded in the Korana River and its tributaries) are protected on both national (Nature Protection Act OG no. 80/13), and European level (listed in Appendix III of the Bern Convention).

The Drava River is a 719 km long tributary of the Danube River, with its source in Italy. It flows through Austria, Slovenia, Croatia and Hungary, and has a mouth into the Danube River in Croatia, near the city of Osijek. In the upper reaches of the river (up to the Donja Dubrava impoundment in Croatia, rkm 254), more than 20 dams have been constructed. The lower reaches of the Drava River have been considerably regulated with embankments and channels (Hudina et al. 2009). The discharge of the Drava River in Croatia averages around 500 m^3/s (Rabi et al. 2015). Two ICS were recorded in the Drava River basin in Croatia: *A. astacus*, which was more numerous in the upper reaches of the Drava River in Croatia, and *P. leptodactylus*, which was more numerous in its lower reaches (Maguire and Gottstein-Matočec 2004).

For this study, data gathered via a comprehensive literature search and our own fieldwork were used. The literature search included studies examining the distribution of *P. leniusculus* in Croatia (Maguire et al. 2008, 2018; Hudina et al. 2009, 2012, 2013, 2017; Rebrina et al. 2015). This was followed by conducting a field study on the Korana and Drava Rivers. Throughout this study, invasion core refers to population established for some time now and with higher crayfish abundance, while invasion front refers to recently established populations from the very edge of the range with significantly lower relative crayfish abundance. In both the Korana and the Drava River, the fieldwork was conducted during the period of increased crayfish activity of both sexes (i.e. before mating period), in the early autumn of 2018. In the Korana River *P. leniusculus* distribution was analysed at a total of 8 sites distributed along 33 km of the watercourse: two invasion core sites, two invasion front sites from 2015 (upstream and downstream invasion front from a study by Hudina et al. 2017; hereon former invasion fronts) and four additional sites – one upstream and three downstream from these former invasion fronts. In the Drava River, range expansion was examined at a total of 17 sites downstream from the last invasion site recorded in the literature (Maguire et al. 2011). Sampling covered 128 km of the Drava River watercourse. Upstream dispersal of *P. leniusculus* through the Drava River was not monitored within this study, since recent research by Maguire et al. (2018) found no *P. leniusculus* records in this section of the river (upstream from the confluence with the Mura River). Smaller number of sites was investigated in the Korana River compared to the Drava River since *P. leniusculus* range expansion in the Korana River has been more frequently monitored (last monitoring: 2015 in the Korana River, 2011 in the Drava River) and since recorded dispersal rates are approximately 9 times lower in the Korana compared to the Drava River (Hudina et al. 2009, 2017).

Crayfish were captured using baited LiNi traps (Westman et al. 1978). At least five traps were exposed per 100 m of the watercourse (Maguire 2014) at each site and were left in the water overnight. All captured crayfish were identified to the species level. We counted the number of caught individuals per trap and determined their sex. Upon data collection, captured ICS were returned to the same location where they were caught, while NICS were taken to the laboratory for further analyses within the frame of the project STRIVE (https://www.pmf.unizg.hr/strive/en) which included the analyses of crayfish plague presence (Pavić et al. 2020). Based on the collected data, we calculated the catch per unit effort (CPUE, equal to the number of crayfish captured per LiNi trap per trapping night) for every trapping session for each site and each species. CPUE is a frequently used measure of relative crayfish abundance (Dana et al. 2010) and was used to compare the relative abundance of *P. leniusculus* between the sites and relative abundance of different crayfish species (ICS *vs.* NICS) within

the sites, in the case of mixed populations. After each fieldwork, the traps were washed in disinfectant solution using Chlormax (Genera, Croatia) in order to prevent potential cross-contamination and spreading of the crayfish plague between the sites.

Position of sampling sites was recorded using a Garmin GPS map 60CSx (projection WGS84). Collected data were analysed using descriptive statistic, and geographical presentations of crayfish distribution were performed by ArcGis 10.1 program package (ESRI Inc.).

Results

The Korana River

In the Korana River, a total of 523 crayfish were caught, belonging to two species: *P. leniusculus* (NICS: 415 individuals, 79.3% of all captured crayfish) and *P. leptodactylus* (ICS: 108 individuals, 20.7% of all captured crayfish). *Pacifastacus leniusculus* was recorded at 6 (75%) of the examined sites, while *P. leptodactylus* was found at 5 (62.5%) sites. At 3 sites (sites 1, 10 and 11; Figure 1) mixed populations of the invasive *P. leniusculus* and the native *P. leptodacytlus* were recorded (Table 1). Since 2015, *P. leniusculus* range increased by the total of 5 km: 4 km in the upstream direction (dispersal rate: 1.3 km/yr) and 1 km in the downstream direction (dispersal rate: 0.3 km/yr) from the former invasion fronts (Figure 1). The invasion range of *P. leniusculus* now stretches along a total of 30 km of the lower course of the Korana River, covering approximately 22.4% of the entire watercourse.

Out of all captured *P. leniusculus* individuals, 362 individuals (87.2%) were captured at the invasion core (sites 4 and 8; Figure 1), while 23 individuals (5.5%) were captured at each of the former invasion fronts (sites 2 and 10; Table 1). At new upstream invasion front (site 1; Figure 1) 3 individuals (0.7% of all caught *P. leniusculus*) were captured, while 4 individuals (1%) were captured at new downstream invasion front (site 11; Figure 1). The new downstream invasion front is 1.8 km upstream from the confluence with the Mrežnica River (site 12; Figure 1). No NICS were recorded downstream from the confluence with the Mrežnica River (Table 1).

The native *P. leptodactylus* was recorded at both *P. leniusculus* invasion fronts (sites 1 and 11; Figure 1). In both cases, *P. leptodactylus* were more numerous than *P. leniusculus* (12.9 times higher CPUE at the downstream invasion front and 2.5 times higher CPUE at upstream invasion front; Table 1). Finally, *P. leptodactylus* abundance was 6 times higher at the downstream invasion front, compared to its abundance at the upstream invasion front of *P. leniusculus*. No *P. leptodactylus* were recorded at sites with very high *P. leniusculus* relative abundance (invasion core: sites 4 and 8; Figure 1).

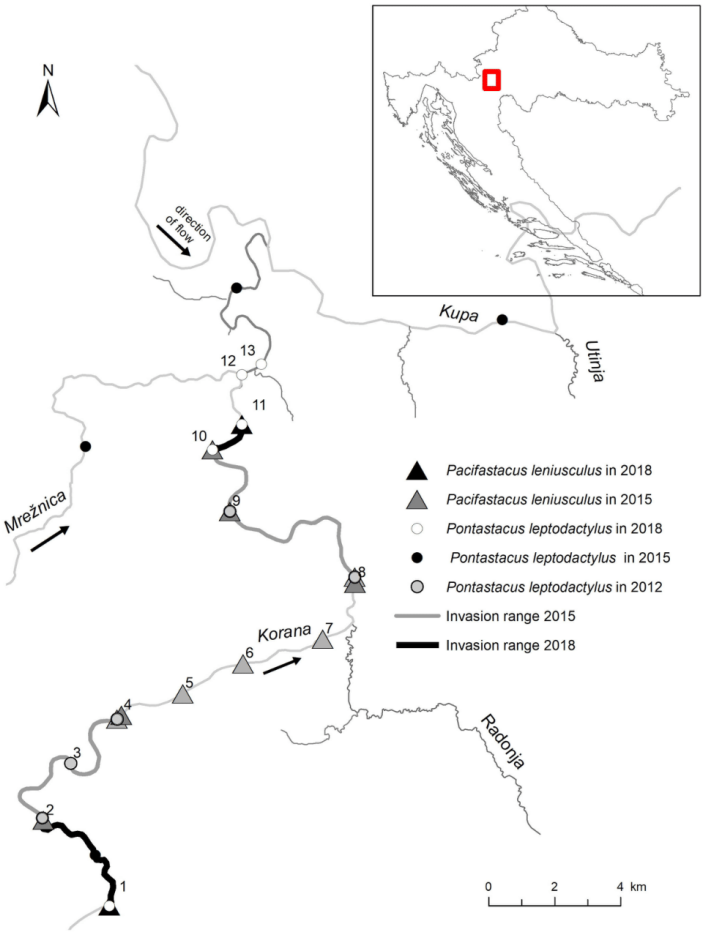

Figure 1. The distribution of *Pacifastacus leniusculus* in the Korana River, Croatia. The bold black line represents the increase in invasion range since 2015 (invasion range 2018), while the bold grey line represents the increase in invasion range from 2012–2015 (i.e. invasion range 2015). Site numbers are presented for both literature data and the fieldwork conducted within this study. Sites where *P. leniusculus* was recorded are labelled with triangles, while *P. leptodactylus* records are labelled with circles. Fieldwork in 2018 covered sites 4 and 8 (invasion core), 2 and 10 (former invasion fronts), 1 and 11 (new invasion fronts determined within the study), and sites 12 and 13 (no signal crayfish recorded).

The Drava River

During the study performed in the autumn of 2018, no ICS and only 4 NICS individuals were caught in the Drava River (Table S1): *P. leniusculus* (3 individuals; 75% of all captured crayfish) and *F. limosus* (1 indivudual, 25% of all captured crayfish). *Pacifastacus leniusculus* were captured at 3 sites, while *F. limosus* was recorded only at 1 site (Table 1; Table S1). In seven years, the invasion range of *P. leniusculus* has increased by approximately 148.9 km in the downstream direction from the former invasion front

Table 1. Number of captured crayfish individuals per site and catch per unit effort (CPUE; the number of crayfish captured per LiNi trap per trapping night) in the Drava and Korana Rivers during fieldwork in 2018. Crayfish numbers (No. Pl, Fl and Plept) and CPUE (CPUE Pl, Fl, Plept) are presented separately for each species of NICS: *P. leniusculus* (*Pl*) and *F. limosus* (*Fl*), and ICS *P. leptodactylus* (*Plept*). Site codes with associated GPS coordinates (X, Y) correspond to codes in Figures 1 and 2.

Water body	X (WGS84)	Y (WGS84)	Site code	No. *Pl*	No. *Fl*	No. *Plept*	CPUE *Pl*	CPUE *Fl*	CPUE *Plept*
Drava	45.920623	17.59068	2	1	0	0	0.33	0	0
Drava	45.858044	17.64762	3	1	0	0	0.2	0	0
Drava	45.699705	18.391038	8	1	0	0	0.2	0	0
Drava	45.60079	18.589261	12	0	1	0	0	0.2	0
Korana	45.319775	15.517625	1	3	0	8	0.043	/	0.107
Korana	45.343504	15.491309	2	23	0	0	1.1	/	0
Korana	45.371911	15.52147	4	134	0	0	8.93	/	0
Korana	45.408823	15.611404	8	228	0	0	12	/	0
Korana	45.445223	15.5578	10	23	0	13	1.577	/	1.39
Korana	45.451736	15.566728	11	4	0	75	0.106	/	1.366
Korana	45.465374	15.566272	12	0	0	11	0	/	2.2
Korana	45.468247	15.573795	13	0	0	1	0	/	0.25

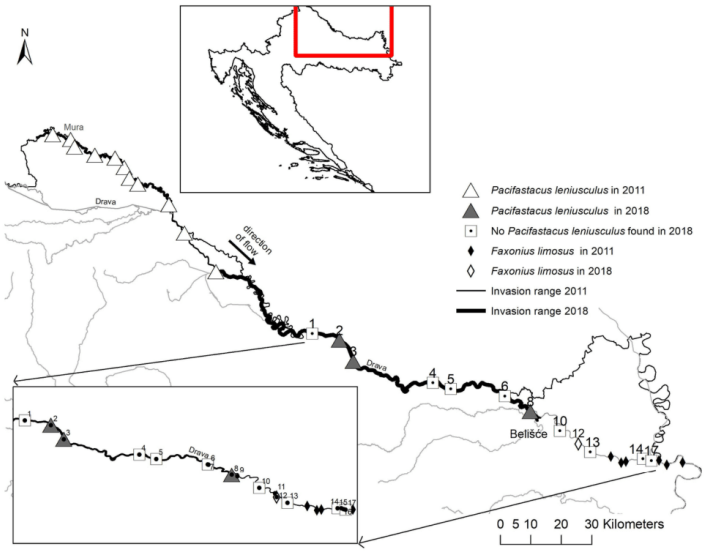

Figure 2. The invasion range of the signal crayfish in the Drava River, Croatia. The bold line along the river represents the invasion range recorded in 2018. Sites examined in 2018 are labelled by numbers (1–17). The figure shows both literature records of *P. leniusculus* (white triangles) and fieldwork records from 2018 (grey triangles). *Faxonius limosus* literature data for the Drava River (black diamond shapes) and 2018 fieldwork records (white diamond shapes) are also shown. The new *P. leniusculus* invasion front recorded in this study is located at site 8.

established in 2011 (Figure 2), which corresponds to dispersal rate of 21.3 km/yr. The new invasion front is located only 3.3 km upstream from the predicted point of contact between *P. leniusculus* and *F. limosus* by Hudina et al. (2009).

Only one individual of *F. limosus* was captured in the Drava River, at the Karašica River mouth (site 12, Figure 2), 15.3 km upstream from its former invasion front in the Drava (Hudina et al. 2009; Figure 2). This latest *F. limosus* record is located 25 km downstream from the current *P. leniusculus* invasion front.

Discussion

Range expansion constitutes a crucial element of invasion success and understanding its dynamics is a prerequisite for assessment of NICS regional and global impacts, as well as prioritization of sites and species for NICS management (Manfrin et al. 2019). While range expansion of one of the most successful NICS invaders in Europe, *P. leniusculus*, has been explored and monitored all over Europe (UK: Peay 1997; Peay and Rogers 1999; Bubb et al. 2004, 2005; Portugal: Bernardo et al. 2001, 2011; Croatia: Hudina et al. 2009, 2013, 2017; Maguire et al. 2011, 2018; Austria: Weinländer and Füreder 2009, 2012; Finland: Erkamo et al. 2010; Jussila et al. 2014; Ruokonen et al. 2018; Poland: Dobrzycka-Krahel et al. 2017) only several studies regularly monitored its long-term spread or prioritized sites for its potential management.

In this study, we present the results of a decade long monitoring effort of range expansion of *P. leniusculus*. The obtained results show that 10 years after its first record in Croatia, *P. leniusculus* continues to successfully expand its range in Croatian freshwater ecosystems. Out of all NICS currently recorded in Croatia, *P. leniusculus* is the most widespread due to its high dispersal rates and wide invasion range which covers a large portion of continental part of Croatia. Also, the proximity of its populations to endangered and protected ICS populations, as well as to protected areas (Plitvice Lakes National park), and the recorded presence of crayfish plague pathogen in both Drava and Korana populations (Maguire et al. 2016; Pavić et al. 2020) highlight its prominent threat to native Croatian astacofauna.

In the Korana River, the invasive range of *P. leniusculus* has increased by a total of 5 km in three years and now stretches along a total of 30 km of the river. *P. leniusculus* is expanding its range both upstream and downstream from the invasion core, but at approximately nine times slower rate than *P. leniusculus* populations in the Drava River. Korana River has much lower average discharge (29 m^3/s) compared to the Drava (500 m^3/s) and has numerous natural and man-made cascades along the whole course of its length (cf. Hudina et al. 2017), and both of these factors may influence both downstream and upstream invasion (Holdich et al. 2014). Thus, slower dispersal rates could be attributed to the river hydromorphology, but also to the differences in invasion pathways – natural dispersal in the Korana River versus undiscerned invasion pathway in the Drava (discussed in the later sections).

The recorded downstream dispersal rates of *P. leniusculus* in the Korana River (0.3 km/yr) are somewhat lower compared to other studies (1–7 km/yr: Peay 1997; Peay and Rogers 1999; Bubb et al. 2004, 2005; Weinländer and Füreder 2009; Bernardo et al. 2011), while upstream dispersal (1.3 km/yr) is similar to existing literature data (0.35–4 km/yr: Bubb et al. 2005; Weinländer and Füreder 2009; Bernardo et al. 2011). Since the last study in

2015 (Hudina et al. 2017), *P. leniusculus* dispersal rate has decreased in both downstream (6.5 times) and upstream (2 times) direction. Previous studies have reported that *P. leniusculus* dispersal rates are not constant (cf. Holdich et al. 2014) and that natural barriers can slow down both the upstream and the downstream invasion of *P. leniusculus* (Peay and Rogers 1999; Rosewarne et al. 2013; Bubb et al. 2004). In general, downstream dispersal is expected to be faster than movement upstream (Bubb et al. 2005), however recorded dispersal rates in the Korana River were higher in the upstream than in the downstream direction. Differences between upstream and downstream dispersal rates typically depend on the river characteristics (i.e. river gradient and the existence of barriers; Bubb et al. 2005; Bernardo et al. 2011) and in some cases are only weakly biased in downstream direction (Holdich et al. 1995; Guan and Wiles 1999). Also, high discharge events can facilitate downstream dispersal of juvenile crayfish (cf. Bubb et al. 2002; Bernardo et al. 2011), but factors other than river hydromorphology, such as limiting resources, predation and competitive pressure will also drive the dynamics range expansion (Bernardo et al. 2011).

Therefore, we believe that in the case of the Korana River, the observed lower downstream dispersal rates could potentially be due to high competitive pressure from the native *P. leptodactylus* at downstream invasion front. *Pontastacus leptodactylus* has been considered as the most robust ICS since it has better adaptive plasticity than the other native crayfish species (Lucić et al. 2012; Perdikaris and Georgiadis 2017), may exhibit partial resistance to the *A. astaci* infection (Svoboda et al. 2012; Kokko et al. 2012, 2018) and is the only ICS showing range expansion in water bodies of Croatia (Maguire et al. 2011, 2018) and other European countries (Kouba et al. 2014). Therefore, its high population abundance at the downstream invasion front (13 times higher CPUE than *P. leniusculus*) could be slowing down the downstream range expansion of *P. leniusculus*. The populations of the native *P. leptodactylus* at the downstream invasion front have much higher relative population abundance (6 times higher) than its populations at the upstream front due to its relatively recent spread to the Korana from the Mrežnica River (Maguire et al. 2011, 2018). Thus, the uneven competitive pressure exerted by the native ICS may be contributing to the observed differences in dispersal rates of *P. leniusculus* at two invasion fronts. Nevertheless, such dispersal rate reduction will probably be short term, since the invasive *P. leniusculus*, even in the absence of crayfish plague (disease transmission), is competitively advantageous over the native European crayfish. This was demonstrated in previous laboratory and field studies on other ICS (*A. astacus*: Söderbäck 1991, 1995; Westman et al. 2002), as well as for *P. leptodactylus* both experimentally (Hudina et al. 2016) and in the field (the Korana River; Hudina et al. 2017), where *P. leptodactylus* was gradually displaced from

the invasion core populations. At the invasion core, *P. leptodactylus* were present in 2012 (Hudina et al. 2013, 2017). Since then, the *P. leniusculus* populations abundances have increased over 20 times. Additionaly, we have recently confirmed that the small proportion (6%) of the entire *P. leniusculus* population in the Korana River is *A. astaci*-positive (Pavić et al. 2020). Therefore, in addition to competitive advantage, crayfish plague transmission might be contributing to the observed decline of *P. leptodactylus* in the Korana River.

In the Drava River, based on the current study, it seems that from 2011 the invasion range of *P. leniusculus* has increased by approximately 148.9 km in the downstream direction with a dispersal rate of 21.3 km/yr. The dispersal rate matches the rates recorded in a study a decade ago (18–24.4 km/yr; Hudina et al. 2009). However, it is important to emphasize that dispersal rate calculations within this study are based on a low number of captured *P. leniusculus* individuals in the Drava River. Therefore, we cannot rule out the possibility that such fast dispersal rate in the Drava River is a consequence of human translocations, especially since voids in *P. leniusculus* distribution were recorded (no *P. leniusculus* recorded between sites 3 and 8). Currently, the evidence of human-mediated translocations of crayfish in Croatia is scarce (e.g. *A. astacus* in the Adriatic Sea drainage: Maguire et al. 2011, 2018; *A. astacus* in Plitvice Lakes region: Maguire et al. 2013; *P. leniusculus* in the Korana River: Hudina et al. 2013; Mijošek et al. 2017), most probably because crayfish are not commercially important species in Croatia (no crayfish farms exist in Croatia and crayfish are not sold in local markets nor used as baits). Also, recorded voids in distribution may be attributable to the river size (e.g. in this section the Drava is 200 m wide and crayfish distribution in the river is not uniform but patchy; Hudina et al. 2008). The high dispersal rate may potentially be attributed to hydrological conditions of the Drava River – high average discharges in combination with hydromorphological changes due to upstream hydropower plant constructions (cf. Hudina et al. 2009). Finally, the very low number of caught *P. leniusculus* indicates a low population density (which is expected at invasion fronts), and "standard" crayfish traps such as the ones used in this study (LiNi traps) are less effective compared to other trapping methods (i.e. baited stick catch – approximately 5 times more effective, artificial refuge traps – approximately 4 times more effective; Policar and Kozak 2005; Green et al. 2018). Therefore we hypothesize that, regardless of the invasion pathway (natural or human mediated), the low number of caught crayfish as well as voids in distribution are potentially a consequence of a patchy distribution in a large river and low population density in combination with lower trapping efficiency of "standard" crayfish traps. We used "standard" baited crayfish traps since: i) the Drava River is a large, deep and relatively turbid river and alternative trapping methods are not a viable option in this habitat and ii) their use remains commonplace and allows comparisons

with data from previous studies performed on this river (Hudina et al. 2009) as well as with other studies (Larson and Olden 2016; Green et al. 2018). Taking all above into consideration, the recorded dispersal rates as well as invasion pathways need to be further explored in order to discern whether the downstream spread in the Drava River is natural or human-mediated and to examine the effect of hydrological factors (e.g. the effects of high discharges and hydromorphological changes) on dispersal rates of *P. leniusculus*.

It was estimated that by 2018, *P. leniusculus* would encounter the invasive *F. limosus* in the Drava River and that the encounter area would be 50 km upstream from the confluence of the Drava and the Danube River (Hudina et al. 2009). While in this study *P. leniusculus* was recorded only 3.3 km upstream from the predicted encounter area by Hudina et al. (2009), *F. limosus* was absent from this newly established *P. leniusculus* invasion front. This might be due to the slower than expected upstream dispersal rate of *F. limosus*. However, due to low number of both captured NICS this cannot be discerned and further monitoring of this river section is required in order to determine population dynamics of the two invaders in this river stretch.

In the case of ICS, the lack of native *P. leptodactylus* records in the Drava River during our study and during the survey by Maguire et al. (2018) suggests its displacement by both *P. leniusculus* and *F. limosus*. The findings from both the Korana and the Drava Rivers and from previous studies (Hudina et al. 2009, 2017; Maguire et al. 2018) suggest that, even though currently robust, *P. leptodactylus* might in the long-term become completely displaced from large rivers and their tributaries in the continental part of Croatia (the Danube, the Drava and the Korana) due to the competitive pressure by NICS. Thus, the status of *P. leptodactylus* in Croatia should be monitored and re-examined – even though it is the only ICS undergoing range expansion in some water bodies, it is also currently the ICS species most adversely affected by the spread of the NICS in Croatia.

Other species threatened by *P. leniusculus* dispersal are the populations of the protected ICS (*A. astacus* and *A. torrentium*) in the Korana River and its tributaries. *A. torrentium* populations are located 46 km upstream of the invaded area in a tributary of the Korana River, while both *A. astacus* and *A. torrentium* populations are present 109 km upstream from the upstream invasion front, in the most well-know Croatian National Park, the Plitvice Lakes (Maguire et al. 2013; Hudina et al. 2013), where springs and upper course of the Korana River are located. Due to higher dispersal rates of *P. leniusculus* in the Korana River in upstream direction, as well as the existence of several populations of protected ICS upstream in the Korana and its tributaries, the dispersal rate of *P. leniusculus* in the upstream direction should be prioritized and carefully monitored, as well as any intentional or unintentional translocation of crayfish (Bohman et al.

2006; Diéguez-Uribeondo 2006; Ruokonen et al. 2018). Finally, due to recorded presence of crayfish plague pathogen *Aphanomyces astaci* in the invaded range of *P. leniusculus* in the Drava (Maguire et al. 2016) and the Korana (Pavić et al. 2020), disinfection protocols for fishing equipment should be put in place in order to prevent unintentional crayfish plague transmission to other parts of the rivers and their tributaries.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Records of native and non-native crayfish in Drava and Korana rivers.

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2020/Supplements/BIR_Dragicevic_etal_SupplementaryMaterial.xlsx

Supplementary material

Table S1. Records of native and non-native crayfish in Drava and Korana rivers.

Year	River	Species	Invasive/native	Site code*	X (HTRS96)	Y(HTRS96)	X(WGS84)	Y (WGS84)
2018	Drava	*Pacifastacus leniusculus*	invasive	2	5087332.864	584599.8053	45.920623	17.59068
2018	Drava	*Pacifastacus leniusculus*	invasive	3	5080439.949	589116.4401	45.858044	17.64762
2018	Drava	*Pacifastacus leniusculus*	invasive	8	5063941.867	647261.1153	45.699705	18.391038
2018	Drava	*Faxonius limosus*	invasive	12	5053333.206	662984.0148	45.60079	18.589261
2018	Korana	*Pacifastacus leniusculus*	invasive	1	5020449.99	422982.64	45.319775	15.517625
2018	Korana	*Pacifastacus leniusculus*	invasive	11	5035068.79	427002.32	45.451736	15.566728
2018	Korana	*Pontastacus leptodactylus*	native	1	5020449.99	422982.64	45.319775	15.517625

**Site codes correspond to codes in the manuscript and site labels on Figures.*

Znanstveni rad br. 2

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REVIEW

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Microbial pathogens of freshwater crayfish: A critical review and systematization of the existing data with directions for future research

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Abstract

Despite important ecological role and growing commercial value of freshwater crayfish, their diseases are underresearched and many studies examining potential crayfish pathogens do not thoroughly address their epizootiology, pathology or biology. This study reviews over 100 publications on potentially pathogenic viruses, bacteria, fungi and fungal-like microorganisms reported in crayfish and systematizes them based on whether pathogenicity has been observed in an analysed species. Conclusions on pathogenicity were based on successful execution of infectivity trials. For 40.6% of examined studies, microbes were successfully systematized, while for more than a half (59.4%) no conclusion on pathogenicity could be made. Fungi and fungal-like microorganisms were the most studied group of microbes with the highest number of analysed hosts, followed by bacteria and viruses. Our analysis demonstrated the need for: (a) inclusion of higher number of potential host species in the case of viruses, (b) research of bacterial effects in tissues other than haemolymph, and (c) more research into potential fungal and fungal-like pathogens other than *Aphanomyces astaci*. We highlight the encountered methodological challenges and biases and call for a broad but standardized framework for execution of infectivity trials that would enable systematic data acquisition on interactions between microbes and the host.

KEYWORDS

bacteria, fungi and fungal-like microorganisms, infectivity trials, microbes, potential crayfish pathogens, viruses

1 | INTRODUCTION

Freshwater crayfish (Crustacea, Decapoda, Astacidea) belong to a diverse order of decapod crustaceans which encompasses marine, freshwater and semiterrestrial crustaceans with over 10,000 described species (Hobbs, 2001). The infraorder Astacidea (crayfish) are distributed nearly worldwide and include a high number of species (669 species) classified into five families and 38 genera (Crandall & De Grave, 2017). Crayfish are keystone species of freshwater ecosystems and an important component of freshwater food webs due to their large size, relatively long life span and high activity which includes burrowing, aggressive behaviour and omnivory (Reynolds et al., 2013). They are ubiquitous in freshwater ecosystems and inhabit diverse environments including subterranean and semiterrestrial habitats, as well as brackish waters (Edgerton et al., 2004). At the same time, they are among the most successful aquatic invaders (Holdich et al., 2009) that may adversely impact the structure, function and biodiversity of freshwater ecosystems (Edgerton et al., 2004; Holdich et al., 2009; Reynolds et al., 2013). Additionally, populations of native crayfish in the wild are increasingly endangered

and facing decline (Holdich et al., 2009; Lodge et al., 2000; Richman et al., 2015; Taylor, 2002) as a direct consequence of multiple anthropogenic stressors on the freshwater ecosystems, which include climate change, hydromorphological changes, and introduction of non-indigenous crayfish species often carrying novel pathogens. As these pressures are expected to increase, there is an urgent need for better understanding of their contribution to extinction risks for the native crayfish populations (Richman et al., 2015), with emphasis on research into occurrence and emergence of diseases and pathogens within these populations.

Besides their key role in freshwater ecosystems, crayfish are also economically important, and are cultivated in aquaculture, both for food and ornamental purposes (Holdich, 2002). The majority of crustaceans farmed in aquaculture are marine species such as prawns and shrimp (Bondad-Reantaso et al., 2012). However, freshwater crayfish aquaculture is also on the rise (Harlıoğlu & Farhadi, 2017; Holdich, 1993) and a growing number of studies focuses on optimising crayfish production (Harlıoğlu & Farhadi, 2017; Momot, 1991). Despite this, crustaceans still account for only a small proportion of aquaculturally produced animals (FAO, 2018). Consequently, crayfish diseases and pathogens have received substantially less attention compared to other aquaculturally important organisms, primarily fish (Altinok & Kurt, 2003; Assefa & Abunna, 2018; Idowu et al., 2017) and only a few crayfish diseases have been extensively studied. We briefly present some of the most well-established crayfish diseases. The crayfish plague is one of the most researched and the most detrimental crayfish diseases that causes significant declines of crayfish populations in Europe and other regions (Martín-Torrijos et al., 2017, 2018). The causative agent of this disease, oomycete *Aphanomyces astaci* Schikora, 1906, spreads through water by viable zoospores (Oidtmann et al., 2002). Its natural hosts are the North American crayfish species that are usually chronically infected and can serve as vectors that transmit the pathogen to susceptible species, such as European indigenous crayfish species (ICS) in regions where North American species have been translocated and co-occur with ICS (Martín-Torrijos et al., 2018). Symptoms of the disease are brown melanization spots on the exoskeleton and whitening of the tail muscle in severely affected areas as well as limb loss or abdominal paralysis (Edgerton et al., 2002). In some populations of ICS, *A. astaci* infection can cause 100% mortality (Edgerton et al., 2002). Another well-researched disease, the white spot disease (WSD), is caused by the *White spot syndrome virus* (WSSV) which displays a low degree of host specificity at the order level and therefore may have the ability to infect all decapod crustaceans (Longshaw, 2016). Clinical symptoms, although often absent in infected individuals of many species (Edgerton et al., 2002), include small white spots on the inside surface of the cuticle of the carapace and appendages, lethargy, sudden reduction in food consumption, red discoloration of the body and appendages, and a loose cuticle (Sánchez-Paz, 2010). Crayfish, as many other decapod crustaceans show highly variable morbidity and mortality as a consequence of WSSV infection (Lo & Kou, 1998). Another serious crayfish disease is thelohaniasis, or the porcelain disease, caused by the fungi *Thelohania contejeani* Henneguy, 1892 (Dunn et al., 2009; El-Matbouli & Soliman, 2006). Infection occurs through ingestion of *T. contejeani* spores and causes an opaque and milky appearance of abdominal muscles, while clinical signs in advanced stages of infection include sluggishness of crayfish and a lack of tail-flip response (El-Matbouli & Soliman, 2006). The porcelain disease can result in considerable mortality of crayfish populations (Dunn et al., 2009; El-Matbouli & Soliman, 2006).

Better understanding of the effects of crayfish pathogens and their interaction with the host is of a paramount importance both for successful crayfish aquaculture and for the conservation of vulnerable native crayfish populations in the wild (cf. Evans & Edgerton, 2002). In aquaculture, disease outbreaks are not uncommon since animals are often kept in stressful conditions such as high density and poor water quality, and pathogens may also spread easily due to cannibalistic nature of crayfish and addition of the wild broodstock (Hajek & Shapiro-Ilan, 2018). Even in the case of non-lethal diseases, gross symptoms may sometimes cause considerable economic losses since they make the crayfish unappealing and hence unmarketable (Vogt, 1999). Furthermore, cross-species transmissions of pathogens may occur when multiple crustacean species are farmed together (Kibenge, 2016). Pathogens may also be transferred to wild populations via contaminated equipment (Alderman & Polglase, 1985) and via the escape of animals from aquaculture (Alderman et al., 1990; Lodge et al., 2000). In crayfish populations in the wild, pathogens are an important limiting factor of population growth since disease outbreaks may cause severe declines of host populations (Kinne, 1980; Vogt, 1999). In the context of the invasive species, diseases pose a serious threat during intentional or unintentional introduction of alien species into novel environment, since novel pathogens may also be introduced with their hosts. In such cases, due to lack of resistance to the new disease, populations of native congeners may be completely eradicated by the introduced pathogen (Vogt, 1999). This was evident in the spread of *A. astaci*: translocation of North American crayfish species to Europe, Asia and Australia introduced novel highly virulent *A. astaci* strains (e.g. genotype group B) to these regions and reduced the populations of native crayfish species, while having little or no effect to the North American crayfish carriers (Jussila et al., 2014; Oidtmann et al., 2002; Svoboda et al., 2017).

To date, numerous studies examining potentially pathogenic microbes in crayfish have been published, and many papers and books have reviewed crayfish diseases (Bateman & Stentiford, 2017; Cerenius & Söderhäll, 1992; Edgerton, 2002; Edgerton et al., 2002, 2004; Evans & Edgerton, 2002; Johnson, 1983; Longshaw, 2011, 2016; Unestam, 1973; Vey, 1986; Vogt, 1999). However, except for the relatively few well-established pathogens already mentioned above (i.e. *A. astaci:* Unestam, 1969; Vey et al., 1983; Diéguez-Uribeondo et al., 1995; Aydin et al., 2014; Svoboda et al., 2017; Jussila et al., 2017; WSSV: Shi et al., 2000; Corbel et al., 2001; Huang et al., 2001; Edgerton, 2004; Jiravanichpaisal et al., 2004; Bateman et al., 2012; *T. contejeani:* Quilter, 1976; Graham & France, 1986; Lom et al., 2001; El-Matbouli & Soliman, 2006; Imhoff et al., 2012; Pretto

et al., 2017), many microbes that have been isolated from crayfish showing gross disease symptoms have never been thoroughly investigated. Most often, they were often only recorded without thoroughly addressing their epizootiology, pathology, biology and the causal relationship between the microbe and specific damage observed in the host has never been explored (e.g. *Aeromonas sobria* Popoff and Véron, 1981: Wong et al., 1995; Bowater et al., 2002; Longshaw et al., 2012; Edgerton et al., 1995; Krugner-Higby et al., 2010). Thus, identifying the causality and onthology of a disease, and determining potential effects of pathogens and identifying their hosts, is an essential task in disease management and prioritization of interventions for crayfish conservation and astaciculture. First steps in this task are: (a) a comprehensive systematization of current knowledge on relationships between microbes and their hosts, (b) identification of gaps in the existing data, (c) prioritization of future data collection, and (d) development of general and standardized methodological frameworks that will enable meaningful comparisons between the studies.

This study aims to give such critical and comprehensive review and systematization of the existing research on (potential) crayfish pathogens. In the systematization of heterogeneous studies and available data, we used the concept of a pathogen according to Casadevall and Pirofski (2003). Pathogen is defined as a microbe that is capable of causing damage (or virulence) in the susceptible host, where damage can be measured at any level of host's biological organization (i.e., in an individual or its organs, tissues, cells) and may induce adverse outcomes for host's fitness. Damage to the host may be the result of both microbe effects and host response (i.e. immune response) acting together and can be quantified in the host.

This review is focused on microbes that have been recognized as the most prevalent pathogens in crustaceans: viruses, bacteria, fungi and fungal-like microorganisms. By thorough analysis of the existing research, we aimed to make a systematic review by determining for which microbes' pathogenicity has been observed and susceptible crayfish host identified, and for how many the data are still insufficient to make such systematization.

2 | METHODS

2.1 | Database organization

In order to make a comprehensive review, we analysed 117 available publications (publication year ranging from 1940 to 2020), including existing review papers, which reported on microbes potentially associated with crayfish diseases. From these publications, we extracted a total of 454 entries belonging to three groups of microbes frequently associated with crayfish diseases: (a) viruses, (b) bacteria, (c) fungi and fungal-like microorganisms. Each entry is a combination of a (potentially pathogenic) microbe and a (potential) crayfish host species. Thus, in our database, the same microbe species analysed in different hosts are considered as separate entries. Identically, different isolates or strains of the same microbial species or same microbial species from different publications (due to lack of confirmation of their common identity) were treated as a separate entry. We classified the recorded entries based on whether pathogenicity has been observed and/or susceptible host identified according to the scheme presented in Figure 1. Microbes/entries for which straightforward information regarding pathogenicity was lacking were classified as Data Deficient (Figure 1).

2.2 | Defining pathogenicity/virulence

In this review, we define *pathogenicity* as "the capacity of a microbe to cause damage in the host" (Casadevall & Pirofski, 1999). *Virulence* is defined as the adverse outcome of interaction between the microbe and the host which can be recorded at any biological level of the host's organization (cells, tissues, organs; Méthot & Alizon, 2014), that is the amount of damage that is manifested in the host or the decrease in host fitness due to the infection (Pirofski & Casadevall, 2012). When measuring virulence, it is hard to define the threshold between "low" and "high" virulence, and what is the threshold for a microbe to be declared a pathogen (Méthot & Alizon, 2014). Due to lack of such

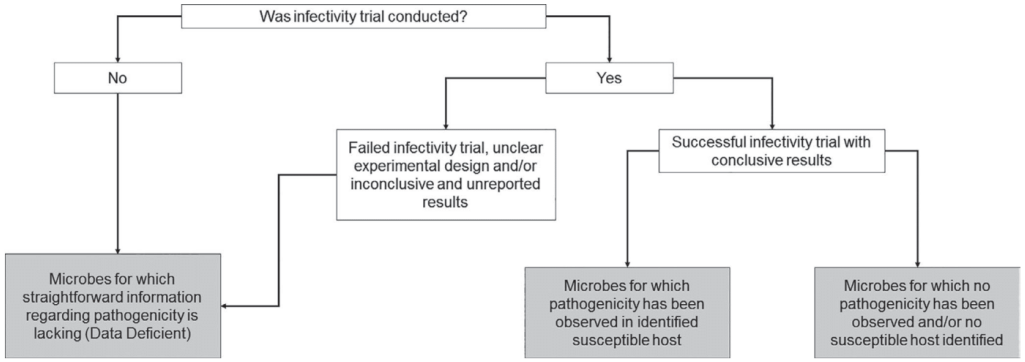

FIGURE 1 Scheme for systematization of microbes applied in this study

agreement, in the context of our study, we decided to apply a more "liberal" approach: any microbe that caused any level of virulence in a susceptible host (i.e., significant negative effect on any level of biological organization) was considered a microbe for which pathogenicity has been observed and susceptible host identified, while in cases of zero level virulence (i.e., no negative effect on any level of biological organization), it was considered that no pathogenicity has been observed and/or that no susceptible host has been identified for that microbe. Therefore, in our review recorded virulence equals pathogenicity, and the terms are used interchangeably.

2.3 | Systematization of microbes/entries

We used the execution of infectivity trials as a main principle in systematic review of available literature data (Figure 1). In brief, the first step in our analysis was to determine whether the pathogenicity of the microbe in a host has been observed after experimental testing, that is whether the crayfish were experimentally infected and developed any symptoms related to the infection with a particular microbe. Due to multitude of approaches and a lack of standardized procedures for experimental infection (hereon referred to as infectivity trial) within analysed publications, we applied a flexible definition of what constitutes an infectivity trial, including any of the following: (a) inoculation of the microbe into crayfish tissue, (b) feeding the crayfish with infected tissue or feed, or (c) exposing the crayfish to the microbe in the water or by cohabitation with infected individuals. The duration, pathogen load or the number of repetitions of infection was not taken into account; however, the existence of a control group was considered a prerequisite.

If infectivity trial was successfully conducted, and the methodology and results of the experiment were clearly stated and conclusive, microbe/entry was classified into one of the two categories: pathogenicity observed or pathogenicity not observed (Figure 1). In these cases, crayfish were successfully exposed to the microbe and results were compared to control animals. If symptoms and negative effects (including mortality) at any biological level of organization of the host (cells, organs, tissues, individual level etc.) were observed and clearly reported, and their occurrence was significantly more frequent than in the control group, the respective entry was classified as "pathogenicity observed in identified susceptible host" (Figure 1; Tables 1-3: marked with yes). Symptoms ranged from behavioural, morphological, physiological, histopathological or any noticeable sign of reduced fitness and negative effect on the crayfish health. If the microbe did not impair animal health in any way (i.e. no negative effects at any level of biological organization/no significant difference from the control group recorded), it was classified as "no pathogenicity observed and/or no susceptible host identified" (Figure 1; Tables 1-3: marked with no).

Microbes/entries for which straightforward information regarding pathogenicity was lacking were classified in the third group—Data Deficient. This group includes the cases (a) when the infectivity trial has not been conducted (Appendices S1–S3), and (b) when the infectivity trial, for any reason, has not been successfully executed, symptoms not reported and/or compared to control group and the details of the experimental design and results were lacking or were not clearly presented in the study, which consequently prevented straightforward interpretation of results (Tables 1-3).

3 | RESULTS AND DISCUSSION

This study systematically reviews existing literature on potentially pathogenic microbes reported in freshwater crayfish by focusing on (a) identification of pathogenicity as the capacity of a microbe to cause damage in the host at any level of biological organization, and (b) successful execution of infectivity trials as a method of quantifying damage, that is method of observation of pathogenicity. As such, it provides a list of the most prevailing microbes responsible for damage observed within freshwater crayfish and, for the first time, a list of microbes with clearly demonstrated pathogenicity in a particular crayfish host. Moreover, it highlights microbes with a recorded wide host range as well as those that have been shown to affect multiple host tissues. Systematization such as the one presented here represents the first step towards identification of gaps in research and development of risk assessment procedures.

Results of the performed systematization are presented in Tables 1-3 and Appendices S1–S3. Results for specific groups of microbes are presented in: Table 1 and Appendix S1—viruses; Table 2 and Appendix S2—bacteria; Tables 3 and Appendix S3—fungi and fungal-like microorganisms. Entries provided within the tables (i.e., microbial taxa extracted from the literature with potential pathogenicity towards freshwater crayfish) have been further separated in those for which infectivity trials have been conducted (Tables 1-3) and those for which infectivity trials have not been conducted (Appendices S1–S3).

Out of a total of 117 analysed studies reporting on (putative) crayfish pathogens, in only 40.6% a valid infectivity trial (according to criteria postulated in Methods section) has been performed, based on which pathogenicity of a microbe has been successfully systematized, that is classified as either pathogenicity observed/susceptible host identified or pathogenicity not observed and/or susceptible host not identified (Figure 2a). In over a half of examined studies (59.4%), the straightforward information regarding pathogenicity was not available and these studies were classified as Data Deficient (Figure 2a). Studies falling under Data Deficient category were usually the health surveys of crayfish populations, where microbes, isolated from dead or moribund crayfish, were mostly identified to genus or species level, with no further inspection of its pathogenicity and execution of infectivity trials (97.2% of Data Deficient studies). Alternatively, in 2.8% of the Data Deficient studies infectivity trials were performed but lacked description of the experimental design, or lacked control groups and/or details regarding symptoms, or results were inconclusive or not clearly reported. The fact that the majority of studies were classified in the Data Deficient category demonstrates that body of research investigating the effects of microbes on crayfish hosts is still limited.

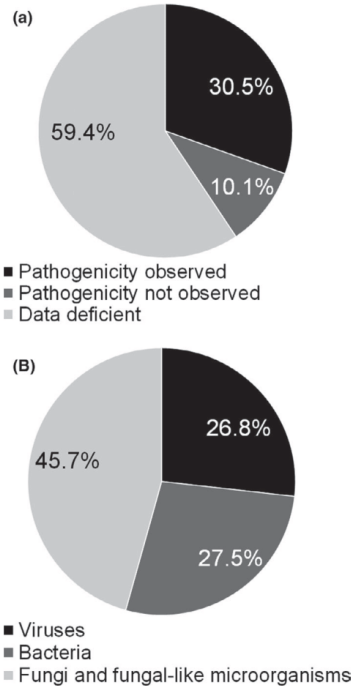

FIGURE 2 Pie charts showing the percentage of analysed studies containing reports on microbes with observed/not observed pathogenicity, or those belonging to Data Deficient category (a), and the percentage of studies exploring viruses, bacteria, and fungi and fungal-like microorganisms (b)

As visible from Tables 1-3, Appendices S1–S3, and Figure 2b, from all studies analysed in this review, the majority (45.7%) investigated fungi and fungal-like microorganisms, which makes them the most studied group of microbes in the context of crayfish diseases, followed by bacteria and finally viruses, as the least frequently researched group of potential crayfish pathogens (Figure 2b).

We first report our findings for each group of microbes and then discuss encountered methodological challenges and general gaps, biases and issues related to pathogen definition and research, as well as future directions for research of crayfish pathogens.

3.1 | Viruses

Even though viruses are recognized as important pathogens in aquatic ecosystems (Ahne, 1994), their pathogenicity in crayfish hosts has been scarcely explored (Edgerton et al., 2002). Viruses were the least frequently studied group of microbes, with the lowest number of entries (42) and identified studies (34; Figure 2b) but also the group with the highest percentage of entries (52.4%) with successfully determined pathogenicity status. The small number of identified entries related to viral infections might a consequence of: (a) the rarity of many viral infections with many often being asymptomatic in crustaceans, (b) the lack of appropriate molecular primers and suitable cell lines to culture viruses and (c) general lack of expertise in crustacean pathology (Edgerton, 2003; Longshaw, 2016). Out of all entries for 47.6% pathogenicity has been observed and susceptible hosts identified (Figure 3a). Noteworthy, this was the group of microbes where taxonomical status was unresolved for many entries. Only a few crustacean viruses, such as WSSV, have been described, named and classified following the guidelines of ICTV (Bateman & Stentiford, 2017), while for many others these guidelines were not followed, which resulted in only presumptive taxonomy and their appearance under different names throughout the studies without the actual confirmation of their (common) identity, for example, *Cherax baculovirus* (CBV) or *Cherax quadricarinatus baculovirus* (CqBV) or *intranuclear bacilliform virus* (IBV) *of Cherax quadricarinatus*) (Anderson & Prior, 1992; Claydon et al., 2004; Davidovich et al., 2019; Edgerton & Owens, 1997; Edgerton et al., 1995; Groff et al., 1993; Kent Hauck et al., 2001; Romero & Jiménez, 2002).

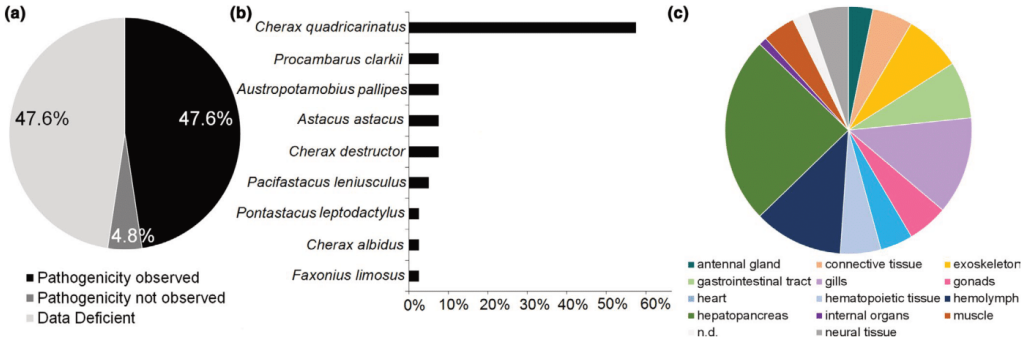

FIGURE 3 The percentage of entries for viruses (a) classified in each of the groups (pathogenicity observed/not observed, Data Deficient), (b) analysed in different potential crayfish hosts and (c) reported from different tissues

TABLE 1 List of viruses of crayfish with executed infectivity trials and determined pathogenicity status according to the developed systematisation system

Taxonomic affiliation	Virus	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	Reference
Birnaviridae	*Infectious pancreatic necrosis virus* (IPNV)	*Astacus astacus*	Haemolymph, muscle, gastrointestinal tract, gills, hepatopancreas, heart, gonads, antennal gland	Yes[a]	Mortality	Halder and Ahne (1988)
Iridoviridae	*Cherax quadricarinatus iridovirus* (CqIV)	*Cherax quadricarinatus*	Haematopoietic tissue, haemolymph, gills	Yes	Mortality; anorexia; lethargy; cessation of feeding; flaccidity; virions in the cytoplasm of haematopoietic tissue cells and gill cells	Xu et al. (2016)
	Cherax quadricarinatus iridovirus (CqIV)	*Procambarus clarkii*	Haematopoietic tissue, haemolymph, gills	Yes	Mortality; anorexia; lethargy; cessation of feeding; flaccidity; virions in the cytoplasm of haematopoietic tissue cells and gill cells	Xu et al. (2016)
Nimaviridae	*White spot syndrome virus* (WSSV)	*Austropotamobius pallipes*	Gills, heart, gonads, connective tissue, neural tissue, exoskeleton, haemolymph	Yes	Mortality; white spots in tissues; enlarged wssv-infected nuclei with marginalized chromatin containing distinct eosinophilic inclusion body; inclusions in heart connective tissue and muscle; signs of infection in haemocytes and connective tissue	Bateman et al. (2012)
	White spot syndrome virus (WSSV)	*Pacifastacus leniusculus*	Gills, heart, gonads, connective tissue, neural tissue, exoskeleton, haemolymph	Yes	Mortality; white spots in tissues; enlarged WSSV-infected nuclei with marginalized chromatin containing distinct eosinophilic inclusion body; inclusions in heart connective tissue and muscle; signs of infection in haemocytes and connective tissue	Bateman et al. (2012)
	White spot syndrome virus (WSSV)	*Pontastacus leptodactylus*	Haemolymph	Yes	Mortality; reduction in food intake; lethargy	Corbel et al. (2001)
	White spot syndrome virus (WSSV)	*Faxonius limosus*	Haemolymph	Yes	Mortality; reduction in food intake; lethargy	Corbel et al. (2001)
	White spot syndrome virus (WSSV)	*Cherax albidus*	Exoskeleton, connective tissue, heart, neural tissue, gonads, muscle, hepatopancreas, antennal gland	Yes	Mortality; laying on the back waving pleopods; viral inclusions in multiple tissues	Edgerton (2004)
	White spot syndrome virus (WSSV)	*Procambarus clarkii*	Haemolymph	Yes	Sluggish movement; stopped feeding; unresponsive to outside stimulation; haemolymph not coagulating	Huang et al. (2001)
	White spot syndrome virus (WSSV)	*Astacus astacus*	Haematopoietic tissue	Yes	Mortality	Jiravanichpaisal et al. (2004)
	White spot syndrome virus (WSSV)	*Pacifastacus leniusculus*	Haematopoietic tissue	Yes	Mortality	Jiravanichpaisal et al. (2004)
	White spot syndrome virus (WSSV)	*Cherax quadricarinatus*	Haemolymph, gills, gastrointestinal tract, exoskeleton, connective tissues, hepatopancreas	Yes	Mortality; loss of appetite; redder in colour; hypertropic nuclei of infected cells; viral particles in haemolymph and tissues (gills, stomach, cuticle, hepatopancreas)	Shi et al. (2000)

(Continues)

TABLE 1 (Continued)

Taxonomic affiliation	Virus	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	Reference
Nodaviridae	*Macrobrachium rosenbergii nodavirus* (MrNV)	*Cherax quadricarinatus*	n.d.	Yes[a]	Mortality; inability to swim normally; reduced appetite; pale exoskeleton; muscle necrosis and myositis; growth changes	Hayakijkosol et al. (2011)
Nudiviridae	*Cherax quadricarinatus bacilliform virus* (CqBV) (old nomenclature: CBV)	*Cherax quadricarinatus*	Hepatopancreas	Yes	Inclusions in hepatopancreas	Edgerton and Owens (1997)
	Intranuclear bacilliform virus (IBV) *of Cherax quadricarinatus* (old nomenclature: CqBV)	*Cherax quadricarinatus*	Hepatopancreas	Yes[b]	Hypertrophic nuclei with inclusions in hepatopancreatic cells; hepatopancreatic lesions	Claydon et al. (2004)
Parvoviridae	*Cherax quadricarinatus densovirus* (CqDV) (old nomenclature: CqPV)	*Cherax quadricarinatus*	Gills, exoskeleton	Yes	Mortality; white spots on the inside of the carapace; reddish colouration of the abdomen and pereiopods; migration to the surface; branchial membrane blistering; morbidity	Bochow (2016)
	Cherax quadricarinatus densovirus (CqDV) (old nomenclature: CqPV)	*Cherax destructor*	n.d.	No[c]	Mortality	Bochow (2016)
	Cherax quadricarinatus parvo-like virus (CqPV)	*Cherax quadricarinatus*	Gills, exoskeleton, gastrointestinal tract, connective tissue, antennal gland, haematopoietic tissue, gonads	Yes	Mortality; lethargy; weakness; disorientation; anorexia; red discoloration of the carapace before dying; avoided other crayfish and sheltered; lacked aggression when challenged by other crayfish; no responding to tapping on the aquarium; oedema between the inner and outer cuticle of the carapace in the area over the gills	Bowater et al. (2002)
	Infectious hematopoietic necrosis virus (IHHNV)	*Procambarus clarkii*	Gills, haemolymph, hepatopancreas	Yes	Mortality; enlarged nuclei in gills	Chen et al. (2018)

(Continues)

TABLE 1 (Continued)

Taxonomic affiliation	Virus	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	Reference
	Penaeus merguiensis densovirus (PmergDNV)	*Cherax quadricarinatus*	Gills, hepatopancreas	Data Deficient[d]	Mortality; lethargic; tendency to lie on their sides; weakened or failed tail-flip response; reduced appetite; unable to right themselves when placed on their back; moribund crayfish laying on their backs waving their pleopods for several hours prior to death; the appendages and mouthparts became reddened prior to death	La Fauce and Owens (2007)
Reoviridae	*Hepatopancreatic reovirus* (Australian strain)	*Cherax quadricarinatus*	Hepatopancreas	Yes	Mortality; smaller size and weight; lethargy; weakened tail-flip response; reduced appetite; appendages and mouthparts became reddened; necrosis of hepatopancreocytes and inflammatory cells in hepatopancreatic tubules	Hayakijkosol and Owens (2011)
Roniviridae	*Yellow head virus* (YHV) type 1	*Cherax quadricarinatus*	Haemolymph	No	None	Soowannayan et al. (2015)
unclassified	*Cherax Giardiavirus-like virus* (CGV)	*Cherax quadricarinatus*	Hepatopancreas	Yes	Inclusions in hepatopancreas	Edgerton and Owens (1997)

[a]The authors stated that the viral titre was very low after the infection of experimental animals, suggesting that the virus is potentially not replicating in the tested hosts. However, damage (mortality) in the animals was recorded.

[b]Infectivity trials included different treatment groups: group which was already infected with IBV and then additionally orally infected by more IBV showed a higher number of viral inclusions, while group which was not initially infected with IBV but was only infected once did not have any inclusions. Authors suggest that some individuals may have developed a resistance to the virus, since this crayfish species shows high levels of inter-population variability.

[c]The infected animals showed no signs of disease and the recorded mortality was not significantly higher than the mortality in the control group. Thus, pathogenicity has not been observed.

[d]The authors stated that the observed symptoms are not characteristic for this virus and that it is not replicating in tested host. Furthermore, they suggest that the symptoms and mortality are a consequence of a co-infection by another microbe.

Viruses were the group with the lowest number of analysed hosts (9; Figure 3b); however, almost all examined hosts (88.9%) were identified as susceptible (i.e. in 8 species of the recorded potential hosts pathogenicity has been observed). By far, the most studied species was *Cherax quadricarinatus* (von Martens, 1868) (57.1% of all entries), a species of high aquaculture interest (Rigg et al., 2020; Saoud et al., 2013), which resulted in the discovery of a number of *C. quadricarinatus*-specific viruses (Table 1).

Finally, viruses were recorded in the highest number of diverse crayfish tissues (Figure 3c), but most frequently (24.5% of all entries) in hepatopancreas, followed by gills (12.8%) and haemolymph (11.7%; Figure 3c). When analysing only entries where pathogenicity has been observed, the pattern was similar (Table 1). These findings reflect the current knowledge: viral infections are known to affect multiple crayfish tissues with most viruses being tissue specific and most often recorded in hepatopancreatic or gut cells but also in gills, haemocytes, heart and connective tissue (Edgerton et al., 2002; Evans & Edgerton, 2002; Longshaw, 2016).

Although viruses are mostly species-specific, some, such as WSSV, exhibit low host specificity and these viruses present one of the most threatening disease agents for crustaceans (Shi et al., 2000). Therefore, future research within this group of microbes should include a wider range of hosts in order to detect other potential non-species-specific viruses in a timely manner and to expand existing knowledge regarding crayfish viruses. Also, further research into crayfish viruses is required as they, similar to fish, potentially harbour a high number of new/undiscovered viruses (Bačnik et al., unpublished study; Geoghegan et al., 2018).

3.2 | Bacteria

Bacteria were the second most studied group of microbes in the context of crayfish diseases in terms of the total number of entries (159) and number of recorded publications (35; Figure 2b). This was not surprising since bacteria have a long history of research and, due to well-defined culture and isolation methods, are frequently reported in studies (Isenberg, 2003). However, they were the group with the highest percentage of Data Deficient entries (82.4%; Figure 4a), probably due to the fact that numerous identified studies represented only health survey reports and monitoring screenings from crayfish farms in which crayfish were only screened for the presence of microbes without further inspection of their pathogenicity or execution of infectivity trials (considered as crucial parameters in our analyses).

Bacteria were the group with the second highest number of analysed hosts (12 crayfish species; Figure 4b), out of which 5 species were identified as susceptible hosts. The most analysed and at the same time the most susceptible hosts recorded in the literature were commercially important crayfish species *Procambarus clarkii* (Girard, 1852), *Pacifastacus leniusculus* (Dana, 1852), and species from genus *Cherax*, that are of high aquaculture interest. None of the native European crayfish species were among the identified susceptible hosts for bacteria within the literature and were only recorded from Data Deficient studies, showing the lack of studies of effects of this group of microbes in species with no economical but high ecological value.

Finally, similar to viruses, bacteria were recorded in diverse tissues (Figure 4c). This is not surprising since bacteria are known to be ubiquitous, and many bacterial species form an integral part of the organism's microbiome. Also, bacterial infections can occur anywhere in the body since they can enter through minor wounds of the exoskeleton, gastrointestinal tract or other routes, gaining further access to the haemocoel and haemolymph (Davis & Sizemore, 1982; Tubiash et al., 1975). As visible from the Table 2, Appendix S2 and Figure 4c, most bacteria in analysed studies were isolated from haemolymph, and this is especially evident for those entries where pathogenicity has been observed, making it by far the most analysed tissue in this group (Table 2; Figure 4c). While some studies suggested that haemolymph should normally be sterile and that occurrence of bacteria in the haemolymph is a sign of stress or disease (Bang, 1970; Lee and Pfeifer, 1975; Johnson, 1976), others suggest that the

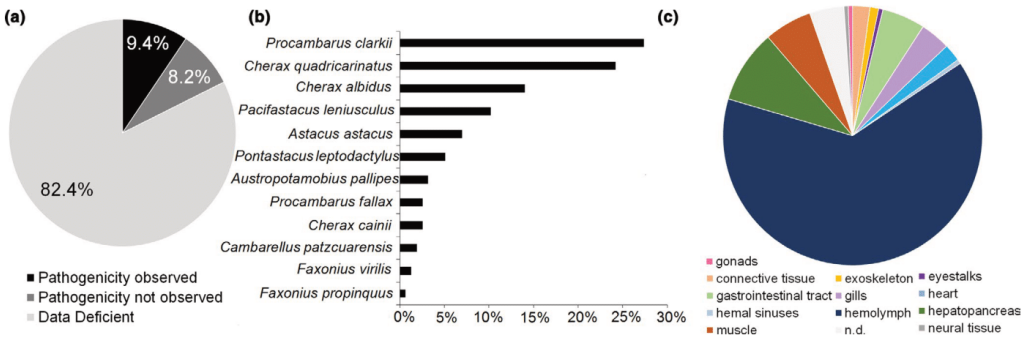

FIGURE 4 The percentage of entries for bacteria (a) classified in each of the groups (pathogenicity observed/not observed, Data Deficient), (b) analysed in different potential crayfish host and (c) reported from different tissues

TABLE 2 List of bacteria with executed infectivity trials and determined pathogenicity status according to the developed systematisation system

	Taxonomic affiliation	Bacterium	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
Bacteroidetes	Flavobacteriaceae	*Flavobacterium* sp.	*Procambarus clarkii*	n.d.	Data Deficient[a]	Mortality	Amborski et al. (1975)
	Weeksellaceae	*Chryseobacterium* sp. B2	*Pacifastacus leniusculus*	Haemolymph	Yes	Mortality	Jiravanichpaisal et al. (2009)
Firmicutes	Bacillaceae	*Bacillus mycoides*	*Cherax cainii*	Gastrointestinal tract	No	None	Ambas et al. (2015)
		Bacillus sp.	*Cherax cainii*	Gastrointestinal tract	No	None	Ambas et al. (2015)
		Bacillus spp.	*Faxonius virilis*	n.d.	No	None	Davidson et al. (2010)
		Bacillus subtilis	*Cherax cainii*	Gastrointestinal tract	No	None	Ambas et al. (2015)
	Staphylococcaceae	*Staphylococcus aureus*	*Pacifastacus leniusculus*	Gills, hepatopancreas	No[b]	Nodular reactions of haemocytes in the observed tissues including gill, heart, hepatopancreas and hemal sinuses	Jiravanichpaisal et al. (2009)
Proteobacteria	Aeromonadaceae	*Aeromonas hydrophila* B1	*Pacifastacus leniusculus*	Haemolymph, hepatopancreas	Yes	Mortality; lethargy; unresponsiveness; tail curved down to the abdomen; extensive necrotic lesions with pyknotic nuclei in various tissues including gill, heart, interstitial tissue of hepatopancreas and the blood circulatory system; massive haemocytes aggregated in the hemal sinuses with presence of many pyknotic nuclei	Jiravanichpaisal et al. (2009)
		Aeromonas hydrophila B1	*Pacifastacus leniusculus*	Haemolymph, hepatopancreas	Yes	Mortality	Gizem Korkut et al. (2018)
		Aeromonas sp.	*Procambarus clarkii*	n.d.	Data deficient[a]	Mortality	Amborski et al. (1975)
	Coxiellaceae	*Coxiella burnetii*	*Cherax quadricarinatus*	Hepatopancreas, muscle	Yes	Loss of body mass; bacteraemia and inflammatory cells; exhibiting some form of systemic granuloma or inclusion bodies; swollen or abnormal nuclei within the hepatopancreas and evidence of forced tubule separation	Powell (2013)
		Coxiella cheraxi sp. nov. (strain TO−98)	*Cherax quadricarinatus*	Hepatopancreas, eyestalks, gills, connective tissue	Yes	Lethargy before death; reddened colour; putrefied, with eyes of highly infected crayfish necrotized and the hepatopancreas liquefied	Tan and Owens (2000)

(Continues)

TABLE 2 (Continued)

	Taxonomic affiliation	Bacterium	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
	Enterobacteriaceae	*Citrobacter braakii*	*Procambarus clarkii*	Gastrointestinal tract	Data Deficient[c]	n.d.	Chen et al. (2017)
		Citrobacter freundii	*Procambarus clarkii*	n.d.	Data Deficient[a]	Mortality	Amborski et al. (1975)
		Citrobacter freundii	*Procambarus clarkii*	Gastrointestinal tract	Yes	Mortality	Chen et al. (2017)
		Citrobacter freundii HP5	*Pacifastacus leniusculus*	Hepatopancreas	No[b]	Several melanized spots in hepatopancreas as a sign of mounted defence	Jiravanichpaisal et al. (2009)
		Citrobacter gillenii HP10	*Pacifastacus leniusculus*	Hepatopancreas	No[b]	Several melanized spots in hepatopancreas as a sign of mounted defence	Jiravanichpaisal et al. (2009)
		Citrobacter murliniae/freundii HP9	*Pacifastacus leniusculus*	Hepatopancreas	No[b]	Several melanized spots in hepatopancreas as a sign of mounted defence	Jiravanichpaisal et al. (2009)
		Citrobacter werkmanii	*Procambarus clarkii*	Gastrointestinal tract	Yes	Mortality	Chen et al. (2017)
		Escherichia coli	*Procambarus clarkii*	n.d.	Data Deficient[d]	None	Amborski et al. (1975)
		Escherichia coli K12	*Pacifastacus leniusculus*	Gills, hepatopancreas, heart, hemal sinuses	No[b]	Nodular reactions of haemocytes in the observed tissues including gill, heart, hepatopancreas and hemal sinuses	Jiravanichpaisal et al. (2009)
	Moraxellaceae	*Acinetobacter* sp. N1	*Pacifastacus leniusculus*	Haemolymph	Yes	Mortality	Jiravanichpaisal et al. (2009)
	Pseudomonadaceae	*Pseudomonas gessardii*	*Pacifastacus leniusculus*	Haemolymph	Yes	Mortality	Gizem Korkut et al. (2018)
		Pseudomonas guinea/peli B3	*Pacifastacus leniusculus*	Haemolymph	No[b]	Several melanized spots in hepatopancreas as a sign of mounted defence	Jiravanichpaisal et al. (2009)
		Pseudomonas libanensis/ gessardii BLT	*Pacifastacus leniusculus*	Haemolymph	Yes	Mortality	Jiravanichpaisal et al. (2009)
		Pseudomonas sp.	*Cherax albidus*	Haemolymph	Yes	Mortality; acute bacteraemic infection; reduced circulating blood cells before death	McKay and Jenkin (1969)
		Pseudomonas sp.	*Procambarus clarkii*	n.d.	Data Deficient[a]	Mortality	Amborski et al. (1975)

(Continues)

TABLE 2 (Continued)

	Taxonomic affiliation	Bacterium	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
		Pseudomonas sp. N2	*Pacifastacus leniusculus*	Haemolymph	no[b]	Several melanized spots in hepatopancreas as a sign of mounted defence	Jiravanichpaisal et al. (2009)
	Shewanellaceae	*Shewanella* sp.	*Cherax cainii*	Gastrointestinal tract	No	None	Ambas et al. (2015)
Tenericutes	Spiroplasmataceae	*Spiroplasma eriocheiris*	*Procambarus clarkii*	Haemolymph, heart, gills	Yes	Clinical signs of disease	Ding, et al. (2014)
		Spiroplasma mirum	*Procambarus clarkii*	n.d.	No	None	Bi et al. (2008)
		Spiroplasma penaei	*Faxonius virilis*	Haemolymph	Yes	Mortality	Davidson et al. (2010)
		Spiroplasma sp.	*Procambarus clarkii*	n.d.	Yes	Mortality; tremor of pereiopods	Bi et al. (2008)
		Spiroplasma-like organism	*Procambarus clarkii*	Haemolymph, connective tissue, exoskeleton, gastrointestinal tract, hepatopancreas, neural tissue, heart, gills	Yes	Weakness symptoms	Wang et al. (2005)

[a]The study is missing the information on the existence of a control group in the experiment, as well as tested tissue and additional details explaining the methods used in conducting the infectivity trials. Thus, the microbe was classified as Data Deficient even though mortality has been recorded.

[b]The observed symptoms are not necessarily a consequence of the infection by the bacteria tested during infectivity trials, and the authors stated that these bacteria are not pathogenic.

[c]The symptoms and the results of the infectivity trials were not clearly stated.

[d]The study is missing the information on the existence of a control group in the experiment, as well as tested tissue and additional details explaining the methods used in conducting the infectivity trials.

haemolymph is normally non-sterile (Colwell et al., 1975; Madetoja & Jussila, 1996; Scott & Thune, 1986; Welsh & Sizemore, 1985; Wong et al., 1995). Our analysis (Table 2) shows that some bacteria (e.g., some members of the genera *Bacillus*, *Citrobacter*, *Escherichia*, *Pseudomonas*, *Shewanella*, *Spiroplasma* and *Staphylococcus*) can be present in the haemolymph without causing observable damage in the crayfish (Ambas et al., 2015; Bi et al., 2008; Davidson et al., 2010; Jiravanichpaisal et al., 2009). At the same time, the most commonly recorded genera in haemolymph for the entries with successfully determined pathogenicity were *Bacillus*, *Citrobacter*, *Spiroplasma*, and *Pseudomonas*. Frequent analyses of bacteria from haemolymph could be due to the fact that haemolymph extraction does not require animal sacrifice, and/or that haemolymph was most often the focus of research due to the occurrence of bacterial septicaemia (excessive multiplying of specific bacteria in the haemolymph) and researchers' attempts to identify bacteria responsible for this condition (Bowater et al., 2002; Edgerton et al., 1995; Jones & Lawrence, 2001; Longshaw et al., 2012; Madetoja & Jussila, 1996; Scott & Thune, 1986; Wong et al., 1995).

In conclusion, the lack of research on other tissues may lead to non-recognition of bacterial infections in tissues other than haemolymph. Thus, research on crayfish bacteria should be expanded to include more diverse tissues, which would potentially allow the detection of a wider range of bacterial diseases of crayfish.

3.3 | Fungi and fungal-like microorganisms

Our results show that fungi and fungal-like microorganisms were the most studied group of microbes in the context of crayfish diseases (Figure 2b), with a total of 253 entries recorded in a total of 58 publications (Table 3; Appendix S3). This was also the group with high number of Data Deficient entries which accounted for 79.9% of all recorded entries (Figure 5a). Given that research of fungi and fungal-like microorganisms throughout the history has focused most frequently on *A. astaci* as one of the most destructive crayfish pathogens (Longshaw, 2016), a high number of reported species of fungi and fungal-like microorganisms (other than *A. astaci*) without analysed pathogenicity status is not surprising. Similar to bacteria, other potential reasons for a high number of Data Deficient taxa could be the fact that many conducted studies represented either health monitoring surveys of crayfish individuals, or performed only screening for the presence of fungi and fungal-like microorganisms, without further examining their effects on the host. Since it is known that fungi cause large-scale epizootics in wild and cultured populations of crustaceans (Johnson, 1983), the number of studies and studied taxa for this group is still disproportionately small.

In addition, fungi and fungal-like microorganisms were the group with the highest number of analysed hosts (31 crayfish species; Figure 5b), with the majority of entries reported for species *P. clarkii* and *P. leniusculus*, the two commercially important crayfish species that are also among the most successful crayfish invaders, followed by the native and endangered European species, *Austropotamobius pallipes* (Lereboullet, 1858) and *Astacus astacus* (Linnaeus, 1758) (Kouba et al., 2014). When analysing only susceptible hosts (i.e. those hosts in which pathogenicity has been observed), this was also the group of microbes with the highest number of reported susceptible hosts (20 species), dominated by three European ICS (*A. astacus*, *Pontastacus leptodactylus* (Eschscholtz, 1823) and *Austropotamobius torrentium* (Schrank, 1803)) along with the invasive *P. leniusculus*. Differences in frequency of reports between all potential hosts (Figure 5b) and susceptible hosts is probably due to the already mentioned research focus on *A. astaci* and its effects on native European crayfish.

Compared to other two microbial groups, fungi and fungal-like microorganisms were isolated in lower number of tissues, and most frequently from exoskeleton (58.2%; Figure 5c). Fungi often enter the body through wounds and mostly infect the exoskeleton, particularly the soft cuticle (Edgerton et al., 2002; Evans &

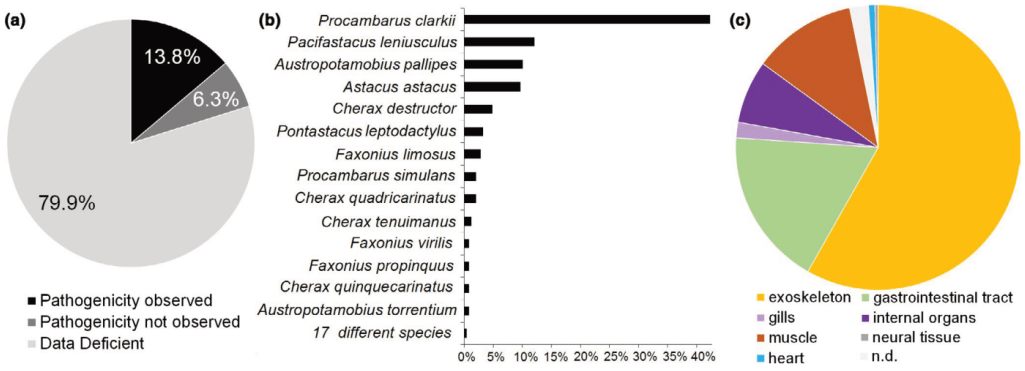

FIGURE 5 The percentage of entries for fungi and fungal-like microorganisms (a) classified in each of the groups (pathogenicity observed/not observed, Data Deficient), (b) analysed in different potential crayfish host and (c) reported from different tissues

TABLE 3 List of fungi and fungal-like microorganisms with executed infectivity trials and determined pathogenicity status according to the developed systematization system

	Taxonomic affiliation	Fungi or fungal-like microorganism	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
Ascomycota	Didymellaceae	*Epicoccum nigrum*	*Astacus astacus*	Exoskeleton	No	None	Makkonen et al. (2013)
	Nectriaceae	*Fusarium avenacuem*	*Astacus astacus*	Exoskeleton	Yes	Mortality; trauma sites on carapace	Makkonen et al. (2013)
		Fusarium oxysporum 1,215	*Pontastacus leptodactylus*	Gills, exoskeleton	Yes	Mortality; brownish spots on gills	Maestracci and Vey (1988)
		Fusarium oxysporum 1,235	*Austropotamobius pallipes*	Gills, exoskeleton	Yes	Mortality; brownish spots on gills; orange spots on carapace	Maestracci and Vey (1988)
		Fusarium solanii	*Pontastacus leptodactylus*	Exoskeleton	Yes[a]	Scarred animals: mortality; punctured animals: mortality; melanized spots on punctured area; weakness	Chinain and Vey (1987)
		Fusarium solanii	*Pacifastacus leniusculus*	Exoskeleton	Yes[a]	Scarred: no mortality; fungus completely eliminated; punctured: mortality; melanized spots	Chinain and Vey (1987)
		Fusarium solanii	*Pontastacus leptodactylus*	Exoskeleton	Yes	Mortality; melanized cuticle	Chinain and Vey (1988)
		Fusarium solanii	*Pacifastacus leniusculus*	exoskeleton	yes	mortality; melanized cuticle	Chinain and Vey (1988)
Basidiomycota	Trichosporonaceae	*Trichosporon jirovecii*	*Procambarus clarkii*	Exoskeleton	Yes	Mortality; whitish discoloration around the site of injection that consequently developed black melanization zone	Abdallah et al. (2018)
Chytridiomycota	Rhizophydiales (order)	*Batrachochytrium dendrobatidis*	*Procambarus alleni*	Gastrointestinal tract, gills	Yes	Mortality; weight loss; reduced growth rate	McMahon et al. (2012)
Microsporidia	Pleistophoridae	*Vavraia parastacida*	*Cherax tenuimanus*	Muscle	Yes	Muscle lesions; white patches on muscles	Langdon and Thorne (1992)
		Vavraia parastacida	*Cherax albidus*	Muscle	Yes	Lesions in cephalothorax, legs and tail; anorexia; weakness	Langdon and Thorne (1992)
	Thelohaniidae	*Thelohania contejeani*	*Faxonius virilis*	Muscle	No[b]	n.d.	Graham and France (1986)

(Continues)

TABLE 3 (Continued)

	Taxonomic affiliation	Fungi or fungal-like microorganism	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
		Thelohania contejeani	*Austropotamobius pallipes*	Muscle	No	None	Imhoff et al. (2012)
		Thelohania contejeani	*Pacifastacus leniusculus*	Muscle	No	None	Imhoff et al. (2012)
		Thelohania sp.	*Cherax quadricarinatus*	Muscle	Yes[c]	Adult: none; juvenile: skeletal muscle largely replaced by masses of spores; cardiac muscle exhibited focal myocarditis and granulocyte infiltration; limited focal inflammation of the gills; ovary discoloration; spores and pansporoblasts in the cardiac and cephalothoraxic muscle tissue, gills, nerve cord and gut wall	Herbert (1988)
Oomycota (class)	Saprolegniaceae	*Aphanomyces astaci* strain As (genotype A)	*Austropotamobius torrentium*	Exoskeleton	Yes[d]	Mortality	Jussila et al. (2017)
		Aphanomyces astaci strain As (genotype A)	*Astacus astacus*	Exoskeleton	Yes	Mortality	Jussila et al. (2017)
		Aphanomyces astaci strain As (genotype A)	*Pacifastacus leniusculus*	Exoskeleton	No	None	Jussila et al. (2017)
		Aphanomyces astaci strain As (genotype A)	*Pacifastacus leniusculus*	Exoskeleton	Yes	Mortality; gross symptoms	Aydin et al. (2014)
		Aphanomyces astaci strain Pc (genotype B)	*Astacus astacus*	Exoskeleton	Data Deficient[e]	n.d.	Diéguez-Uribeondo et al. (1995)
		Aphanomyces astaci strain PsI (genotype B)	*Austropotamobius torrentium*	Exoskeleton	Yes	Mortality	Jussila et al. (2017)
		Aphanomyces astaci strain PsI (genotype B)	*Astacus astacus*	Exoskeleton	Yes	Mortality	Jussila et al. (2017)

(Continues)

TABLE 3 (Continued)

Taxonomic affiliation	Fungi or fungal-like microorganism	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
	Aphanomyces astaci strain PsI (genotype B)	*Pacifastacus leniusculus*	Exoskeleton	Yes	Mortality	Jussila et al. (2017)
	Aphanomyces astaci strain PsI (genotype B)	*Pacifastacus leniusculus*	Exoskeleton	Yes	Mortality; gross symptoms	Aydin et al. (2014)
	Aphanomyces astaci strain Si (genotype B)	*Astacus astacus*	Exoskeleton	Yes	Mortality; melanized cuticle	Vey et al. (1983)
	Aphanomyces astaci strain Si (genotype B)	*Faxonius limosus*	Exoskeleton	Yes	Mortality; melanized cuticle	Vey et al. (1983)
	Aphanomyces astaci strain Si (genotype B)	*Astacus astacus*	Exoskeleton	Yes	Mortality; melanized cuticle	Persson and Söderhäll (1983)
	Aphanomyces astaci strain Si (genotype B)	*Pacifastacus leniusculus*	Exoskeleton	Yes	Mortality; melanized cuticle	Persson and Söderhäll (1983)
	Aphanomyces astaci strain Si (genotype B)	*Euastacus kershawi*	exoskeleton	yes	mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Euastacus spinifer*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Euastacus crassus*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Cherax destructor*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Cherax quinquecarinatus*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Cherax papuanus*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)

(Continues)

TABLE 3 (Continued)

Taxonomic affiliation	Fungi or fungal-like microorganism	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
	Aphanomyces astaci strain Si (genotype B)	*Geocharax gracilis*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Astacopsis gouldi*	Exoskeleton	Yes	Mortality; melanized cuticle	Unestam (1975)
	Aphanomyces astaci strain Si (genotype B)	*Astacopsis franklinii*	Exoskeleton	Yes	MORTALITY; MELANIZED CUTICLE	Unestam (1975)
	Aphanomyces frigidophilus	*Austropotamobius pallipes*	Exoskeleton	No[f]	NONE	Ballesteros et al. (2006)
	Aphanomyces laevis strain A	*Procambarus clarkii*	n.d.	Data Deficient[g]	n.d.	Smith (1940)
	Aphanomyces laevis strain B	*Procambarus clarkii*	n.d.	Data Deficient[g]	*n.d.*	Smith (1940)
	Aphanomyces repetans strain An	*Cherax destructor*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Aphanomyces repetans strain An	*Astacus astacus*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Aphanomyces repetans strain Ar	*Cherax destructor*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Aphanomyces repetans strain Fa	*Cherax destructor*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Aphanomyces repetans strain Fb	*Cherax destructor*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Aphanomyces repetans strain Fb	*Astacus astacus*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Aphanomyces repetans strain Se	*Cherax destructor*	Exoskeleton	No[f]	None	Royo et al. (2004)

(Continues)

TABLE 3 (Continued)

Taxonomic affiliation	Fungi or fungal-like microorganism	Tested host	Tissue in which microbe was recorded	Pathogenicity observed	Type of damage recorded	References
	Aphanomyces repetans strain Se	*Astacus astacus*	Exoskeleton	No[f]	None	Royo et al. (2004)
	Saprolegnia australis	*Faxonius rusticus*	Exoskeleton	No[b]	None	Krugner-Higby et al. (2010)
	Saprolegnia australis	*Faxonius propinquus*	Exoskeleton	No[b]	None	Krugner-Higby et al. (2010)
	Saprolegnia parasitica	*Procambarus clarkii*	Exoskeleton	Yes	Mortality; melanized cuticle	Diéguez-Uribeondo et al. (1994)
	Saprolegnia parasitica	*Pacifastacus leniusculus*	Exoskeleton	Yes	Mortality; melanized cuticle	Diéguez-Uribeondo et al. (1994)
	Saprolegnia parasitica	*Astacus astacus*	Exoskeleton	Yes	Mortality; melanized cuticle	Diéguez-Uribeondo et al. (1994)

[a]The infectivity trials included two different treatments of crayfish—animals' exoskeleton was either scarred or punctured before the experimental infection and the recorded symptoms were different for each treatment.

[b]The authors stated that there was no transmission of spores in the water, and animals were not infected. However, the natural route of infection for freshwater fungi and fungal-like microorganisms is through water transmission and feeding, which were used as infection methods in these experiments.

[c]Only the juvenile animals developed the symptoms of the disease.

[d]The authors stated that the microbe was not pathogenic. However, a mortality of 40% was recorded.

[e]The symptoms and the results of the infectivity trials were not clearly stated.

[f]Although histopathological examination has not been performed, no gross symptoms were observed on the exoskeleton and no mortality has been recorded. Since this microbe is targeting primarily the exoskeleton and not the tissues inside the body, we concluded that the pathogenicity has not been observed.

[g]The study is missing the information on the tested tissue and additional details explaining the methods used in conducting the infectivity trials.

Edgerton, 2002; Longshaw, 2016), which is also visible from our records: 78.9% of all entries with identified pathogenicity status (Table 3) were recorded from exoskeleton. Fungi are known to cause secondary infections in wounded or immunocompromised crustaceans (Longshaw, 2016) and may also infect the cuticle in combination with other pathogenic microbes, such as bacteria, and cause the co-infection manifested as shell disease (Johnson, 1983; Sindermann, 1990; Edgerton et al., 2002). Besides cuticle, fungi and fungal-like organisms were frequently reported in literature from muscles and gills (Edgerton et al., 2002; Evans & Edgerton, 2002; Longshaw, 2016) which is also evident from our analysis (11.8% of entries from muscle, however only 1.8% from gills; Figure 5c). Also, we recorded a high number of Data Deficient entries from gastrointestinal tract (17.9%) which were almost exclusively Data Deficient.

For a long time, *A. astaci* has been the focus of many studies due to the high mortality rates it caused in infected farmed and wild crayfish populations, which made it one of the most studied crayfish pathogens (Edgerton et al., 2002, 2004; Svoboda et al., 2017). As a consequence, the emphasis and focus on *A. astaci* research has led to potentially false attributions of some unexplained crayfish mortalities to crayfish plague and has curtailed assessments and research into other potential crayfish pathogens (Edgerton et al., 2004; Longshaw, 2016). However, our results show that other species of the genus *Aphanomyces*, as well as some other genera such as *Fusarium* and *Saprolegnia*, are beginning to receive more attention in research of crayfish pathogens. The inclusion of a wider range of fungi and fungal-like microorganisms is of paramount importance for crayfish disease research.

3.3.1 | Methodological challenges encountered

In this Review, we considered the execution of infectivity trials as one of the critical principles in analysis and systematization of available literature data on potentially pathogenic crayfish microbes. However, methodological approaches to examining pathogenicity differed substantially in analysed studies, both in the execution of infectivity trials and measurements of impacts on the host, which may consequently affect the outcome of observations of pathogenicity. In some cases, the applied infection methods did not necessarily mimic the microbe's natural route of infection. For example, for WSSV the natural infection route is by feeding, which was used as a method of infection in some studies (Bateman et al., 2012; Corbel et al., 2001; Edgerton, 2004; Jiravanichpaisal et al., 2004). However, several studies (Table 1) also infected the crayfish with WSSV also by intramuscular inoculation (Bateman et al., 2012; Corbel et al., 2001; Edgerton, 2004; Huang et al., 2001; Jiravanichpaisal et al., 2004; Shi et al., 2000). Similarly, the pathogen load used in infectivity trials and the number of repeated infections was frequently either not reported or varied substantially between the studies and could also significantly affect the outcomes of the infectivity trial and the amount of recorded damage to the host. These methodological differences may have resulted in identification of some microbes as those with demonstrated pathogenicity even though the infection routes, concentrations or their presence in certain tissues may not occur naturally. In addition, we detected a substantial difference in the number of parameters used to measure damage to the host between the studies, since for many studies defining pathogenicity of a specific microbe was not a primary focus. For example, certain studies have focused on testing the difference in virulence between strains of a particular pathogenic species, comparing only the difference in mortality (e.g., Diéguez-Uribeondo et al., 1995; Jussila et al., 2017), while others examined only histopathological symptoms related to an effect of water temperature on the virulence of specific pathogen in only one particular target tissue (Jiravanichpaisal et al., 2004).

Due to the observed large discrepancies in the implementation and methodology of infectivity trials, we suggest that a general framework for execution of such trials would be beneficial in future inspection of interactions between microbes and the host (summarized in Box 1). Primarily, studies involving infectivity trials should contain a control group and clearly report methodology and results and provide details into which disease symptoms were measured and how, since many studies report only that disease symptoms were measured without specifying them. Similarly, studies should provide quantitative rather than qualitative ("less or more virulent than") comparisons of virulence. Furthermore, when possible, a dose–response relationship between the concentrations (pathogen load) would be beneficial parameter to record, as well as description whether the infection method mimics potential natural infection routes. Finally, it would be advantageous to measure at least one other parameter of damage to the host other than mortality (i.e. the highest possible amount of damage to the host), because even smaller amounts of damage at lower levels of biological organization can impair host's fitness and therefore microbe could be considered as pathogenic.

3.3.2 | General issues in systematic analysis of pathogens

The binary view on classification of a microbe as either pathogen or non-pathogen is nowadays under significant debate since pathogenicity is increasingly recognized not as a property of the microbe itself, but rather as a function of the host, the microbe, and their interactions which are mediated by their environment and constitute an interactive system in which the ecological role of microbes and hosts can be changed (Méthot & Alizon, 2014; Pirofski & Casadevall, 2012). We discuss the major issues regarding a binary view of pathogenicity in the context of analysed studies on microbes recorded in freshwater crayfish, following the framework of Méthot and Alizon (2014).

Changes in the host–microbe interactions, microbial evolution and effects of the host immune system

It is argued that due to adaptations of both microbes and the host to the changes in their environment, non-pathogens or commensals can become pathogens and reversely, pathogens can become commensals. Furthermore, since host–microbe interactions change in response to diverse selective pressures present in the environment, and since microbes readily adapt to these changes, the virulence and consequently pathogenicity of a microbe are subject to change (Méthot & Alizon, 2014). In addition, pathogenicity is a result of interaction between the microbe and host's immune system, which may have an exaggerated response (i.e., exhibit "immunopathology") and can also adapt the level of response to an infection. Such adaptation in host–microbe interactions is visible in the case of effects of *A. astaci* on some European ICS: in the past, certain crayfish plague strains expressed high virulence in populations of highly susceptible native crayfish, however, chronically infected ICS populations were observed over the last few years (e.g., *A. astacus*: Jussila et al., 2011; Makkonen et al., 2012; *A. pallipes*: Martín-Torrijos et al., 2017; *A. torrentium*: Kušar et al., 2013; *P. leptodactylus*: Kokko et al., 2018). However, none of the studies analysed the underlying causes of observed adaptations to the host–*A. astaci* co-existence, but only speculate that they could be driven by changes in the *A. astaci* virulence, host response or both (Kokko et al., 2012; Kušar et al., 2013; Martín-Torrijos et al., 2017).

Due to reasons discussed above, we made no differentiation between opportunistic pathogens and pathogens. Opportunists may be ubiquitous, but rarely pathogenic in immunocompetent organisms (Hajek & Shapiro-Ilan, 2018). In immunocompromised organisms, or those already infected with a pathogen, they can cause secondary infection (Edgerton et al., 2002; Johnson, 1983; Longshaw, 2016) and thus overload the immune system, which can lead to mortality that would not necessarily result from a primary infection alone. For example, in the case of crayfish shell disease, bacteria or fungi that are not the primary causes of the disease can take advantage of the presence of lesions, enter the body of crayfish and have a detrimental effect on crayfish health (Edgerton et al., 2002; Johnson, 1983). In our analysis, such microbes were also classified as those with observed pathogenicity if they met the criteria we set: successful infection and records of significant damage to the host compared to control group. We argue that systematization and recording of information regarding the circumstances under which a certain microbe has or has not demonstrated pathogenicity will be essential for broadening our understanding of host–microbe interactions in the future.

The effects of multiple infections and environment on pathogenicity

Infection of the crayfish with several microbes, which individually cause none or only mild negative effects, can induce detrimental effects in an individual (Dunn et al., 2012; Edgerton et al., 1995, 2004). Thus, damage to the host may occur due to co-infection by microbes that can engage in antagonistic or synergistic interactions. Furthermore, multiple infections may either elicit a strong immune response, or disrupt immune response and ultimately cause host's mortality (Méthot & Alizon, 2014). A strong immune response to one pathogen may reduce the host's ability to effectively respond to infection by other pathogens, as identified in a study by Jiravanichpaisal et al. (2004). Upon infection of *P. leniusculus* and *A. astacus* with WSSV, mortality in *P. leniusculus* occurred more rapidly than in *A. astacus*, which was attributed to the presence of the crayfish plague causing agent, *A. astaci*, in *P. leniusculus* (Jiravanichpaisal et al., 2004). Due to its presence in the cuticle of *P. leniusculus*, this crayfish host has a permanent high level of transcripts coding for prophenoloxidase, an important innate immunity protein, and such strong immune response to *A. astaci* was considered responsible for higher susceptibility of *P. leniusculus* to WSSV and secondary infection (Jiravanichpaisal et al., 2004). On the other hand, *A. astacus* is not a carrier of *A. astaci*, and therefore its immune system can respond more efficiently to the WSSV infection in the early stage. However, infection with WSSV ultimately led to mortality in both crayfish species. Interactions between microbes may not only increase, but also decrease the amount of damage to the host. We have recently shown that certain bacterial isolates from the crayfish cuticle can inhibit the growth of *A. astaci* in vitro (Orlić et al., In press). Furthermore, infection by one pathogen may sometimes reduce the opportunity for co-infection by other pathogens, as demonstrated in amphipods (Rauque & Semenas, 2012).

Research in laboratory conditions has shown that in addition to biotic factors, abiotic factors may also affect host–microbe interactions and the occurrence of pathogenicity by decreasing the microbe's virulence or by decreasing the host's immunocompetence (Méthot & Alizon, 2014). For example, temperature strongly affects the virulence of WSSV: at low water temperature, freshwater crayfish can serve as a reservoir of WSSV, while at high water temperature WSSV can replicate and cause mortality (Jiravanichpaisal et al., 2004). Besides temperature, other water quality parameters, such as dissolved oxygen levels and presence of pollutants (e.g., chemicals, drugs and antibiotics) may affect the susceptibility of crayfish and other aquatic organisms to pathogens (Diéguez-Uribeondo et al., 1994; Hajek & Shapiro-Ilan, 2018; Hernández-Pérez et al., 2020). This shows that numerous factors can influence the outcome of infectivity trials and host–microbe interactions and pinpoints that due to complexity of interactions between the microbes, host and the environment, experimental research in laboratory conditions, as performed in infectivity trials, may record different effects on the host than the ones that would occur naturally. Infectivity trials may not necessarily be a realistic representation of the effects of a microbe in nature, but due to a large gap in understanding of host–microbe interactions, we believe they are currently the best methodological approach for analysis of possible outcomes of such interactions.

Virulence

In the past, virulence was considered a property of a pathogen and was assumed that pathogens can be distinguished from non-pathogens due to the presence of virulence factors (i.e., traits that are responsible for the occurrence of virulence) (Méthot & Alizon, 2014).

However, virulence is not a fixed but rather an emergent property, which occurs only in the host (in which it can also be measured), while the microbe itself outside the host does not express virulence (Pirofski & Casadevall, 2012). As such, it is a relative property that occurs and changes from host to host and depends on multiple determinants: microbe, host, their environment and interaction between the three, therefore the microbe can be both pathogen and commensal in a different host (Méthot & Alizon, 2014; Pirofski & Casadevall, 2012), as discussed earlier. Even among microbes of the same species, differences in virulence may occur between strains: certain strains express high virulence in certain host species, while other strains express no virulence in the same host. As reported by Wong et al. (1995), mortality of *Cherax albidus* Clark, 1936 and *C. quadricarinatus* infected with different strains of *Vibrio mimicus* Davis et al. 1982 ranged between 0% and 100%, depending on the *V. mimicus* strain. Another well-known example of variation in virulence among different strains is *A. astaci* (Becking et al., 2015). Research has shown that some strains are highly virulent in certain hosts, while others less so, for example group A strains, which have been present in Europe for many years, appear to be less virulent for ICS than strains which were introduced more recently, with crayfish imports from North America (Svoboda et al., 2017). Furthermore, Aydin et al. (2014) reported that *P. leniusculus*, the North American carrier of *A. astaci*, is susceptible to the European *A. astaci* strain belonging to the group A. Hence, closely related crayfish populations, belonging to the same or different (sub)species, can develop resistance to different strains of the same microbe depending on which microbes occur in their environment (Claydon et al., 2004). Given these differences in virulence among strains of a single microbe and among different hosts, it is possible that potentially some microbes for which pathogenicity in a specific host was not observed, may express pathogenicity in a different closely related host. Thus, recording and analysing effects of a specific microbe in multiple different hosts is required in the study of potential crayfish pathogens. In conclusion, occurrence of virulence (and pathogenicity) is dependent upon multiple determinants, can vary experimentally or spontaneously, and be enhanced, lost and restored (Méthot & Alizon, 2014).

3.3.3 | More research on potential pathogens in native and endangered crayfish populations in the wild required

Within this analysis, we identified a bias in the selection of host crayfish species for the disease studies and infectivity trials. As visible in the Tables 1-3, Appendices S1–S3 and Figures (3–5b), some species of crayfish, that is *C. quadricarinatus* and *P. clarkii*, are much more represented in this type of studies than the other species, such as European ICS. As already observed by Longshaw (2016), most research on crayfish diseases has been conducted on aquaculturally important species while pathogens and diseases in crayfish populations in the wild remain understudied. Furthermore, the high number of observed microbes that cause pathogenicity in commercially important host species does not indicate that these species are more susceptible to infection, but is a consequence of research focus on these species of aquaculture interest (Longshaw, 2016). This is especially visible in the case of viruses which are, as obligate parasites, mostly species-specific. Although large numbers of viruses remain unexplored in both farmed and wild crayfish populations, focusing on viruses only in commercially important crayfish species further contributes to the imbalance between known and unknown crayfish viruses and their impact on crayfish populations. Some viruses, such as CBV, have been researched in multiple studies (Anderson & Prior, 1992; Claydon et al., 2004; Davidovich et al., 2019; Edgerton & Owens, 1993, 1997; Edgerton et al., 1995; Groff et al., 1993; Kent Hauck et al., 2001; Romero & Jiménez, 2002) since its host *C. quadricarinatus* is a species farmed worldwide. On the other hand, viruses of less commercially important crayfish species, or native crayfish species of no economic but high ecological value are rarely the subject of research. For example, only three entries for viruses have been recorded in *A. pallipes* (Bateman et al., 2012; Edgerton, 2003; Grandjean et al., 2019), an endangered native European crayfish, as opposed to, for example, *C. quadricarinatus*, in which 24 entries have been recorded (Table 1 and Appendix S1). Furthermore, the bias is visible not only in the case of research focus on hosts but also in the case of microbes: some common and well-established pathogenic microbes are more often studied than some other, less prevalent ones. Such is the case with *A. astaci*: the extreme emphasis on the importance and threat of *A. astaci* for a long period of time resulted in overseeing other, less widespread crayfish pathogens, as well as in underdevelopment of crayfish pathology and diagnostic methods (Edgerton et al., 2004; Longshaw, 2016). Underdevelopment of research of other crayfish pathogens may directly influence the effectivity of management decisions aimed at conserving European ICS, since the importance and presence of other less researched pathogens, especially those occurring in the wild populations, is rarely taken into account (Edgerton et al., 2004).

Box 1 Guidelines for standardized execution of infectivity trials

- Existence of a control group
- Clear description which disease symptoms were measured
- Information whether applied infection route mimics natural infection routes
- Information regarding applied pathogen load and number of executed infections

If possible:

- Measurements of at least one additional parameter of damage to the host other than mortality
- A dose–response relationship between the applied pathogen load and measured parameters of damage to the host

In comparison to farmed animals, detection and monitoring pathogens in wild animal populations is a very demanding task, since it depends on the sophistication of sampling design and diagnostic techniques (Scott, 1988). Often only a disease with very clear and extensive symptoms can be detected, while for the detection of diseases causing lesser effects (such as delayed age at sexual maturity, reduced growth or stamina) a detailed and continuous monitoring of the population is required (Scott, 1988). Furthermore, it is often not easy to define whether, for example, a crayfish lethargy is caused by a pathogen or some other factor, such as environmental stress and habitat conditions (e.g., oxygen level, temperature, water quality) (Evans & Edgerton, 2002). Additional difficulties may arise if the probability of trapping sick animals is reduced, since the sampled animals will not give the accurate representation of the level of disease in a wild population (Wiger, 1977). Therefore, detecting and quantifying pathogens causing mortality in wild animal populations presents a major challenge. Even though prompt detection of disease and recognition of its impact in wild animal populations, especially in endangered species, could aid in species conservation, disease research in wild animal populations has been relatively scarce and often related to those diseases that have serious zoonotic and economic implications (Mörner et al., 2002; Thorne & Williams, 1988). Finally, development of non-invasive approaches for pathogen monitoring is required for native and endangered crayfish populations in the wild, in order to avoid unnecessary killing of individuals for screening purposes. eDNA coupled with sensitive molecular techniques such as qPCR (e.g. Robinson et al., 2018; Strand et al., 2019; Wittwer et al., 2018) are increasingly being used in monitoring of crayfish plague, and non-invasive approach to *A. astaci* detection from cuticle swabs has recently been developed that circumvents the killing of crayfish for *A. astaci* detection purposes (Pavić et al., 2020). Again, it must be acknowledged that such approaches have been designed for monitoring and detection of the most well-studied disease in crayfish, the crayfish plague, while methodological developments for detection of other diseases remain sparse.

4 | CONCLUSION

This review provides systematization of studies that report on potential crayfish pathogens with a detailed list of microbes responsible for damage observed within susceptible freshwater crayfish. It also overviews in detail the frequency of studies and the available information regarding the observed effects of microbes on hosts and as such may represent a base for future risks assessments. The Review also identifies the gaps in knowledge demonstrating the need for: (a) research of higher number of crayfish species in the case of viruses in order to detect and prioritize viruses with wide host range, (b) research of effects in a wider range of tissues in the case of bacteria since due to focus on haemolymph it is possible that bacterial infections in other tissues have not been recognized, and (c) the need for research into other potential pathogens other than *A. astaci* in the case of fungi and fungal-like organisms.

Studies are systematized according to observation of pathogenicity, but these may change over time since it is possible that susceptible hosts have not been identified within these studies, and since host–microbe interaction is a dynamic process subject to change. Due to the fluidity of the boundaries between pathogens and commensals which is also dependent upon multiple other known and unknown factors (e.g., environment, host's immune system, interactions with other microbes; Pirofski & Casadevall, 2012), the classification of microbes into one of these two categories should also be dynamic. Finally, in order to broaden our understanding of host–microbe interactions in crayfish future research should also aim to record and analyse circumstances under which a certain microbe has or has not demonstrated pathogenicity. For such future analyses, we suggest that a broad but standardized framework for execution of infectivity trials should be adopted for systematic data acquisition on interactions between microbes and the host.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Paula Dragičević involved in data collection and analysis, and wrote, reviewed and edited the manuscript. Ana Bielen involved in conceptualization and wrote, reviewed and edited the manuscript. Ines Petrić wrote, reviewed and edited the manuscript. Sandra Hudina involved in conceptualization and data analysis, supervised the study and wrote, reviewed and edited the manuscript.

DATA AVAILABILITY STATEMENT

This MS is a review and analysis of data available within respective publications. Analyses performed through this MS will be available in the text and as supporting information in the Appendix S1–S3 and if required in a repository.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supporting information

Appendix 1. List of viruses of crayfish with undetermined pathogenicity status due to non-execution of infectivity trials, according to the developed systematisation system (Data Deficient).

Taxonomic affiliation	Virus	Tested host	Tissue in which microbe was recorded	Reference
Bunyavirales (order)	*Athtabvirus*	*Cherax quadricarinatus*	muscle, neural tissue	Sakuna et al. (2018)
Iflaviridae	*Chequa iflavirus*	*Cherax quadricarinatus*	muscle, neural tissue	Sakuna et al. (2017)
Nudiviridae	*Astacus astacus baciliform virus* (AaBV)	*Astacus astacus*	hepatopancreas, gastrointestinal tract	Edgerton et al. (1996)
	Austropotamobius pallipes baciliform virus (ApBV)	*Austropotamobius pallipes*	hepatopancreas, gastrointestinal tract	Edgerton (2003)
	Cherax baculovirus (CBV)	*Cherax quadricarinatus*	hepatopancreas, gastrointestinal tract	Edgerton et al. (1995)
	Cherax baculovirus (CBV)	*Cherax quadricarinatus*	hepatopancreas	Anderson and Prior (1992)
	Cherax destructor baciliform virus (CdBV)	*Cherax destructor*	hepatopancreas	Edgerton (1996)
	Cherax quadricarinatus bacilliform virus (CqBV) (old nomenclature: CBV)	*Cherax quadricarinatus*	hepatopancreas	Davidovich et al. (2019)

	Cherax quadricarinatus bacilliform virus (CqBV) (old nomenclature: CBV)	*Cherax quadricarinatus*	hepatopancreas	Romero and Jimenez (2002)
	Cherax quadricarinatus bacilliform virus (CqBV), Utah (USA) strain (old nomenclature: CBV)	*Cherax quadricarinatus*	hepatopancreas	Kent Hauck et al. (2001)
	Cherax quadricarinatus bacilliform virus (CqBV), Utah (USA) strain (old nomenclature: CBV)	*Cherax quadricarinatus*	hepatopancreas	Groff et al. (1993)
	Pacifastacus leniusculus bacilliform virus (PlBV)	*Pacifastacus leniusculus*	hepatopancreas	Hedrick et al. (1995)
Parvoviridae	*Cherax destructor systemic parvo-like virus* (CdSPV)	*Cherax destructor*	gills, hepatopancreas, gastrointestinal tract	Edgerton et al. (1997)
	Cherax quadricarinatus parvo-like virus (CqPV)	*Cherax quadricarinatus*	gills	Edgerton et al. (2000)
	Spawner-isolated mortality virus (SMV)	*Cherax quadricarinatus*	internal organs	Owens and McElnea (2000)
Phenuiviridae	*Bunya-like Brown Spot Virus* (BBSV)	*Austropotamobius pallipes*	exoskeleton	Grandjean et al. (2019)
Reoviridae	*Hepatopancreatic reovirus*	*Cherax quadricarinatus*	hepatopancreas	Edgerton et al. (2000)
unclassified	*Cherax Giardiavirus-like virus* (CGV)	*Cherax quadricarinatus*	hepatopancreas	Edgerton et al. (1995)

	Cherax Giardiavirus-like virus (CGV)	*Cherax quadricarinatus*	hepatopancreas	Edgerton et al. (1994)

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Appendix 2. List of bacteria with undetermined pathogenicity status due to non-execution of infectivity trials, according to the developed systematisation system (Data Deficient).

Taxonomic affiliation		Bacterium	Tested host	Tissue in which microbe was recorded	Reference
Actinobacteria	Corynebacteriaceae	*Corynebacterium* sp.	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Corynebacterium sp.	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Corynebacterium sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
	Micrococcaceae	*Arthrobacter* sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Micrococcus luteus	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Micrococcus luteus	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Micrococcus roseus	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Micrococcus sp.	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Micrococcus spp.	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
	Mycobacteriaceae	*Mycobacterium fortuitum*	*Procambarus clarkii*	hepatopancreas	Ahmed et al. (2010)

		Mycobacterium gordonae	*Cherax quadricarinatus*	hepatopancreas, gills, gonads	Davidovich et al. (2019)
	Nocardiaceae	*Nocardia* sp.	*Austropotamobius pallipes*	muscle	Alderman et al. (1986)
Bacteroidetes	Flavobacteriaceae	*Flavobacterium dormitator*	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Flavobacterium indologenes	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
		Flavobacterium sp.	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Flavobacterium sp.	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
		Flavobacterium sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Flavobacterium spp.	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
	Sphingobacteriaceae	*Sphingobacterium multivorum*	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
	Weeksellaceae	*Weeksella virosa*	*Cambarellus patzcuarensis*	hemolymph	Longshaw et al. (2012)
Firmicutes	Bacillaceae	*Bacillus* sp.	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Bacillus sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)

		Bacillus spp.	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
	Listeriaceae	*Listeria monocytogenes*	*Pontastacus leptodactylus*	muscle	Khamesipour et al. (2013)
		Listeria monocytogenes	*Procambarus clarkii*	muscle	Li et al. (2015)
	Planococcaceae	*Kurthia* sp.	*Cherax albidus*	hemolymph	Wong et al. (1995)
	Staphylococcaceae	*Staphylococcus cohnii*	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Staphylococcus epidermidis	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Staphylococcus epidermis	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Staphylococcus spp.	*Cherax albidus*	hemolymph	Wong et al. (1995)
	Streptococcaceae	*Streptococcus* sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
Proteobacteria	Aeromonadaceae	*Aeromonas hydrophila*	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Aeromonas hydrophila	*Pacifastacus leniusculus*	hemolymph	Longshaw et al. (2012)
		Aeromonas hydrophila	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)

		Aeromonas hydrophila	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
		Aeromonas hydrophila	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Aeromonas hydrophila	*Austropotamobius pallipes*	hemolymph	Quaglio et al. (2006a)
		Aeromonas hydrophila	*Austropotamobius pallipes*	hemolymph	Quaglio et al. (2008)
		Aeromonas sobria	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Aeromonas sobria	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Aeromonas sobria	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Aeromonas sobria	*Procambarus fallax*	hemolymph	Longshaw et al. (2012)
		Aeromonas sobria	*Cambarellus patzcuarensis*	hemolymph	Longshaw et al. (2012)
		Aeromonas sobria	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
		Aeromonas sobria	*Faxonius propinquus*	exoskeleton	Krugner-Higby et al. (2010)
		Aeromonas sp.	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)

		Aeromonas sp.	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
		Aeromonas veroni	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
	Alcaligenaceae	*Alcaligenes* sp.	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Alcaligenes sp.	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Oligella ureolytica	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
	Campylobacteraceae	*Campylobacter* spp.	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
	Chromobacteriaceae	*Chromobacterium violaceum*	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
	Enterobacterales (order)	*Plesiomonas shigelloides*	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Plesiomonas shigelloides	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
	Enterobacteriaceae	*Citrobacter freundii*	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Citrobacter freundii	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Citrobacter freundii	*Cambarellus patzcuarensis*	hemolymph	Longshaw et al. (2012)

		Citrobacter freundii	*Procambarus fallax*	hemolymph	Longshaw et al. (2012)
		Citrobacter freundii	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
		Citrobacter freundii	*Austropotamobius pallipes*	hepatopancreas	Quaglio et al. (2006a)
		Citrobacter freundii	*Procambarus clarkii*	hemolymph	Quaglio et al. (2006b)
		coliform-like organism	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Enterobacter aerogenes	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Enterobacter agglomerans	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Enterobacter cloacae	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Escherichia coli	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Escherichia coli	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
		Klebsiella pneumoniae	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Klebsiella pneumoniae	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)

	Francisellaceae	*Francisella tularensis*	*Procambarus clarkii*	gastrointestinal tract, hepatopancreas	Anda et al. (2001)
	Hafniaceae	*Edwardsiella tarda*	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Hafnia alvei	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Hafnia alvei	*Pacifastacus leniusculus*	hemolymph	Longshaw et al. (2012)
		Hafnia alvei	*Astacus astacus*	gastrointestinal tract	Orozova et al. (2014)
		Hafnia alvei	*Austropotamobius pallipes*	hemolymph	Quaglio et al. (2008)
	Moraxellaceae	*Acinetobacter antitratum*	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Acinetobacter antitratum	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Acinetobacter calcoaceticus	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Acinetobacter calcoaceticus	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Acinetobacter calcoaceticus var. *lwoffi*	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Acinetobacter lwoffi	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)

		Acinetobacter lwoffi	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Acinetobacter sp.	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Acinetobacter sp.	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
		Acinetobacter sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Moraxella sp.	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
	Morganellaceae	*Proteus vulgaris*	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
	Pasteurellaceae	*Pasteurella multocida*	*Procambarus fallax*	hemolymph	Longshaw et al. (2012)
		Pasteurella spp.	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
	Pseudomonadaceae	*Pseudomonas acidovorans*	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
		Pseudomonas alcaligenes	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Pseudomonas cepacia	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Pseudomonas cepacia	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)

		Pseudomonas fluorescens	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
		Pseudomonas fluorescens	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
		Pseudomonas maltophilia	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Pseudomonas maltophilia	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Pseudomonas mendocina	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Pseudomonas putida	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
		Pseudomonas putrefaciens	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Pseudomonas sp.	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Pseudomonas sp.	*Procambarus clarkii*	hemolymph	Quaglio et al. (2006b)
		Pseudomonas spp.	*Cherax quadricarinatus*	hemolymph	Bowater et al. (2002)
		Pseudomonas stutzeri	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Pseudomonas vesicularis	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)

	Rickettsiaceae	*Rickettsia*-like organism	*Cherax quadricarinatus*	hepatopancreas	Edgerton and Prior (1999)
		Rickettsia-like organism	*Cherax quadricarinatus*	connective tissue, hepatopancreas	Jiménez and Romero (1997)
	Shewanellaceae	*Shewanella putrefaciens*	*Cherax albidus*	hemolymph	Wong et al. (1995)
		Shewanella putrefaciens	*Cherax quadricarinatus*	hemolymph	Edgerton et al. (1995)
	Sphingomonadaceae	*Sphingomonas paucimobilis*	*Astacus astacus*	hemolymph	Madetoja and Jussila (1996)
	Vibrionaceae	*Grimontia hollisae*	*Procambarus fallax*	hemolymph	Longshaw et al. (2012)
		Vibrio alginolyticus	*Pacifastacus leniusculus*	hemolymph	Longshaw et al. (2012)
		Vibrio alginolyticus	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
		Vibrio alginolyticus	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Vibrio anguillarum	*Cherax albidus*	hemolymph	Jones and Lawrence (2001)
		Vibrio cholerae	*Cherax quadricarinatus*	hemolymph	Wong et al. (1995)
		Vibrio cholerae	*Cherax albidus*	hemolymph	Wong et al. (1995)

		Vibrio cholerae	*Procambarus clarkii*	hemolymph	Thune et al. (1991)
		Vibrio harveyi	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
		Vibrio mimicus	*Procambarus clarkii*	hemolymph	Thune et al. (1991)
		Vibrio mimicus	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
		Vibrio sp.	*Procambarus clarkii*	hemolymph	Scott and Thune (1986)
		Vibrio vulnificus	*Pontastacus leptodactylus*	muscle	Raissy et al. (2014)
Tenericutes	Spiroplasmataceae	*Spiroplasma eriocheiris*	*Procambarus clarkii*	hemolymph, heart, gills, hepatopancreas, connective tissue	Ding et al. (2014b)

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Appendix 3. List of fungi and fungal-like microorganisms with undetermined pathogenicity status due to non-execution of infectivity trials, according to the developed systematisation system (Data Deficient).

Taxonomic affiliation		Fungi or fungal-like microorganism	Tested host	Tissue in which microbe was recorded	Reference
Ascomycota	Apiosporaceae	*Arthrinium phaeospermum*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Arthrinium sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Aspergillaceae	*Aspergillus album*	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Aspergillus brasiliensis	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Aspergillus clavatus	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Aspergillus clavatus	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Aspergillus flavus	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Aspergillus flavus	*Pontastacus leptodactylus*	exoskeleton	Fard et al. (2011)
		Aspergillus fumigatus	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Aspergillus glaucus	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)

		Aspergillus niger	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Aspergillus sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Aspergillus sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Aspergillus sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Aspergillus sp.	*Procambarus clarkii*	exoskeleton, internal organs	Lahser (1975)
		Aspergillus sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Aspergillus spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2006a)
		Aspergillus terreus	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Aspergillus versicolor	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Penicillium expansum	*Pontastacus leptodactylus*	exoskeleton	Fard et al. (2011)
		Penicillium sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Penicillium sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)

		Penicillium sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Penicillium sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Penicillium spp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Penicillium spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
		Penicillium verrucosum	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Bionectriaceae	*Acremonium chrysogenum*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Acremonium kiliense	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Acremonium persicinum	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Acremonium sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Acremonium sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Acremonium sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Acremonium sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)

		Acremonium spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2006a)
		Clonostachys rosea	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
	Blastocladiaceae	*Allomyces* sp.	*Cherax quadricarinatus*	n.d.	Cannon and Sewell (1994)
	Cephalothecaceae	*Paecilomyces inflatus*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Chaetomellaceae	*Chaetomella raphigera*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Chaetomium sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Cladosporiaceae	*Cladosporium chlorocephalum*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Cladosporium chlorocephalum	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Cladosporium cladosporioides	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Cladosporium cladosporioides	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Cladosporium sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Cladosporium sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)

		Hormodendrum sp.	*Procambarus simulans simulans*	exoskeleton, internal organs	Lahser (1975)
		Hormodendrum sp.	*Procambarus clarkii*	exoskeleton, internal organs	Lahser (1975)
	Cordycipitaceae	*Paecilomyces farinosus*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Dematiaceae	unidentified member of Dematiaceae	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
	Didymellaceae	*Epicoccum nigrum*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Phoma glomerata	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Phoma sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Phoma sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
	Dipodascaceae	*Geotrichum* spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
	Erysiphaceae	*Hormisum* sp.	*Procambarus clarkii*	exoskeleton, internal organs	Lahser (1975)
		Uncinula sp.	*Procambarus simulans simulans*	exoskeleton, internal organs	Lahser (1975)
	Graphiaceae	*Graphium* sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)

	Hypocreaceae	*Trichoderma* sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Trichoderma sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Trichoderma sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Trichoderma spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2006a)
		Trichoderma spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
		Trichoderma viridae	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
	Hypocreales (order)	*Gliocladium* sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Gliocladium spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
	Microascaceae	*Cephalotrichum microsporum*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Scopulariopsis sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Microdochiaceae	*Microdochium bolleyi*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
	Myrotheciomycetaceae	*Emericellopsis* sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)

		Emericellopsis sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Myxotrichaceae	*Oidiodendron flavum*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Nectriaceae	*Fusarium dimerum*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Fusarium graminearum	*Pacifastacus leniusculus*	exoskeleton	Edsman et al. (2015)
		Fusarium negundis	*Pacifastacus leniusculus*	exoskeleton	Edsman et al. (2015)
		Fusarium oxysporum	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Fusarium proliferatum	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Fusarium solanii	*Astacus astacus*	exoskeleton	Makkonen et al. (2013)
		Fusarium sp.	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Fusarium sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Fusarium sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Fusarium sp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2006a)

		Fusarium sp.	*Pontastacus leptodactylus*	exoskeleton	Fard et al. (2011)
		Fusarium sp.	*Procambarus simulans simulans*	exoskeleton, internal organs	Lahser (1975)
		Fusarium sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Fusarium spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
		Fusarium tricinctum	*Pacifastacus leniusculus*	exoskeleton	Edsman et al. (2015)
		Fusarium verticillioides	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Fusarium tabacinum	*Austropotamobius pallipes*	gills	Alderman and Polglase (1985b)
	Ophiocordycipitaceae	*Paecilomyces lilacinum*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Paecilomyces lilacinus	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Pleosporaceae	*Alternaria alternata*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Alternaria alternata	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Alternaria cheiranthi	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)

		Alternaria chlamydospora	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Alternaria chlamydospora	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Alternaria sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Alternaria sp.	*Pontastacus leptodactylus*	exoskeleton	Fard et al. (2011)
		Alternaria sp.	*Procambarus simulans simulans*	exoskeleton, internal organs	Lahser (1975)
		Alternaria sp.	*Procambarus acutus acutus*	exoskeleton, internal organs	Lahser (1975)
		Alternaria sp.	*Creaserinus fodiens*	exoskeleton, internal organs	Lahser (1975)
		Alternaria sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Alternaria spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2006a)
		Alternaria spp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)
		Drechslera sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Ulocladium sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)

	Saccotheciaceae	*Aureobasidium pullulans* var. *melanogenum*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Aureobasidium pullulans var. *pullulans*	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Aureobasidium pullulans var. *pullulans*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Schizoparmaceae	*Coniella* sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Sordariaceae	*Sordaria fimicola*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Sporocadaceae	*Pestalotiopsis guepinii*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Trichocomaceae	*Hemicarpenteles ornatum*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Talaromyces flavus	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Trichosphaeriaceae	*Khuskia oryzae*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	unclassified	*Paecilomyces* sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Paecilomyces sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
Basidiomycota	Cryptococcaceae	*Cryptococcus gammari*	*Austropotamobius pallipes*	n.d.	Goodrich (1956)

		Cryptococcus laurentii	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
	Sporidiobolaceae	*Rhodotorula* sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
	Trichosporonaceae	*Trichosporon beigelii*	*Astacus astacus*	exoskeleton	Söderhäll et al. (1993)
Chytridiomycota	Rhizophydiales (order)	*Batrachochytrium dendrobatidis*	*Orconectes virilis*	gastrointestinal tract	McMahon et al. (2012)
		Batrachochytrium dendrobatidis	*Procambarus clarkii*	gastrointestinal tract	McMahon et al. (2012)
Microsporidia	Burenellidae	*Vairimorpha austropotamobii* sp. nov.	*Austropotamobius pallipes*	muscle	Pretto et al. (2017)
		Vairimorpha cheracis	*Cherax destructor*	muscle	Moodie et al. (2003c)
	Glugeidae	*Cambaraspora floridanus*	*Procambarus paeninsulanus*	muscle, heart	Bojko et al. (2020)
		Cystosporogenes sp.	*Pacifastacus leniusculus*	muscle	Imhoff et al. (2010)
	Mrazekiidae	*Bacillidium* sp.	*Pacifastacus leniusculus*	muscle	Dunn et al. (2009)
	Nosematidae	*Vittaforma corneae*	*Pacifastacus leniusculus*	muscle	Imhoff et al. (2010)
		Vittaforma sp.	*Pacifastacus leniusculus*	muscle	Dunn et al. (2009)

	Pleistophoridae	*Pleistophora soganderesi*	*Cambarellus puer*	muscle	Sogandares-Bernal (1962)
		Pleistophora sp.	*Cherax destructor*	muscle	O'Donoghue et al. (1990)
		Vavraia parastacida	*Cherax tenuimanus*	muscle	Langdon (1991)
	Thelohaniidae	*Thelohania contejeani*	*Austropotamobius pallipes*	muscle	Dunn et al. (2009)
		Thelohania contejeani	*Pacifastacus leniusculus*	muscle	Dunn et al. (2009)
		Thelohania contejeani	*Austropotamobius pallipes*	muscle	Imhoff et al. (2010)
		Thelohania contejeani	*Pacifastacus leniusculus*	muscle	Imhoff et al. (2010)
		Thelohania contejeani	*Paranephrops zealandicus*	muscle	Quilter (1976)
		Thelohania contejeani	*Astacus astacus*	muscle	Lom et al. (2001)
		Thelohania contejeani	*Austropotamobius pallipes*	muscle	Longshaw et al. (2012b)
		Thelohania contejeani	*Austropotamobius pallipes*	muscle, heart, gastrointestinal tract, neural tissue	Pretto et al. (2017)
		Thelohania montirivulorum	*Cherax destructor*	n.d.	Moodie et al. (2003a)

		Thelohania parastaci	*Cherax destructor*	muscle	Moodie et al. (2003b)
		Thelohania sp.	*Orconectes propinquus*	n.d.	Krugner-Higby et al. (2010)
		Thelohania sp.	*Cherax quadricarinatus*	muscle	Herbert (1987)
		Thelohania sp.	*Cherax destructor*	muscle	O'Donoghue et al. (1990)
		Thelohania sp.	*Cherax quadricarinatus*	muscle	O'Donoghue and Adlard (2000)
		Thelohania sp.	*Cherax quinquecarinatus*	muscle	O'Donoghue and Adlard (2000)
		Thelohania sp.	*Cherax tenuimanus*	muscle	O'Donoghue and Adlard (2000)
		Thelohania sp.	*Cherax destructor*	muscle	O'Donoghue and Adlard (2000)
		Thelohania sp.	*Paranephrops planifrons*	muscle	Jones (1980)
	unclassified	*Microsporidium* sp.	*Pacifastacus leniusculus*	muscle	Dunn et al. (2009)
Mucoromycota	Cunninghamellaceae	*Absidia fusca*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)

		Absidia glauca	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Lichtheimiaceae	*Circinella muscae*	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
	Mortierellaceae	*Mortierella* sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Mortierella turficola	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
	Mucoraceae	*Mucor hiemalis*	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Mucor hiemalis	*Astacus astacus*	exoskeleton	Makkonen et al. (2010)
		Mucor plumbeus	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Mucor racemosus	*Astacus astacus*	exoskeleton	Makkonen et al. (2010)
		Mucor sp.	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		Mucor sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Mucor sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		unidentified member of Mucoraceae	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2008)

	Rhizopodaceae	*Rhizopus* sp.	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		Rhizopus stolonifer	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		Rhizopus stolonifer	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
Oomycota (class)	Peronosporaceae	*Phytophthora inundata/humicola*	*Orconectes limosus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
	Pythiaceae	*Phytopythium* sp.	*Astacus astacus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Pythium sp.	*Astacus astacus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
	Saprolegniaceae	*Aphanomyces astaci*	*Austropotamobius pallipes*	exoskeleton	Cammà et al. (2010)
		Aphanomyces astaci strain Pc (genotype D)	*Procambarus virginalis*	exoskeleton	Keller et al. (2014)
		Aphanomyces laevis/repetans	*Orconectes limosus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Aphanomyces repetans	*Austropotamobius pallipes*	exoskeleton	Cammà et al. (2010)
		Achyla sp.	*Cherax quadricarinatus*	gills	Herbert (1987)
		Saprolegnia australis	*Pacifastacus leniusculus*	exoskeleton	Edsman et al. (2015)

		Saprolegnia australis	*Astacus astacus*	exoskeleton	Makkonen et al. (2010)
		Saprolegnia australis	*Astacus astacus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia australis	*Orconectes limosus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia australis	*Austropotamobius pallipes*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia ferax	*Astacus astacus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia ferax	*Orconectes limosus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia ferax	*Austropotamobius pallipes*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia hypogyna	*Astacus astacus*	exoskeleton	Kozubíková-Balcarová et al. (2013)
		Saprolegnia parasitica	*Pacifastacus leniusculus*	exoskeleton	Edsman et al. (2015)
		Saprolegnia parasitica	*Astacus astacus*	exoskeleton	Makkonen et al. (2010)
		Saprolegnia parasitica	*Orconectes limosus*	exoskeleton	Kozubíková-Balcarová et al. (2013)

		Saprolegnia sp.	*Pacifastacus leniusculus*	exoskeleton, internal organs	Geasa (2014)
		Saprolegnia sp.	*Austropotamobius pallipes*	exoskeleton	Quaglio et al. (2006a)
		Saprolegnia sp.	*Pontastacus leptodactylus*	exoskeleton	Fard et al. (2011)
		Saprolegnia sp.	*Procambarus simulans simulans*	exoskeleton, internal organs	Lahser (1975)
		Saprolegnia spp.	*Orconectes limosus*	exoskeleton	Hirsch et al. (2008)
		Scoliolegnia asterophora	*Astacus astacus*	exoskeleton	Makkonen et al. (2010)
unknown	unknown	unidentified yeast	*Procambarus clarkii*	exoskeleton	Dörr et al. (2012)
		unidentified yeasts	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)
		unidentified dematiaceous hyphomycetes	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		unidentified hyaline hyphomycetes	*Procambarus clarkii*	exoskeleton	Quaglio et al. (2006b)
		unidentified mycelia sterilia	*Procambarus clarkii*	gastrointestinal tract	Garzoli et al. (2014)

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RESEARCH ARTICLE

Microbiome of the Successful Freshwater Invader, the Signal Crayfish, and Its Changes along the Invasion Range

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ABSTRACT Increasing evidence denotes the role of the microbiome in biological invasions, since it is known that microbes can affect the fitness of the host. Here, we demonstrate differences in the composition of an invader's microbiome along the invasion range, suggesting that its microbial communities may affect and be affected by range expansion. Using a 16S rRNA gene amplicon sequencing approach, we (i) analyzed the microbiomes of different tissues (exoskeleton, hemolymph, hepatopancreas, and intestine) of a successful freshwater invader, the signal crayfish, (ii) compared them to the surrounding water and sediment, and (iii) explored their changes along the invasion range. Exoskeletal, hepatopancreatic, and intestinal microbiomes varied between invasion core and invasion front populations. This indicates that they may be partly determined by population density, which was higher in the invasion core than in the invasion front. The highly diverse microbiome of exoskeletal biofilm was partly shaped by the environment (due to the similarity with the sediment microbiome) and partly by intrinsic crayfish parameters (due to the high proportion of exoskeleton-unique amplicon sequence variants [ASVs]), including the differences in invasion core and front population structure. Hemolymph had the most distinct microbiome compared to other tissues and differed between upstream (rural) and downstream (urban) river sections, indicating that its microbiome is potentially more driven by the effects of the abiotic environment. Our findings offer an insight into microbiome changes during dispersal of a successful invader and present a baseline for assessment of their contribution to an invader's overall health and its further invasion success.

IMPORTANCE Invasive species are among the major drivers of biodiversity loss and impairment of ecosystem services worldwide, but our understanding of their invasion success and dynamics still has many gaps. For instance, although it is known that host-associated microbial communities may significantly affect an individual's health and fitness, the current studies on invasive species are mainly focused on pathogenic microbes, while the effects of the remaining majority of microbial communities on the invasion process are almost completely unexplored. We have analyzed the microbiome of one of the most successful crayfish invaders in Europe, the signal crayfish, and explored its changes along the signal crayfish invasion range in the Korana River, Croatia. Our study sets the perspective for future research required to assess the contribution of these changes to an individual's overall health status and resilience of dispersing populations and their impact on invasion success.

KEYWORDS invasive species, *Pacifastacus leniusculus*, 16S rRNA gene, microbiome, range expansion

The contribution of the microbiota in maintaining individual health and resilience of animal populations in the wild is being increasingly recognized (1) as well as its role in the context of biological invasions (2–5). Invasive alien species (IAS) are species that

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have been introduced either accidentally or intentionally outside their natural range and whose introduction and spread has negative effects on biodiversity, the economy, or human health in the new environment (6). They are recognized as a major driver of human-induced rapid environmental change (7) because they contribute to biodiversity loss, degradation of ecosystem structure, and impairment of ecosystem services worldwide (8, 9). Recent research demonstrated the effects of IAS on microbial communities in the novel environment. For example, biological invasions affect ecosystem functions, which may consequently drive changes in diversity and shifts in structure of environmental microbial populations (e.g., microbial diversity loss) (1, 5). Also, transmission of novel microbial pathogens is considered one of the main mechanisms through which IAS outcompete their native counterparts and pose a threat to human, animal, and ecosystem health (4, 10).

During the invasion process, successful invaders rapidly disperse within the novel environment (11), and microbes may play an important role in this process since microbial communities present in the novel environment, along with in the host's microbiome, may affect host fitness (12). Although these interactions have been most frequently studied in the case of microbial pathogens, they apply to all microbes, because the effects of microbial community composition on host physiology, immune status, and overall fitness and health have been repeatedly demonstrated (1). For example, several studies suggest that during dispersal into the novel environment, an individual can lose its natural enemies (micropathogens), which may lead to lower prevalence of certain (i.e., pathogenic) microbial taxa in translocated populations of an invader or improve the condition of individuals at invasion fronts (a type of "enemy release") (13–17). Furthermore, dispersing individuals may host microbes that are absent in the novel environment, which may lead to their establishment and spillover to the resident (native) species, giving the dispersing individuals a selective advantage in competition (spillover or novel weapon hypothesis) (18). Dispersing individuals can also acquire local microbes and serve as their reservoir, multiplying their (negative) impact on resident native species (spillback hypothesis) (19) but also with potential negative effects for the dispersing invader itself. Finally, microbial communities of the dispersing invader can contribute to the protection of their host by interfering with the entry of micropathogens into the host's body and by preventing their establishment, growth, and spread (20). Therefore, both dispersal process and the characteristics of the novel environment may affect the structure and composition of a microbiome of a dispersing invader, which may indirectly and directly affect their health and their invasion success.

In this study, we analyzed the microbiome of a signal crayfish *Pacifastacus leniusculus* (Dana, 1852), the most successful crayfish invader in Europe, collected from a recently invaded Korana River in Croatia. We determined the differences between microbiomes of different tissues and examined changes in the microbiome along the signal crayfish invasion range. Invasive crayfish are one of the major threats to freshwater ecosystems (21) because they are among the most frequently translocated aquatic invertebrates that can dramatically modify freshwater communities and ecosystem functioning through combined impacts of consumption, competition, disease transmission, bioturbation, and mechanical destruction (21–24). Their introduction has been followed by rapid range expansion and a high number of documented negative impacts globally (25, 26). The North American signal crayfish is currently the most widespread crayfish invader in Europe, with records from 29 European countries (27) and is listed as a species of EU Concern according to the EU Regulation No. 1143/2014 on invasive alien species (6).

Signal crayfish were first recorded in the lower section of the Korana River in 2011 (28) and have been successfully spreading both upstream and downstream since (29, 30). Previous studies have recorded differences in signal crayfish population characteristics along its invasion range in the Korana River, with invasion fronts being male dominated and containing less aggressive individuals in better body and physiological condition (31, 32), which may be the result of nonrandom dispersal (33) and density-dependent effects. Given

the observed differences between signal crayfish individuals from invasion core and invasion front, in this study, we explored whether such differences occur in its microbiome composition. We hypothesize that the composition of microbial communities of the signal crayfish is also affected by the range expansion and differs significantly along the invasion range. We aim to discern whether microbiomes are affected only by habitat characteristics when signal crayfish spread both upstream and downstream through the river or by a combination of nonrandom dispersal and density-dependent effects in an establishing population at the expanding edges.

To answer these questions, we have analyzed the microbiome of exoskeletal biofilm and multiple tissues (hemolymph, hepatopancreas, and intestine) of *P. leniusculus* using an amplicon sequencing approach based on the gene coding for 16S rRNA. We analyzed the above-mentioned microbiomes collected from four different locations along the signal crayfish invasion range in the Korana River and compared them to microbiomes present in the environment (water and sediment). Results of these analyses may help to understand the differences in microbiome composition of different tissues in crayfish and their changes during species dispersal through the novel environment.

RESULTS

A total of 4,881,556 raw reads were obtained from the samples included in the study. After the DADA2 process (34) and filtering of the resulting feature table, 2,520,310 merged reads from 191 samples were obtained, and a total of 7,041 amplicon sequence variants (ASVs) were identified.

Microbial diversity of crayfish-associated sample groups and the environment. **(i) Alpha and beta diversity.** Overall, taxonomic richness (observed ASVs) and evenness (Pielou's evenness index) differed significantly (Kruskal-Wallis test: $P = 8.76E{-}24$ and $P = 1.03E{-}23$, respectively) between six examined sample groups. Exoskeletal biofilm and sediment samples had the highest number of observed ASVs (Kruskal-Wallis test: $P \ll 0.01$) compared to other sample groups, but no significant difference was recorded between the two (Fig. 1A). Similarly, these sample groups had a significantly higher evenness within a microbiome than other sample types, with significant differences between the sediment and the exoskeletal biofilm ($P = 0.003$) (Fig. 1B). Water samples differed significantly from all other 5 sample groups in the number of observed ASVs ($P \ll 0.01$ in all cases) (Fig. 1A); however, water sample evenness was similar to that of the hepatopancreas and hemolymph (Fig. 1B). Three internal tissues (hemolymph, hepatopancreas, and intestine) exhibited no significant differences in richness or evenness except in the case of hemolymph, which had higher evenness than other internal tissues (Fig. 1B).

Both unweighted and weighted UniFrac showed an overall significant difference (permutational multivariate analysis of variance [PERMANOVA]: $P = 0.001$, pseudo-F = 26.3 and $P = 0.001$, pseudo-F = 50.5, respectively) between microbiomes of all six sample groups. Additionally, beta diversity pairwise tests showed a significant difference between all pairs of sample groups ($P = 0.001$). Intestine and hepatopancreas samples were grouped closely together in the unweighted UniFrac principal coordinates analysis (PCoA) (Fig. 2A) but not in the weighted UniFrac PCoA (Fig. 2B), while the opposite pattern was visible in the case of sediment and exoskeletal biofilm. In both analyses, hemolymph samples were positioned the farthest from other samples (Fig. 2A and B).

Additionally, comparisons of shared and unique ASVs between environmental samples (sediment, water) and each of the crayfish samples showed that the exoskeleton shared the highest number of ASVs with both water and sediment samples followed by the intestine (Fig. S1 in the supplemental material). Hepatopancreas and hemolymph shared the least ASVs with any of the environmental samples and were the sample groups with the highest percentage (70.5% hepatopancreas, 68.4% hemolymph) of unique ASVs compared to environmental samples. Compared to other crayfish samples, hepatopancreas had the lowest percentage of unique ASVs (Fig. S1E).

(ii) Microbial composition. At examined taxonomic levels, 49 phyla and 430 families were detected, with sediment and exoskeleton exhibiting similar composition and

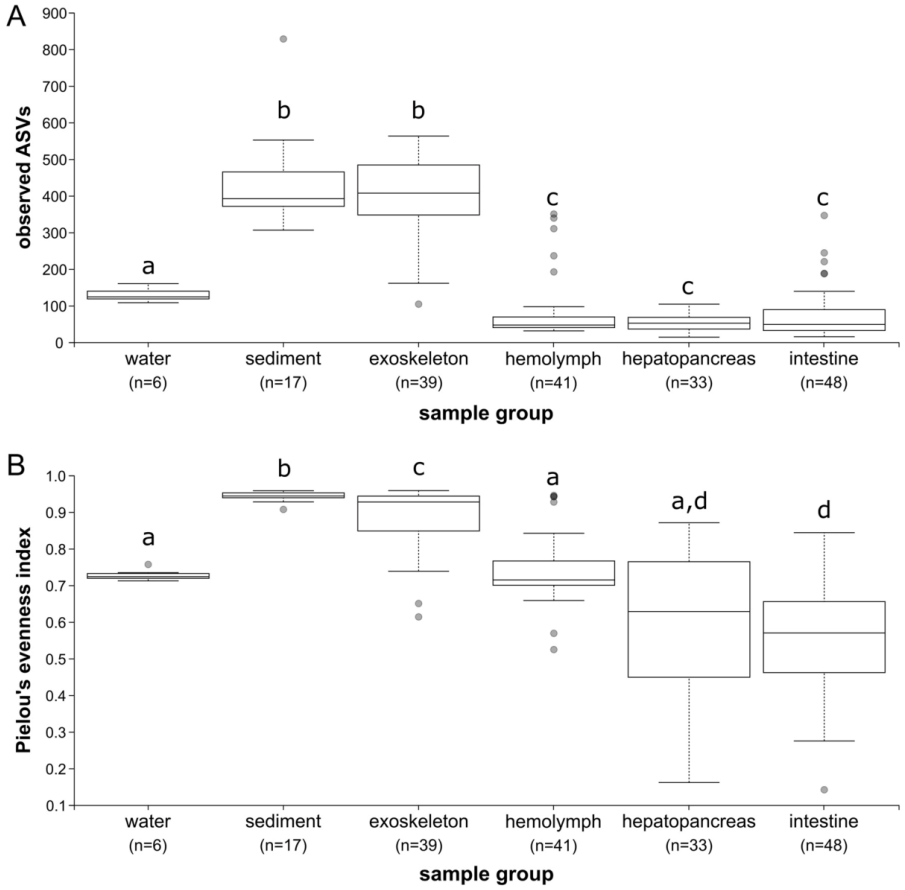

FIG 1 Alpha diversity analyses of microbiomes in different sample groups. (A) Observed ASVs. (B) Pielou's evenness index. Significant differences are marked with different letters.

diversity patterns (Fig. 3 and 4). In addition to *Proteobacteria* (comprising 32.9% of sediment and 33.2% of exoskeletal microbiome), members of the phylum *Planctomycetes* represented 21.7% and 24.7% of the total community abundance in these two groups, with *Pirellulaceae* as the dominant family (Fig. 4). Unlike other sample groups, both sediment and exoskeleton showed notable abundances of the phylum *Cyanobacteria* (13.4% sediment and 11.2%, exoskeleton). Furthermore, sediment samples had the highest number of low-abundant families (54.7%) of all other sample groups (i.e., category 'other', families with abundance less than 3) (Fig. 4).

Intestine and hepatopancreas samples were dominated by the phyla *Tenericutes* (76.7% intestine and 14.9% hepatopancreas) and *Proteobacteria* (16.1% intestine and 62.8% hepatopancreas). At the family level, the intestine was dominated by an unknown family of *Mollicutes* class (68.7%) and the hepatopancreas by *Enterobacteriaceae* (32.8%), *Mycobacteriaceae* (13.6%), and an undetermined family of *Rickettsiales* order (10.6%) (Fig. 3 and 4). The hemolymph microbiome was dominated by members of the phylum *Proteobacteria* (82.9%), with *Sphingomonadaceae* (28.6%) as the dominant family followed by *Pseudomonadaceae* (10.8%). The water microbiome was dominated by *Proteobacteria*

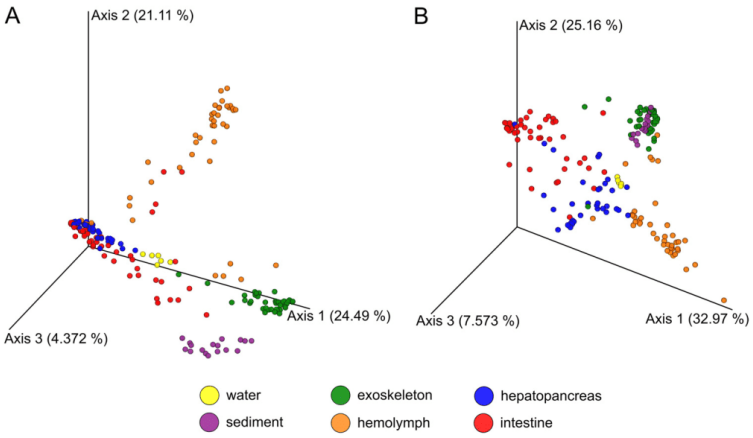

FIG 2 Beta diversity analyses of microbiomes between different sample groups. (A) Unweighted UniFrac. (B) Weighted UniFrac.

(41.3%, with dominant family *Comamonadaceae*) and *Actinobacteria* (38.1%, with dominant unnamed family ACK-M1), both of which were also ubiquitous in all sample groups. Additionally, water samples showed a relatively high abundance of bacteria belonging to the phylum *Bacteroidetes* (12.3%) in comparison to other sample groups, where it comprised 3.1% or less of the microbiome.

Finally, analysis of core features at the ASV level in all sample groups (sediment, exoskeleton, hemolymph, hepatopancreas, and intestine) at 90% sample inclusion identified the phylum *Proteobacteria* as the core feature in all of the sample groups, along with *Planctomycetes* (exoskeleton and sediment), *Verrucomicrobia* (sediment), and *Tenericutes* (intestine) (Table S1). Water samples had the most shared ASVs (47 core taxa at 100%; data

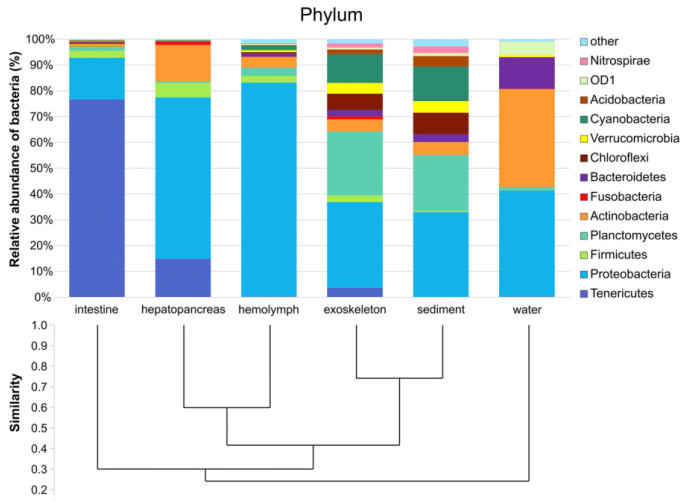

FIG 3 Relative abundance (%) of the overall most prevalent phyla and Bray-Curtis similarity index-based cluster analysis for all six sample groups. Bacterial phyla with an overall abundance of >1% are shown, while the remaining phyla were pooled and marked as "other."

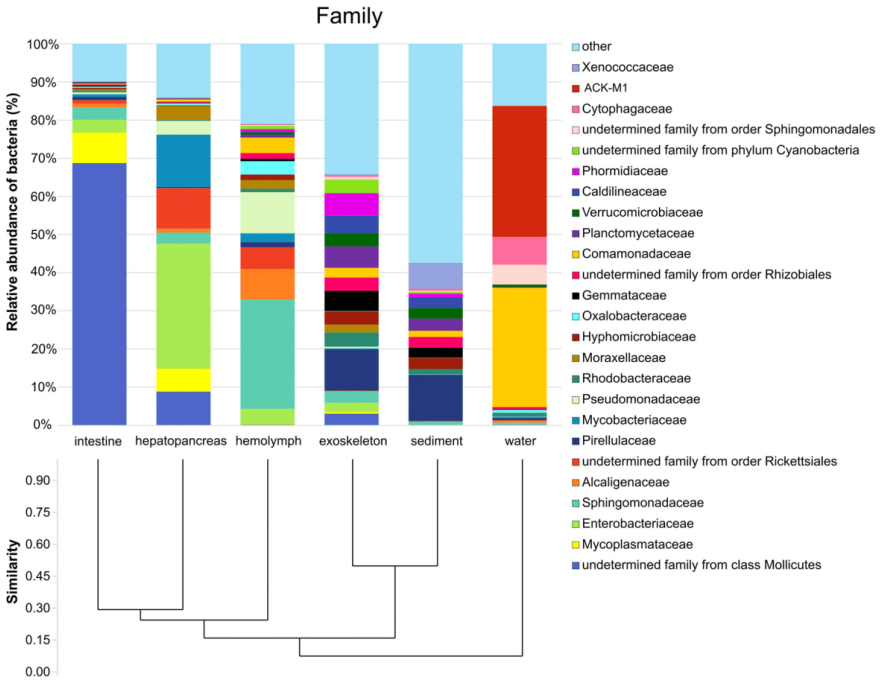

FIG 4 Relative abundance (%) of the overall most prevalent families and Bray-Curtis similarity index-based cluster analysis for all six sample groups. Bacterial families with an overall abundance of >3% are shown, while the remaining families were pooled and marked as "other."

not shown), while hepatopancreas had the smallest (0 core taxa at 100% and 1 core taxa only at 90%).

Variation of the signal crayfish microbiome along its invasion range. Alpha diversity differed significantly only for hemolymph, with significant differences between downstream core and upstream sites (core and front) (Table S2 and Fig. S2). In beta diversity analyses, significant differences were observed for both unweighted (P = 0.001, pseudo-F = 4.072) and weighted UniFrac metrics (P = 0.001, pseudo-F = 9.076) for exoskeletal biofilm samples between all examined locations within the invasion range (upstream and downstream invasion fronts and upstream and downstream invasion cores) (Fig. 5A and B; Table S3). Sediment, intestine, and hemolymph samples did not exhibit any significant differences between examined locations (upstream invasion front [UF], upstream invasion core [UC], downstream invasion core [DC], and downstream invasion front [DF]) for unweighted and weighted UniFrac metrics; thus, they were pooled according to the invasion range (core versus front) and position in the river (upstream versus downstream part of the river). Sediment samples significantly differed between both the invasion range (core versus front) and position within the river (upstream or downstream part of the river) in both unweighted and weighted UniFrac analyses (Table S3).

The hemolymph microbiome differed significantly between upstream and downstream river sections for both unweighted and weighted UniFrac (P = 0.038, pseudo-F = 1.98 and P = 0.003, pseudo-F = 4.43, respectively) (Table S3) but not between invasion core and front. On the contrary, in intestine samples, a significant difference between the microbiomes of core and front populations was observed but only for unweighted UniFrac (P = 0.017, pseudo-F = 2.18) (Table S3), while no significant differences were observed between upstream and downstream segments of the river. A similar pattern was

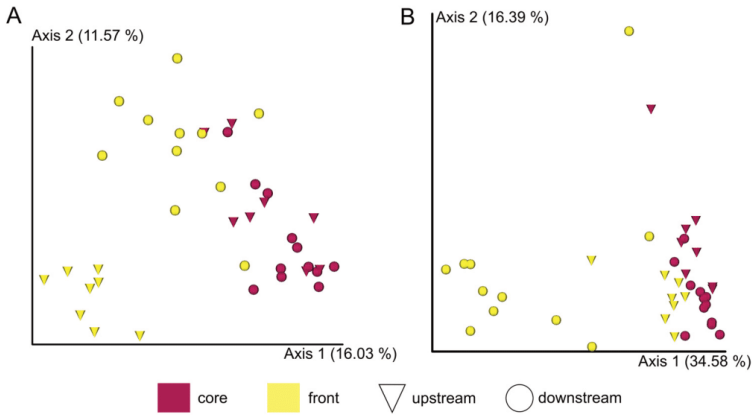

FIG 5 Beta-diversity metrics for the exoskeletal microbiome at all examined locations within the invasion range. Principal coordinate analysis (PCoA) is presented for unweighted (A) and weighted (B) UniFrac distances of individual crayfish.

observed for hepatopancreas, where a significant difference was observed between the upstream invasion front and upstream core, but only in weighted UniFrac ($P = 0.012$, pseudo-F = 4.024), with no significant differences between upstream and downstream parts of the river.

Finally, significant differences in differential abundance of features were recorded in exoskeleton, hemolymph, and hepatopancreas but not in the intestine (Table S4). In the exoskeleton, *Cyanobacteria* and *Firmicutes* exhibited significant differences between examined locations, with the highest abundance of their genera *Acinetobacter*, *Macrococcus*, *Phormidium*, and *Aerococcus* at downstream invasion front (Table S4). In the case of hemolymph and hepatopancreas, genera *Caulobacter*, *Psychrobacter*, and *Salmonella* exhibited significant differences in abundance between examined locations (Table S4).

DISCUSSION

In addition to negative effects on biodiversity, the economy, and human health, biological invasions may drive emergence of (new) diseases and changes in diversity and structure of microbial populations in the novel environment and may also affect population dynamics of invasive species (5, 35). Here, we analyzed differences in the microbiomes of different tissues of the successful freshwater invader the signal crayfish. Also, we examined whether differences in the microbiome occur during the invasion process and whether they are more pronounced along different river sections (downstream versus upstream; a proxy for the effect of microhabitat characteristics) or between crayfish populations of different density and species composition (core versus front; a proxy for the effect of changing population characteristics along the invasion range, that is, nonrandom dispersal and density-dependent effects). As effects of both resident and invader microbiota are increasingly recognized among the drivers of invasion success (35, 36), our results offer a baseline for better understanding their role and dynamics during range expansion.

Composition and diversity of the bacterial communities associated with the signal crayfish. Because there is little comparative information on the crayfish microbiome, except for several studies of single tissues (37–39), we analyzed microbial composition and diversity of different internal organs and tissues (hemolymph, hepatopancreas, and intestine) and exoskeleton and compared them to environmental samples (water and sediment). In all types of crayfish samples, *Proteobacteria* were the dominant phylum, which is consistent with previous research on other crustaceans (40–43) and other aquatic invertebrates (44). This indicates that this phylum is important for the host and ubiquitous in the environment. Out of all analyzed samples, sediment and exoskeletal microbiomes

were the most taxonomically rich and uniform. The identified dominant family *Pirellulaceae* in the exoskeleton and sediment in our study was also among the most represented in other crayfish species (i.e., *Cambarus sciotensis*) (37). Also, exoskeleton samples shared the highest percentage of ASVs with both the sediment and water samples. This is not surprising since the exoskeleton is simultaneously a barrier and a link between crayfish and the environment, and crayfish are in continuous contact with the sediment (bioturbators) (45, 46) during their life span. However, beta diversity analyses showed significant differences between sediment and the exoskeleton, despite their close grouping in weighted UniFrac. Thus, in addition to the characteristics of rich and diverse bacterial communities in the sediment, which were shown to shape the crayfish exoskeletal microbiome (37) and skin microbiome of other aquatic species (i.e., fish [47–50]), other factors such as exoskeleton characteristics (i.e., cuticle morphology and structure, presence of microinjuries, or time since last molt) and population characteristics (i.e., density, structure, number of species present, and their physiological status, which is discussed later in the text) may also influence the microbiome composition.

Internal tissues (hemolymph, hepatopancreas, and intestine) were significantly less rich in ASVs and exhibited lower evenness. Hemolymph had the lowest (albeit not significant) richness out of all internal tissues. While microbial communities in hemolymph are generally considered less rich and abundant than other organs due to its regulation by the host immune response (51, 52), this study, along with some other studies (52), demonstrated similarity in microbial community richness of hemolymph and the hepatopancreas. In addition to harboring potentially pathogenic microbes or opportunistic micropathogens, hemolymph may also contain symbiotic microbes that may help boost the host's immune response or even inhibit pathogen proliferation (52). Beta diversity analyses showed that hemolymph had the most distinct microbiome composition and feature abundance compared to other analyzed crayfish or environmental samples. This could be explained by its specificity compared to other crayfish sample groups (circulating liquid tissue with many antimicrobial components tightly controlled by the host's immune system [51–53]). However, the fact that hemolymph is in direct contact with all internal organs (54) explains the observed significant portion (>80%) of shared ASVs with other crayfish tissues. Additionally, some studies (52, 55) support the hypothesis that microbes may be translocated from the digestive tract (hepatopancreas and intestine) to hemolymph in invertebrates with open circulatory systems.

Intestine samples had the lowest evenness of all samples, since certain ASVs (i.e., members of the class *Mollicutes*) dominated the intestinal microflora. Also, intestine and hepatopancreas samples, albeit being significantly different, shared similar ASVs (unweighted UniFrac analyses) but in different abundances (weighted UniFrac analyses). Intestinal and hepatopancreatic communities are probably partly determined by the type of food since they are both parts of the digestive system, and multiple studies highlight diet as one of the main drivers in shaping the host's intestinal microbiome (38, 56–58). However, the functions of these organs differ significantly, which may explain differences in abundance of shared features, as hepatopancreas is a digestive gland involved in metabolism and absorption of low-molecular-weight nutrients, while the intestine plays a role in digestion, ion osmoregulation, and water uptake (54). The latter is visible from the high number of shared ASVs between the intestine and sediment and water samples observed in this study.

Variation of the signal crayfish microbiome along its invasion range. In concordance with our hypothesis, the crayfish microbiome demonstrated differences along the invasion range; the analyzed signal crayfish samples exhibited both variation in respect to their position along the invasion range (invasion core versus invasion front: exoskeleton, hepatopancreas, and intestine) as well as in respect to their position along the river (upstream or downstream section of the river: exoskeleton and hemolymph). The exoskeletal microbiome varied significantly between all examined locations, while sediment samples exhibited a similar pattern of variation in beta diversity between the core and front as well as upstream and downstream parts of the river. This, in addition to the comparative analyses of alpha diversity

of sediment and exoskeletal samples, corroborates that the exoskeletal microbiome is shaped to a high extent by local environmental characteristics, as recorded in previous studies (37). However, the significant difference in diversity and abundance of sediment and exoskeleton samples (discussed in chapter above) indicates that other factors besides the characteristics of the local environment affect the exoskeletal microbiome composition. As the exoskeletal microbiome is determined by the available regional microbial species pool, which also includes microbes of all host individuals in a given environment (59), we suggest that crayfish density and population structure may significantly affect its composition and lead to the observed high variation in diversity and abundance among all locations and contribute to the observed significant differences from the environmental (sediment) microbiome. In the invasion core populations, only signal crayfish are present in high abundance, while invasion fronts have 7 to 8 times lower signal crayfish abundance than invasion cores and also cooccur with the native congener *Pontastacus leptodactylus* (Eschscholtz, 1823) (30). Aggression plays an important role in the dynamics of crayfish populations (60), and high crayfish abundance has been shown to increase the competition for limited resources and the rate of interaction between individuals (61). Thus, as contact rates between individuals increase with increasing density, this may also elevate the transmission of microbiota between individuals, as established in the case of pathogens (62, 63).

Hemolymph exhibited significant differences in the microbiome composition and feature abundance between upstream and downstream river sections, but not in respect to the position along the invasion range (invasion core versus front). Under favorable conditions, homeostasis exists between the microbial communities of the hemolymph and the host (51), with the composition and abundance of bacterial communities remaining relatively stable (64). However, under stress, significant community changes may occur since many hemolymph bacteria are opportunistic pathogens that may proliferate under stressful conditions, induce bacterial septicemia, and adversely affect crayfish health (42, 65). Because multiple factors (i.e., changes in the environment, host physiology status, microbe-microbe interactions in a tissue, etc.) (11, 59, 66, 67) can lead to changes in the microbiome, the observed differences in the feature abundance and composition of the hemolymph microbiome at the upstream and downstream locations may be driven by differences in characteristics of these two environments, as the upstream section of the studied area of the Korana River flows through the sparsely populated rural region, while the downstream section of the river passes through the industrial zone of the Karlovac city. Future studies should address the observed changes in the hemolymph microbiome along with detailed analyses of the water quality parameters and crayfish immune response at each site to address this question.

The analyses of intestinal and hepatopancreatic microbiomes (unweighted and weighted UniFrac tests) showed significant differences between core and front populations. Hepatopancreatic microbiomes were composed mostly of the same taxa, but with different abundances at (upstream) the invasion core and front. On the contrary, intestinal microbiomes exhibited significant differences in taxonomic composition between invasion core and front populations but had similar abundances. We hypothesize that observed differences in both abundance in the hepatopancreas and diversity of taxa in the intestine are dependent on the crayfish feeding regimen and crayfish condition and driven by both environmental conditions (i.e., type of available food) and density-dependent effects. Crayfish are omnivorous and ingest large amounts of detrital materials during feeding (68), which is also visible from the high number of shared ASVs between sediment and the intestine. In this study, we identified differences in sediment microbiome composition and abundance along the invasion range and river sections, which indicates the potentially different composition of detrital materials ingested between the sites. Also, as already discussed, examined populations at invasion cores and fronts differed significantly in crayfish density. In the latter case, the higher intensity of resource competition in highly abundant populations at invasion cores may affect crayfish diet (i.e., feeding rates, patterns, and preferred food availability) (61). The latter may have a more pronounced impact on the observed differences in abundance of particular groups of intestinal microbes between invasion core and front

populations than the composition of detrital material ingested. Additionally, previous studies have demonstrated the link between individual fitness and the composition of the intestinal microbiota (reviewed in reference 1), while studies on the signal crayfish in the Korana River demonstrated that its condition (measured using hepatosomatic and body condition indices) was lower in the core than in the invasion fronts (32). Inferior crayfish condition along with the potentially limited variety and abundance of food sources (69, 70) at invasion cores may thus affect the observed differences in the intestinal microbiome composition between invasion core and invasion front populations (higher observed diversity at invasion fronts). The impact of an animal's condition on the composition and diversity of the gut microbiota has been corroborated in crayfish (71, 72) as well as other aquatic animals (i.e., shrimps [73–75] and fish [76, 77]). Crayfish condition (i.e., its density dependence) may also be driving the observed changes in hepatopancreas community composition along the invasion range. In crustaceans, the hepatopancreas plays an important role in lipid metabolism as a main energy storage organ that supports key physiological functions, such as reproduction, movement, and growth (54, 78, 79). Due to its role in food degradation and due to its specific organ environment (i.e., low pH, presence of digestive enzymes) (80), it may filter which bacteria will successfully colonize it. This may explain the similarity in the composition of the microbiomes of both core and front populations. Also, in other decapods, the hepatopancreas has been shown to have a more conserved and distinct microbiome than the intestine (41, 74), which could explain that differences were observed in the feature abundance, but not in the microbiome composition, along the invasion range. Further studies involving *in situ* research, behavioral studies, analyses of crayfish condition, diet (stable isotope analyses), and the subsequent analyses of the hepatopancreas and intestinal microbiota are required to link more precisely crayfish diet and density to the individual's relative condition, nutrient assimilation patterns, and gastrointestinal and hepatopancreatic microbiome changes, similar to the studies performed in fish (81).

Finally, we expected to observe differences in abundance of some genera, which include crayfish micropathogenic taxa (82), based on suggested hypotheses of their effect during invader's dispersal (i.e., enemy release, spillover effect, spill back effect) (14, 18, 19). However, genera for which the differences were observed along the invasion range were not among the genera with crayfish micropathogens for the specific tissues known from the literature (82) and were not considered pathogenic or classified as potential micropathogens in other aquatic species (i.e., *Psychrobacter*) (83–86). To further investigate the effect of micropathogens on invasion dynamics of the signal crayfish in the Korana River, targeted monitoring of well-established crayfish micropathogens for which the detection assays are available (i.e., *Aphanomyces astaci*, infectious hematopoietic necrosis virus, *Macrobrachium rosenbergii* nodavirus (MrNV), and white spot syndrome virus [82, 87]) is required. Also, since crayfish diseases are still largely under researched (82), further studies into the potentially pathogenic microbial species in crayfish from the genera identified within this study are needed. This is especially relevant in the case of the hepatopancreas, in which acute idiopathic necrotic hepatopancreatitis has been observed extensively in the signal crayfish from the Korana River and which exhibits significant differences in its incidence and severity along the invasion range (88).

In conclusion, we demonstrated differences in the composition of the signal crayfish microbiome along its invasion range, suggesting that microbial communities may affect and be affected by range expansion. Thus, our study sets the perspective for future research required to assess the contribution of the changes in the microbiome to an individual's overall health status, resilience of dispersing populations, and their invasion success. However, microbiomes associated with different crayfish species and organs are currently largely unknown, and detailed studies are needed to describe the microbiome of healthy animals, which could then be used to detect deviations that could be linked with biotic and abiotic environmental stressors often at play during biological invasions.

MATERIALS AND METHODS

Study area. We sampled the lower reaches of the Korana River, a 134-km-long karstic river located in continental Croatia that belongs to the Sava River basin, where signal crayfish are spreading both upstream and downstream (30). The upstream section of the studied area flows through the sparsely populated rural region, while in the downstream section, the river passes through the industrial zone and flows into the Kupa River in the city of Karlovac. Along the whole length of its course, multiple natural and human-made cascades are present (29). The study area includes sites differing in crayfish community composition (i.e., dense intraspecific populations of *P. leniusculus* and less abundant heterospecific populations of *P. leniusculus* and *P. leptodactylus*) (Fig. 6).

Sampling procedure. Fieldwork was conducted during the period of increased crayfish activity of both sexes (i.e., before the mating period [89]) in the early autumn of 2018. The ongoing context of range expansion allowed us to sample signal crayfish individuals from two distributional ends: (i) invasion core (longer established population with higher crayfish abundance) and (ii) invasion front (recently established population at the edge of the range with lower crayfish abundance). The crayfish were captured at four sites along the 33 km of the Korana lower watercourse, which were previously (30) identified as upstream invasion front (UF), upstream invasion core (UC), downstream invasion core (DC), and downstream invasion front (DF) (Fig. 6). Upstream and downstream invasion cores have 7 to 8 times higher relative *P. leniusculus* abundance than invasion fronts and contain no native crayfish since they were outcompeted from these sites, while at invasion fronts, *P. leniusculus* cooccurs in interspecific populations with the native *P. leptodactylus* (29, 30).

Crayfish were captured using baited LiNi traps (90), which were left in the water overnight. Following capture, individuals were identified to species level by visual inspection. Captured native *P. leptodactylus* were returned to the river, while a total of 110 *P. leniusculus* individuals of both sexes (27 from UF, 23 from DF, 30 from UC, and 30 from DC) were placed in individual containers on ice and taken to the laboratory for tissue sample collection. Additionally, environmental samples were collected at all sites; water was sampled using 1,000-ml sterile bottles (one bottle at each invasion core, two bottles at invasion fronts), and sediment was taken as composite samples (4 to 5 samples at each site, which were collected approximately 1 to 2 m apart, from the surface of the sediment [0 to 5 cm]) using a sterile sampling spoon and immediately transported to the laboratory on ice.

In the laboratory, collected water samples were vacuum filtered through 0.22-μm pore-size membrane (MCE) filters, which were stored at −20°C until DNA extraction. Four types of crayfish samples were taken for each individual crayfish: exoskeletal biofilm, hemolymph, hepatopancreas, and intestine (i.e., midgut and hindgut). Exoskeletal biofilm was sampled by taking cuticle swabs as previously described (91). Briefly, any loosely adhered debris (such as vegetation, mud, or sediment) was manually removed from the crayfish, which were then thoroughly scrubbed with a sterile brush wetted with a 0.1% NaCl and 0.15 M Tween 20 solution. After centrifugation of the suspension at 10,000 $\times$ *g* for 15 min at 4°C, the supernatant was discarded, and the pellet of epibiotic cells was frozen at −20°C. Next, we collected 400 μl of hemolymph in 200 μl of anticoagulant solution (0.49 M NaCl, 30 mM trisodium citrate, 10 mM EDTA) from the base of the individual's walking leg (previously rinsed by 70% ethanol) by using a sterile needle as previously described (42). The collected hemolymph was centrifuged at 10,000 $\times$ *g* for 10 min at 4°C, and the pellet was frozen at −20°C until DNA extraction. For dissected organs (hepatopancreas and intestine), the sampling procedure was the same; the complete organ was removed from the body, placed in a sterile petri dish, and carefully chopped into small pieces using a sterile scalpel and frozen at −20°C. Nondisposable dissecting scissors were alcohol flame sterilized between each individual sample.

DNA extraction. Genomic DNA was extracted from exoskeletal biofilm, hemolymph, hepatopancreas, and intestine using the NucleoSpin microbial DNA kit (Macherey-Nagel, Germany) as per the manufacturer's protocol for Gram-positive and Gram-negative bacteria and with modifications regarding sample lysis by agitation as previously described (91). Genomic DNA from sediment and water samples was extracted using a DNeasy PowerSoil Pro kit (Qiagen, Germany). Three replicates of each composite sediment sample were isolated from invasion cores and six from invasion fronts. To select the samples of highest quality for subsequent Illumina sequencing, we have analyzed the yield of metagenomic DNA samples and also tested the samples for the presence of bacterial DNA. DNA quantity was analyzed in all samples using the QuantiFluor ONE double-stranded DNA (dsDNA) system and the Quantus Fluorometer (Promega, USA). Further, the presence of bacterial DNA in the samples was confirmed by PCR; that is, almost full-length 16S rRNA gene amplification (using primers 27F and 1492R as previously described [92]) was conducted on all samples. Finally, we chose 192 samples from all six sample groups for amplicon sequencing of variable regions 3 and 4 of the 16S rRNA (Table S5 in the supplemental material) based on the following criteria: (i) satisfactory DNA concentration, (ii) successful 27F/1492R PCR amplification of the 16S rRNA gene, and (iii) relatively uniform coverage of different sampling locations.

Library preparation, sequencing, and bioinformatics analysis. Amplification and sequencing of the variable V3-V4 region of the 16S rRNA gene was performed by Microsynth, Switzerland. An Illumina library was prepared using 16S Nextera two-step PCR using forward 341F (5′-CCTACGGGNGGCWGCAG-3′) and reverse 802R (5′-GACTACHVGGGTATCTAATCC-3′) primers and sequenced on an Illumina MiSeq using the MiSeq reagent kit v2 (2 $\times$ 250 bp paired-end). Illumina raw paired-end sequences were analyzed in 'Quantitative Insights Into Microbial Ecology 2' (QIIME2) software (93), release 2019.10. Raw demultiplexed paired-end fastq files were imported into QIIME2 using a manifest file and were then quality filtered, trimmed, dereplicated, denoised, merged, and assessed for chimaeras to produce ASVs using the DADA2 plugin (34). The DADA2-generated feature table was filtered to remove ASVs at a frequency of less than 10 per sample and appearing in less than two samples. Taxonomy was assigned to

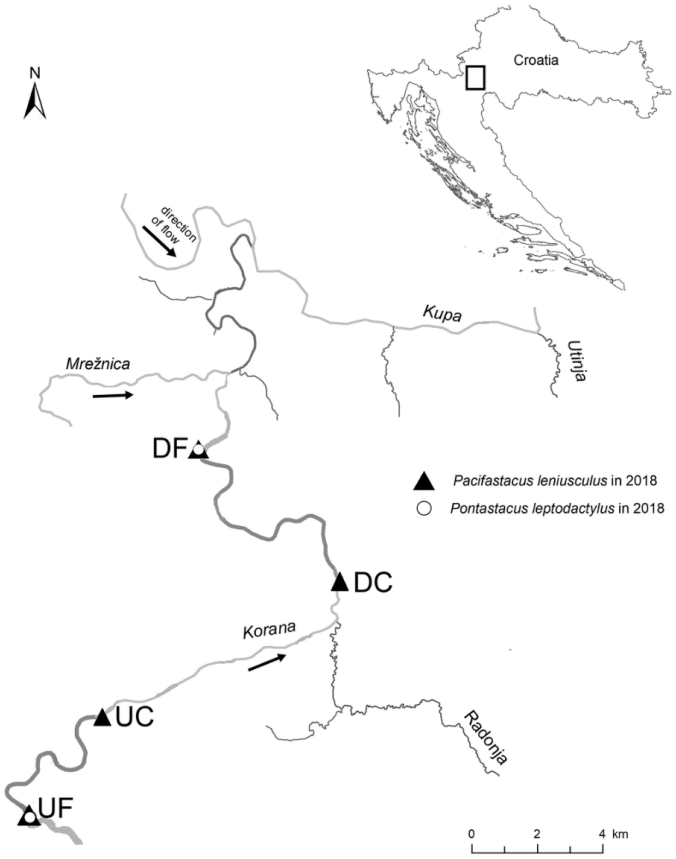

FIG 6 Position of sampling sites along the invasion range of the signal crayfish in the Korana River in 2018. Sampling was performed at both upstream (UF) and downstream (DF) invasion fronts and upstream (UC) and downstream (DC) invasion cores.

ASVs using a pretrained naive Bayes classifier. The classifier was trained on the Greengenes 13_8 99% operational taxonomic unit (OTU) data set, targeting the V3 and V4 region of the 16S rRNA gene using the QIIME2 feature classifier plugin (94). Based on the generated taxonomy, the feature table was filtered to exclude ASVs assigned to the class *Chloroplast*. A phylogenetic tree was generated using fasttree2 based on mafft alignment of ASVs as implemented in the q2-phylogeny plugin. The microbial diversity and richness of all samples were estimated using alpha (Pielou's evenness index and observed ASVs) and beta (unweighted and weighted UniFrac (95) diversity metrics using the diversity plugin within QIIME2. Alpha and beta diversity metrics were calculated for (i) all six groups of samples with the samples of the same type pooled across sites (to analyze the microbial diversity of crayfish tissues and environment; subsampled to 1,402 reads per sample; 184 samples in total) (Table S5A) and (ii) each group of tissue samples separately between four sites (to analyze differences in composition of the microbiome along the invasion range; subsampled to 2,464 reads per sample for exoskeletal biofilm, 2,285 for hemolymph, 1,883 for hepatopancreas, 6,603 for intestine, and 4,909 for sediment; 173 samples in total) (Table S5B). UniFrac diversity metrics were visualized by generating a principal coordinates analysis (PCoA) plot using Emperor (96). Differences along the invasion range and between different river segments were tested with Benjamini-Hochberg corrected Kruskal-Wallis and PERMANOVA tests (97) for alpha and beta diversity, respectively. Since no significant differences between sexes were established for any of the crayfish sample groups (exoskeletal biofilm, hemolymph, hepatopancreas, and intestine) in both alpha and beta diversity analyses, both sexes were pooled. Furthermore, if no significant differences were observed between upstream and downstream invasion fronts or upstream and downstream invasion cores, they were pooled into either the front or core group (differences along the invasion

range) and upstream or downstream group (differences between river segments). Additionally, we determined shared and unique ASVs between environmental samples and each analyzed crayfish sample groups as well as between all crayfish samples by using Venn diagrams, which were visualized using an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Analysis of compositions of microbiomes (ANCOM) tests (98) were used to identify ASVs that are differentially abundant between locations using the composition plugin within QIIME2. Further analysis was performed to establish core features present in high numbers of samples using the feature-table QIIME2 plugin (99). Finally, to reveal the similarities in microbial composition on phylum and family levels, the Bray-Curtis similarity index-based cluster analysis was performed using PAST software (100).

Data availability. The next-generation sequencing data that support the findings of this study are openly available in the EMBL Nucleotide Sequence Data Base (ENA) at https://www.ebi.ac.uk/ena/browser/home, reference number PRJEB43749.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, DOCX file, 0.3 MB.

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Supplemental material

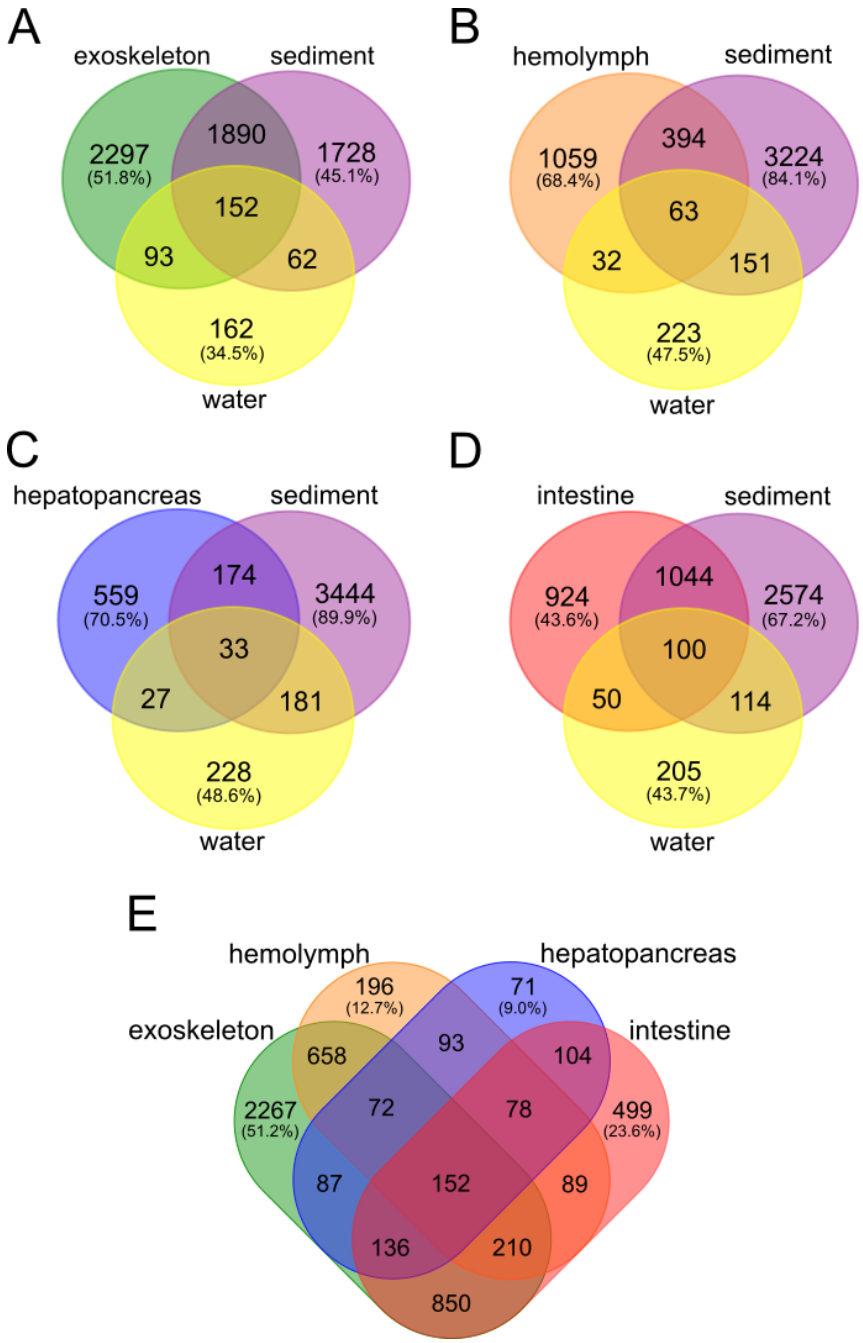

FIG. S1. Venn diagrams showing the numbers of shared and unique ASVs between sediment and water samples, and exoskeletal biofilm (A), hemolymph (B), hepatopancreatic (C) and intestinal (D) samples. The numbers of shared and unique ASVs between four groups of crayfish samples (exoskeletal biofilm, hemolymph, hepatopancreatic, intestinal) are visualized in E.

TABLE S1. Core features at ASV level in sediment, exoskeleton, hemolymph, hepatopancreas and intestine at 90% sample inclusion.

SAMPLE GROUP	CORE FEATURES (at 90% sample inclusion)
exoskeleton	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Rhodobacter; s__
	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__; g__; s__
	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Rhodobacter; s__
	k__Bacteria; p__Planctomycetes; c__Phycisphaerae; o__WD2101; f__; g__; s__
	k__Bacteria; p__Planctomycetes; c__Planctomycetia; o__Pirellulales; f__Pirellulaceae; g__; s__
	k__Bacteria; p__Planctomycetes; c__Planctomycetia; o__Pirellulales; f__Pirellulaceae; g__; s__
	k__Bacteria; p__Planctomycetes; c__Planctomycetia; o__Pirellulales; f__Pirellulaceae; g__; s__
hemolymph	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas; s__
	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas; s__
	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Alcaligenaceae; g__Achromobacter; s__
	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__
	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Cupriavidus; s__
	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__Burkholderia; s__
	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__
	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Gluconacetobacter; s__
hepatopancreas	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas; s__
intestine	k__Bacteria; p__Tenericutes; c__Mollicutes; o__; f__; g__; s__
	k__Bacteria; p__Tenericutes; c__Mollicutes; o__; f__; g__; s__
	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas; s__
	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Alcaligenaceae; g__Achromobacter; s__
sediment	k__Bacteria; p__Planctomycetes; c__Planctomycetia; o__Pirellulales; f__Pirellulaceae; g__Pirellula; s__

	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Sinobacteraceae; g__; s__
	k__Bacteria; p__Verrucomicrobia; c__Verrucomicrobiae; o__Verrucomicrobiales; f__Verrucomicrobiaceae; g__Luteolibacter; s__
	k__Bacteria; p__Planctomycetes; c__Planctomycetia; o__Pirellulales; f__Pirellulaceae; g__; s__
	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Hyphomicrobiaceae; g__; s__

TABLE S2. Results of Kruskal-Wallis test of differences in alpha diversity of observed ASVs between crayfish populations (core-front) and locations (upstream-downstream), with Benjamini-Hochberg FDR correction of P-values. If locations (UF, UC, DC, DF) exhibited significant differences (as for hemolymph samples), they were analyzed separately, if not they were pooled together. Statistically significant results are marked in red.

OBSERVED ASVs			
sample		**p values: core-front**	**p values: upstream-downstream**
exoskeleton		0.715	0.475
hepatopancreas		0.771	0.101
intestine		0.059	0.519
sediment		1.0	0.149
sample	**locations**		**p values**
hemolymph	downstream core (n= 11)	upstream core (n = 7)	0.031
		upstream front (n = 11)	0.031

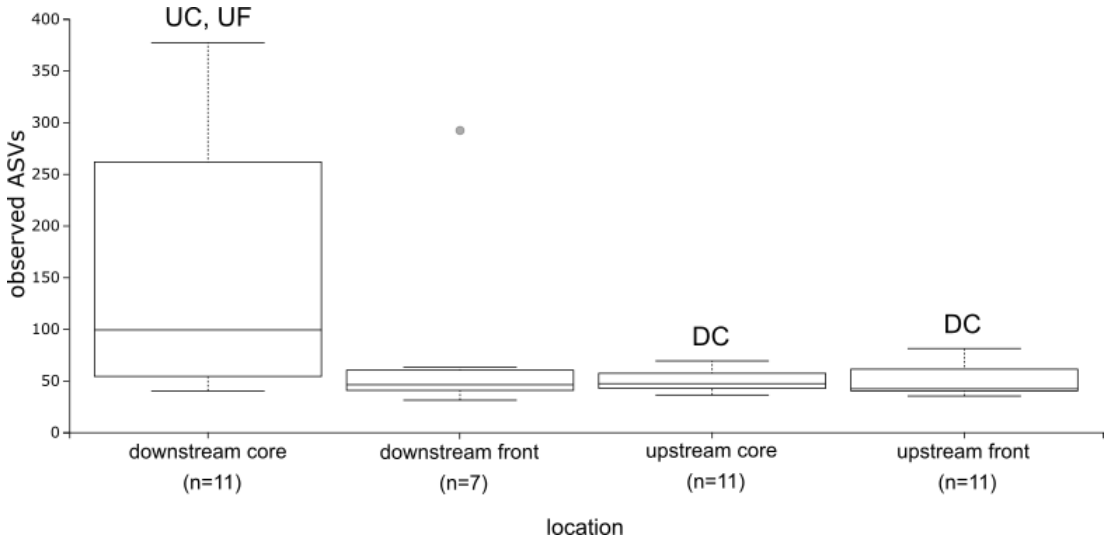

FIG. S2. Alpha diversity analyses of hemolymph microbial communities along invasion range. Downstream core exhibited significantly higher number of observed ASVs in comparison to upstream core and front sites. Significant differences between specific groups were marked with their abbreviations (UC = upstream core, DC = downstream core, UF = upstream front).

TABLE S3. Beta diversity analyses along the invasion range. Samples that differed significantly between locations (UF, UC, DC and DF) are shown in the lower part of the table, while others are grouped by position in the invasion range (core and front) and river section (upstream and downstream). DC= downstream core, DF = downstream front, UC = upstream core, UF = upstream front. Statistically significant results are marked in red.

sample group	unweighted UniFrac				weighted UniFrac			
	core-front		upstream-downstream		core-front		upstream-downstream	
	p-value	pseudo-F	p-value	pseudo-F	p-value	pseudo-F	p-value	pseudo-F
hemolymph	0.233	1.24798	0.038	1.97748	0.211	1.33254	0.003	4.43042
intestine	0.017	2.18175	0.359	1.03873	0.159	1.59869	0.334	1.05177
sediment	0.01	2.22431	0.003	2.80071	0.044	3.07729	0.006	5.10009
hepatopancreas	0.255	1.13066	0.909	0.714999	/	/	/	/

sample group	unweighted UniFrac				weighted UniFrac			
	location		p-value	pseudo-F	location		p-value	pseudo-F
hepatopancreas			/	/	UC	UF	0.0120	4.0241
exoskeleton	UC	DF	0.0012	4.0579	UC	DF	0.0012	14.3280
		UC	0.0050	1.7170		UC	0.0012	3.42621
		UF	0.0012	6.6137		UF	0.0012	10.2810
	DF	UC	0.0012	2.8002	DF	UC	0.0012	8.1536
		UF	0.0012	5.0356		UF	0.0012	10.0947
	UC	UF	0.0012	4.9368	UC	UF	0.0020	6.1673

TABLE S4. List of ASVs showing significantly different abundances between four sample locations. The analysis (ANCOM) was performed at phylum, family and genus levels. Locations with highest ASV abundances were determined based on the median number of ASVs.

ANCOM at phylum level				location with the highest ASV abundance
	Taxon	W-statistic value	clr mean difference	
exoskeleton	k__Bacteria;p__Cyanobacteria	28	53.452	DF
	k__Bacteria;p__Firmicutes	25	8.997	DF
	k__Bacteria;p__OD1	21	14.345	DC
hemolymph	no significant differences in abundance			
hepatopancreas	no significant differences in abundance			
intestine	no significant differences in abundance			
ANCOM at family level				location with the highest ASV abundance
	Taxon	W-statistic value	clr mean difference	
exoskeleton	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae	260	26.483	DF
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	260	38.449	DF
	k__Bacteria;p__Cyanobacteria;c__;o__;f__	259	36.787	DF
	k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Oscillatoriales;f__Phormidiaceae	259	32.882	DF
	k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae	251	33.222	UF
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae	241	22.85	DF
	k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Gomphosphaeriaceae	240	61.247	UF

hepatopancreas	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae	93	10.196	UC
hemolymph	no significant differences in abundance			
intestine	no significant differences in abundance			
ANCOM at genus level				location with the highest ASV abundance
	Taxon	W-statistic value	clr mean difference	
exoskeleton	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__*Acinetobacter*	404	37.812	DF
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__*Macrococcus*	404	26.75	DF
	k__Bacteria;p__Cyanobacteria;c__;o__;f__;g__	404	37.07	DF
	k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Oscillatoriales;f__Phormidiaceae;g__*Phormidium*	404	32.824	DF
	k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;g__*Arthronema*	403	94.609	UF
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__*Aerococcus*	372	22.868	DF
	k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales;f__Synechococcaceae;g__*Paulinella*	366	46.812	UF
hemolymph	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__*Caulobacter*	112	8.284	DC

hepatopancreas	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__*Psychrobacter*	217	16.231	UF
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Salmonella*	216	14.351	DF
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__	210	21.505	UC
intestine	no significant differences in abundance			

TABLE S5. Number of sequenced and analyzed samples per site and sample type.

A	**number of samples in analyses of differences between all sample groups pooled together by site = 184**					
	exoskeletal biofilm	hemolymph	hepatopancreas	intestine	sediment	water
TOTAL	39	41	33	48	17	6
B	**number of samples in analyses of differences across invasion range (DF, DC, UC, UF) for each sample type = 173**					
	exoskeletal biofilm	hemolymph	hepatopancreas	intestine	sediment	water
upstream front	8	11	9	10	6	excluded from these analyses
upstream core	8	11	10	12	3	
downstream core	12	11	9	12	2	
downstream front	11	7	5	10	6	
TOTAL	39	40	33	44	17	0

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Immune Response in Crayfish Is Species-Specific and Exhibits Changes along Invasion Range of a Successful Invader

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Simple Summary: A tiny fraction of all introduced species worldwide successfully establishes viable populations that rapidly spread and negatively impact native biodiversity and ecosystem functioning—i.e., become invasive. Even so, invasive species exert adverse environmental, economic, and human health impacts globally. Thus, identification of successful invaders is one of the immediate challenges in invasion biology. Recent studies highlight species' immunity as an important component of invasion success since it enables invaders to adapt to the novel conditions as they expand their range. Here, we analyzed whether the immune response of the successful crayfish invader, the signal crayfish, changes as the invader encounters different environmental conditions and novel counterparts. We used several parameters, frequently applied to assess crayfish immunological status, and compared their cumulative pattern between sites along the signal crayfish invasion range in the Korana River, Croatia, as well as between the invasive signal crayfish and the native narrow-clawed crayfish. Immune response differed between native and invasive species and exhibited variations along the invasion range, which were mostly affected by water temperature and crayfish density. Our results indicate that changes in immunity may occur during range expansion, and imply that immunity could have a role in invasion success of invertebrate invaders.

Abstract: Immunity is an important component of invasion success since it enables invaders' adaptation to conditions of the novel environment as they expand their range. Immune response of invaders may vary along the invasion range due to encountered parasites/microbial communities, conditions of the local environment, and ecological processes that arise during the range expansion. Here, we analyzed changes in the immune response along the invasion range of one of the most successful aquatic invaders, the signal crayfish, in the recently invaded Korana River, Croatia. We used several standard immune parameters (encapsulation response, hemocyte count, phenoloxidaze activity, and total prophenoloxidaze) to: (i) compare immune response of the signal crayfish along its invasion range, and between species (comparison with co-occurring native narrow-clawed crayfish), and (ii) analyze effects of specific predictors (water temperature, crayfish abundance, and body condition) on crayfish immune response changes. Immune response displayed species-specificity, differed significantly along the signal crayfish invasion range, and was mostly affected by water temperature and population abundance. Specific immune parameters showed density-dependent variation corresponding to increased investment in them during range expansion. Obtained results offer baseline insights for elucidating the role of immunocompetence in the invasion success of an invertebrate freshwater invader.

Keywords: immune response; crayfish abundance; water temperature; body condition; hepatosomatic index

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1. Introduction

Invasive alien species (IAS) are characterized by fast dispersal, successful population establishment in a novel environment, and generation of negative effects on biodiversity, ecosystem structure, and functioning, as well as on human health and the economy [1–4]. Multiple studies suggest that the immune system of IAS is an important component of invasion success, since it is in a constant interaction with microbes/parasites and the environment during range expansion [5–8]. Therefore, variations in the immune response of invaders along the invasion range can be caused by both existing or novel parasites/microbial communities, local environmental conditions, and ecological processes during the range expansion [9–12]. For example, if during range expansion IAS encounter high pathogen and/or competitive pressure from congeners, or if environmental stress is high in the novel environment, IAS may redirect resources into the immune system instead of investing in processes that boost population growth and expansion [5,10,13,14].

Mounting the immune defense is energetically expensive, and thus IAS may use two possible strategies of immune response investment during range expansion [5]. The first one, a reduced investment in the immune response, assumes that the rapid dispersal of individuals at the invasion front will reduce the incidence of 'enemies' (e.g., parasites, pathogens, competitors or predators from their native range) within the population, especially if the first dispersers are healthier (i.e., less infected) and more fit [15]. Such a scenario would result in reduced pathogen presence at distribution edges, which is consistent with the enemy release hypothesis [16], and would allow for reduced energy investment in immunity and increased investment in life history traits promoting population growth and dispersal, i.e., faster individual growth and reproduction (evolution of increased competitive ability hypothesis (EICA)) [5,16–18]. In addition, the lower density of available hosts at the distribution edge (i.e., invasion front) may also potentially result in lower pathogen transmission rates and fewer immune challenges for hosts, which may further reduce the need for a strong immune response [7,15]. Conversely, the second strategy assumes an increased investment in the immune response during range expansion. At the invasion front, individuals are likely to come into contact with new unknown pathogens which may accumulate in the invader's body (i.e., spillback hypothesis) [19], resulting in the need for increased investment in the immune response [5,20–23]. Furthermore, assuming that the dispersal of individuals is not random (i.e., spatial sorting theory) [24], and the less-infected individuals spread first, a better physiological status of such dispersers may indicate their higher immune potential [20,25]. Therefore, the stronger immune response at the invasion front could also appear as an indirect consequence of non-random dispersal of individuals in better condition [20]. Since dispersing individuals at the invasion front could benefit from both reduced and increased investment in the immune response, it is not immediately evident which of these opposing strategies will prevail during range expansion.

In order to investigate the changes in the immune response during range expansion, we selected one of the most successful IAS of freshwater invertebrates in Europe, the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), which is listed among the Invasive Alien Species of Union concern (the Union list) according to the EU Regulation on invasive alien species No. 1143/2014. Invasive crayfish species have advantageous life history traits, such as fast growth, early maturation, high fecundity, and higher aggression in competitive interactions [26–31], which contribute to their rapid establishment and range expansion, and their negative impacts on freshwater ecosystems [32,33]. The population of signal crayfish selected for this research was first recorded in the Korana River, Croatia, in 2011 [34] and has been spreading successfully in both upstream and downstream directions ever since [35,36]. Differences between individuals along the invasion range have already been observed in this population by previous studies [37,38] and were similar to those described for other freshwater invaders (i.e., round goby) [39,40]: invasion fronts contained less aggressive individuals in better bodily and physiological condition, and females with better energetic status of hepatopancreas and gonads compared with the invasion core. Furthermore, the invasion range of the signal crayfish in the Korana River covers

over 30 km of the watercourse [36], with segments potentially characterized by different environmental conditions: upstream (sparsely populated rural area) and downstream (industrial zone at outskirts of the Karlovac City). Considering the above-mentioned differences in fitness and their possible relation to immune potential [25], as well as potentially different environmental conditions along the Korana River, our goal was to examine whether differences in the immune response occur along the signal crayfish invasion range.

Additionally, we compared the immune response of the invasive signal crayfish with the native narrow-clawed crayfish, *Pontastacus leptodactylus* (Eschscholtz, 1823). The narrow-clawed crayfish is a native species that is gradually increasing its range in the Korana and Mrežnica Rivers [41]. However, it has also been gradually outcompeted by the signal crayfish, and completely displaced from the signal crayfish invasion core [35,36]. The narrow-clawed crayfish co-occurs with the signal crayfish at the invasion fronts, where the signal crayfish populations are less abundant [35,36].

Like all invertebrates, crayfish lack adaptive immunity and rely upon the mechanisms of the innate immune system, such as melanin synthesis [42], coagulation system [43], and the production of antimicrobial peptides [44] as a response to parasite entry [45]. Melanin synthesized during crayfish immune response plays an important role in encapsulation of the microorganisms invading the hemocoel [42,46]. At the entry of an invading microorganism or foreign particle into the body, the prophenoloxidaze (proPO)-activating system is triggered. Hemocytes recognize the foreign particle (such as lipopolysaccharides, peptidoglucans, beta-1,3-glucans, i.e., parts of bacteria, fungi, etc.), which leads to the aggregation of other hemocytes and formation of a capsule surrounding the foreign particle (i.e., encapsulation) [42,47]. Simultaneously, proPO, the inactive precursor of phenoloxidaze (PO), is released from the hemocytes (granulocytes and semigranulocytes) into the hemolymph by exocytosis, where it is transformed into its active form (i.e., PO) by the serine protease [48,49]. The PO then catalyzes the synthesis of melanin, which is deposited in the capsule, resulting in capsule hardening, isolation of the foreign particle from the rest of the body, and infection localization [47,49–51]. In light of these processes, we measured the immune response in signal and narrow-clawed crayfish by using several standard immune parameters: strength of encapsulation response, total number of hemocytes in the hemolymph, PO activity, and total proPO. Activation of PO causes a drop in the level of total proPO [52], and indicates that there is a currently active, ongoing immune reaction in the individual. At the same time, the number of hemocytes drops because they are mobilized for the processes of encapsulation, coagulation, and/or degranulation in order to release more proPO into the hemolymph [53]. Consequently, the strength of encapsulation response (i.e., the level of melanization) measured at the site of infection should be proportional to the PO activity, and inversely proportional to the total proPO levels and number of hemocytes.

We aimed to (i) explore and compare the immune response of the native and invasive crayfish in their mixed populations by using the abovementioned immune parameters and (ii) to investigate in more detail the potential changes in the immune response of the expanding signal crayfish invader by comparing the immune response in individuals from populations of different age and crayfish abundance (invasion core and invasion front), as well as between individuals from upstream and downstream segments of the Korana River.

2. Materials and Methods

2.1. Study Area

The fieldwork was conducted in the Korana River, a 134 km-long karstic river located in continental Croatia, belonging to the Sava River basin. The study area covers approximately 33 km of the lower watercourse of this river, where the signal crayfish is spreading in both upstream (through the sparsely populated rural region) and downstream directions (through the industrial zone of the Karlovac City) [36]. The crayfish were sampled along the study area at sampling sites differing in crayfish community composition and cray-

fish abundance: (i) the invasion core sites which are characterized by longer-established, dense populations of the signal crayfish, and (ii) the invasion front sites which include recently established, less abundant signal crayfish populations, which co-occur with the native narrow-clawed crayfish [35,36]. Invasion front sites had 4 to 7 times lower relative total crayfish abundance (native narrow-clawed crayfish and invasive signal crayfish) in comparison with invasion core sites [36]. Invasion core sites contained no native crayfish, since they were outcompeted by the signal crayfish [35,36].

2.2. Sampling Procedure

The sampling was performed during the period of increased crayfish activity of both sexes of the signal and the narrow-clawed crayfish (i.e., before the mating period) [54], in the early autumn of 2020. The crayfish were captured at four sites along the lower reaches of the Korana River: upstream invasion front (UF), upstream invasion core (UC), downstream invasion core (DC), and downstream invasion front (DF; Table 1), previously identified by [36]. Only adult crayfish were caught using baited LiNi traps [55]—approximately 30 traps per site were left in the water overnight. Water temperature was measured at each site at the time of crayfish capture. Both signal crayfish individuals (captured at invasion cores and fronts, N = 138) and narrow-clawed crayfish individuals (captured only at invasion fronts, N = 13) of both sexes were used in the following analyses. Catch per unit effort (CPUE; i.e., equal to the number of crayfish captured per LiNi trap per trapping night) was calculated for each site based on the collected data. CPUE is a frequently used measure of relative crayfish abundance [56] and was used for population abundance comparisons among sites.

Table 1. Geographic coordinates of sampling sites along the invasion range of the signal crayfish in the Korana River in 2020.

Location	X (WGS84)	Y (WGS84)
upstream front (UF)	45.320915	15.518373
upstream core (UC)	45.371918	15.521505
downstream core (DC)	45.411808	15.609231
downstream front (DF)	45.451355	15.567030

2.3. Immune Response Analyses

Immune response analyses included several standard immune parameters which are frequently analyzed in crustaceans: (i) the strength of the encapsulation response, i.e., the amount of synthesized melanin [57,58], (ii) total hemocyte count (THC) [59–63], and (iii) enzyme activity of phenoloxidaze (PO) and total prophenoloxidaze (proPO) [42,61,64,65]. These four parameters were analyzed together as the immune response of crayfish.

2.3.1. The Encapsulation Response Analyses

The experimental immune challenge was conducted on a total of 126 captured signal crayfish individuals (approximately 30 at each sampling site: UF, UC, DC, and DF; Supplementary Table S1), and a total of 13 captured narrow-clawed crayfish individuals (captured at both invasion fronts, i.e., UF and DF; Supplementary Table S2A). A sterile nylon monofilament implant method was used immediately upon capture in the field to induce the encapsulation response and to obtain a standardized measure of the encapsulation response strength, which is strongly related to the defense against parasites [66–69]. A nylon monofilament (i.e., fishing line, Jaxon Satori, Japan; from here on referred to as implant) was roughened with sandpaper, tied into a knot, and cut to the desired length under the knot. Prior to insertion, the implants (4 mm long, 0.22 mm in diameter) were stored in 90% ethanol to ensure sterility. Implants, representing novel and standardized pathogens, were inserted through a small puncture in the first joints of each of the fifth

pair of walking legs using forceps [57,58]. Each individual was then placed in a perforated plastic container (18 × 18 × 9 cm; with numerous perforations approximately 0.7 cm in diameter) that allowed water circulation. Containers with crayfish were then submerged in the river at the exact site where crayfish were caught and left for 48 h. After the 48 h period, the crayfish in containers were put on ice and taken to the laboratory for implant extraction, measurement, and hemolymph sample collection.

In the laboratory, the implants were retrieved from individuals' walking legs using forceps and stored at −20 °C. In further analyses, the two implants from walking legs of each crayfish individual were placed on a white background along with a sterile implant and photographed from two different sides using a digital camera connected to a light microscope (Stemi 305, Zeiss, Germany). In order to quantify the strength of the encapsulation response (i.e., the degree of melanization), the image-processing program (ImageJ, ver. 1.53f, https://imagej.nih.gov/ij/index.html, accessed on 3 November 2020) was used to determine the gray values of reflecting light of the melanized implants [57,58]. Encapsulation response strength was determined by subtracting the mean of the two gray values of a melanized implant from the gray value of a sterile (clear) implant [66]. Finally, the encapsulation response strength per individual was determined by calculating the mean gray value of both inserted implants.

2.3.2. Hemolymph Sampling Procedure

Following implant removal, the individuals were measured (total length (TL), length of the postorbital part of the carapace (POCL)) was weighed, and their hemolymph was sampled. Using a sterile needle, minimally 500 µL of hemolymph was collected from the base of the individual's walking leg, of which: (i) 100 µL was diluted in 400 µL of 1% formalin for total hemocyte count (THC), and (ii) 400 µL was diluted in 800 µL of crayfish saline solution (CFS: 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) [48] for the analyses of PO activity and total proPO. The hemolymph samples collected for PO and proPO analyses were immediately centrifuged at 10,000× *g* for 10 min at 4 °C to prevent coagulation. Next, they were put on ice and sonicated for 10 s with gradually increasing power to 50% (Sonoplus HD 2070, Bandelin, Germany) in order to completely lyse the hemocytes and release the stored proPO into the diluted plasma. Finally, the samples were centrifuged again at 15,000× *g* for 25 min at 4 °C to separate cell debris, and the supernatant was stored at −80 °C. After hemolymph sampling, each individual was killed according to available guidelines for humane killing of crayfish (rapid cut of nerve cord from thorax to the end of abdomen; however, no institutional or national ethical guidelines exist for crayfish) [70]. Each animal was then dissected, and their hepatopancreases were carefully removed and weighed for the analyses of body condition parameters (described below).

2.3.3. Total Hemocyte Count

The hemolymph samples collected for THC were stored at 4 °C for hemocyte fixation until further analyses. The hemocytes were counted by using the Bürker-Türk counting chamber and Zeiss Standard RA Light Microscope [71,72]. The number of hemocytes per milliliter was calculated after taking into account the dilution of the hemolymph during sampling [72,73].

2.3.4. PO Activity and Total proPO

From each individual, hemolymph concentrations of active PO and total proenzyme proPO were measured spectrophotometrically in supernatant samples (containing both active PO and inactive proPO released from the hemocytes) prepared in the previous step, following a modified version of the method by [74]. Briefly, to measure the PO activity, 50 µL of the L-3,4-dihydroxyphenylalanine substrate (L-DOPA; 3 mg/mL, dissolved in Milli-Q water) were mixed with 50 µL of each sample in a microplate (in triplicates), and the absorbance was measured at 490 nm for 25 min. In order to quantify the proenzyme

proPO, all the available proPO in the samples first had to be converted into their active form (enzyme PO). Therefore, in another spectrophotometrical assay, 50 µL of each sample were preincubated with 50 µL of trypsin (acting as an elicitor; 1 mg/mL, dissolved in Milli-Q water) in a microplate for 3 min at room temperature. Afterwards, 50 µL of L-DOPA were added to the reaction mix and the absorbance was measured at 490 nm for 25 min again. The amount of total proPO in samples was calculated as total proPO measured in the trypsin treatment minus the PO activity measured before trypsin treatment [52,74,75]. Finally, in order to standardize the enzyme activity per mg of protein [46], total protein content was measured using the method by [76], as in [77]. Enzyme activity was expressed as the change in absorbance at 490 nm per min and mg of protein (ΔA_{490}/min/mg protein).

2.4. Body Condition Parameters

Since immune response is considered an important fitness component [78] and dependent upon animal (physiological) condition, we measured several condition parameters in addition to immune parameters: (i) Fulton's condition factor (FCF = $W/TL^3 \times 100$; where W = weight (g), and TL = total body length (mm) of the individual), which is used as a proxy for individual's body condition [79], and (ii) hepatosomatic index (HSI = HW/BW; where HW = hepatopancreas weight (g), and BW = body weight (g)), which is indicative of an individual's energy status [80]. These indices are frequently used to determine health and fitness of crayfish individuals [37,81–84] and are also used as proxy measures of fitness in aquatic animals [85].

2.5. Statistical Analyses

2.5.1. Comparisons of Changes of the Signal Crayfish Immune Response along Invasion Range and Their Potential Drivers

To explore the effects of specific predictors on the changes in the signal crayfish immune response, the Partial Least Squares Regression approach (PLS-R) was used. In the present study, the explanatory variables (predictors, X) were water temperature, relative crayfish abundance (i.e., CPUE), and crayfish condition indices (FCF, HSI), while the response variables (Y) were measured immune parameters (encapsulation response, THC, PO activity, and total proPO). The PLS scores associated with the first two PLS components, generated in the model, are new variables summarizing the X variables. Scores contain the information about the objects and their similarity [86] and were therefore used for the interpretation of the PLS-R model. We reported model quality indices Q^2(cum), R^2Y(cum), and R^2X(cum) parameters and calculated standardized coefficient to examine how changes in predictors (water temperature, CPUE, FCF, HSI) affect response variables (immune response: encapsulation response strength, THC, PO activity, total proPO) and which predictors have a greater effect on the response variables. Additionally, in order to examine which of the predictors have the highest explanatory power for the construction of the immune response, we performed a variable importance for the projection (VIP) procedure. Parameters with a VIP value > 1 were considered relevant for explaining the response variables (Y) and contributed significantly to the model, while parameters with a VIP value < 0.8 contributed little [87–89]. Furthermore, we performed generalized linear model (GLM) analysis fitted with aov function on PLS scores to test for the significance in the relationship between response variables and predictors towards sites along the invasion range (DF, DC, UF, UC), upstream (UF, UC) and downstream (DF, DC) river segments, invasion core (UC, DC) and invasion front (UF, DF) sites, and sex. Analyses were performed using statistical software R v. 3.6.2 [90]. Exceptionally, the PLS-R analysis was partly performed using the "plsdepot" package according to [91] in statistical software R, and partly using the XLSTAT version 2018.3 software for data analysis and visualization of radar of correlation provided by Microsoft Excel by Addinsoft. The "ggbiplot" package [92] in R was used for visualization of the PLS-R score plots and principal component analysis (PCA) biplot, while basic R "stats" package was used to perform GLM on PLS scores. In all analyses, the significance threshold was set at $p < 0.05$.

2.5.2. Comparisons of Immune Response between the Invasive Signal Crayfish and the Native Narrow-Clawed Crayfish

PCA was used for comparison of immune response between the two species (invasive signal crayfish and native narrow-clawed crayfish) from their mixed populations at invasion fronts in order to illustrate the importance of immune variables (i.e., encapsulation response strength, THC, PO activity, and total proPO) for the separation of the species. For this analysis, signal crayfish individuals were selected from the pool of all collected individuals (Supplementary Table S2B) so that the sex ratio and body size were kept similar between the species, and were compared to the collected narrow-clawed crayfish individuals (Supplementary Table S2). To test for the significance of the influence of the immune variables in species separation, a GLM fitted with aov function was performed on PCA scores (using basic R "stats" package). A threshold of $p < 0.05$ was considered significant.

3. Results

3.1. Comparisons of Changes of the Signal Crayfish Immune Response along Its Invasion Range and Their Potential Drivers

Using the PLS-R multivariate technique, the relationship between the immune parameters and specific predictors, i.e., water temperature, relative crayfish abundance (CPUE), Fulton's condition factor (FCF) and hepatosomatic index (HSI), was determined. The results of the GLM (Table 2) showed that, based on specified predictors, immune parameters significantly differed ($p < 0.05$) between sites along the invasion range and between upstream and downstream river segments (Figure 1a,b, Table 2). However, the immune parameters did not exhibit significant separation between invasion core and front sites (Figure 1c, Table 2). No clustering was observed for sexes, showing no difference in immune response between males and females among or within all inspected groups (Table 2).

Table 2. Generalized linear model (GLM) fitted with aov on PLS-R scores of immune parameters of signal crayfish. Significant differences ($p < 0.01$) are indicated with **. Df = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean squares.

MODEL	Df	Sum Sq	Mean Sq	F Value	*p* Value
sites along invasion range					
sites	3	625	208.24	4.37	0.006 **
sex	1	12	12.32	0.26	0.61
sites:sex	3	133	44.22	0.93	0.43
residuals	106	5052	47.66		
downstream-upstream					
downstream-upstream	1	410	409.9	8.35	0.004 **
sex	1	12	12.2	0.25	0.62
downstream-upstream:sex	1	0	0	0	0.99
residuals	110	5399	49.1		
core-front					
core-front	1	112	111.5	2.2	0.14
sex	1	2	2.38	0.05	0.82
core-front:sex	1	149	148.95	2.95	0.08
residuals	110	5559	50.5		

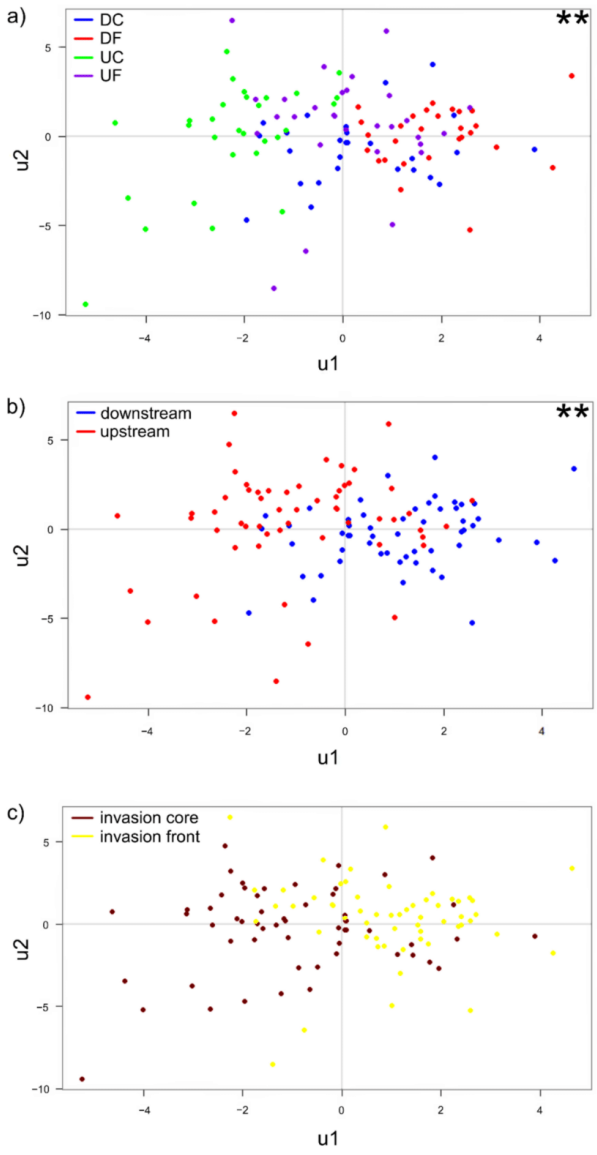

Figure 1. Partial least squares regression (PLS-R) score plots of signal crayfish immune parameters, based on y components (u1 and u2). Plots represent the relationship between response variables (immune parameters) and predictors (water temperature, relative crayfish abundance (CPUE), Fulton's condition factor (FCF), and hepatosomatic index (HSI)) according to sites along invasion range (**a**), upstream and downstream river segments (**b**), and invasion core and front sites (**c**). Significant effect ($p < 0.01$) in 'response-predictor' relation (performed by using the generalized linear model on PLS-R scores) is indicated with **. DC = downstream invasion core, DF = downstream invasion front, UC = upstream invasion core, UF = upstream invasion front.

In the PLS-R model, the first component was calculated with the Q^2(cum), R^2Y(cum), and R^2X(cum) parameters of 0.12, 0.13, and 0.42, respectively and the second component was calculated with the Q^2(cum), R^2Y(cum), and R^2X(cum) parameters of 0.14, 0.17, and 0.54, respectively (Supplementary Figure S1).

The relationship between blocks of predictor and response variables is visually presented in the form of a radar of correlation (Figure 2a), where positively correlated variables are presented close to each other and for negative correlation, variables are located far from one another. PLS-R multivariate analysis showed that encapsulation response strength had the strongest correlation with temperature ($r = 0.66$) and relative crayfish abundance (CPUE; $r = -0.68$), while other immune parameters exhibited measurable to moderate correlations in predictor-response relation (Figure 2a; Supplementary Table S3). Phenoloxidaze (PO) activity exhibited a similar pattern of correlation as encapsulation response strength (positive correlation with water temperature and negative correlation with relative crayfish abundance: $r = 0.12$ and $r = -0.07$, respectively), while total proPO exhibited an inverse correlation pattern (water temperature: $r = -0.24$; CPUE: $r = 0.22$; Figure 2a). Standardized coefficients (Figure 2b) also showed that both water temperature and CPUE had a stronger effect on immune response than crayfish condition in the case of encapsulation response strength and PO activity, while total hemocyte count (THC) and total proPO were more influenced by the crayfish condition: HSI in the case of THC ($r = 0.16$) and both HSI and FCF in the case of total proPO ($r = -0.21$, $r = -0.1$, respectively). Additionally, water temperature and CPUE generally exhibited the highest explanatory power for the construction of the immune response (Figure 2c).

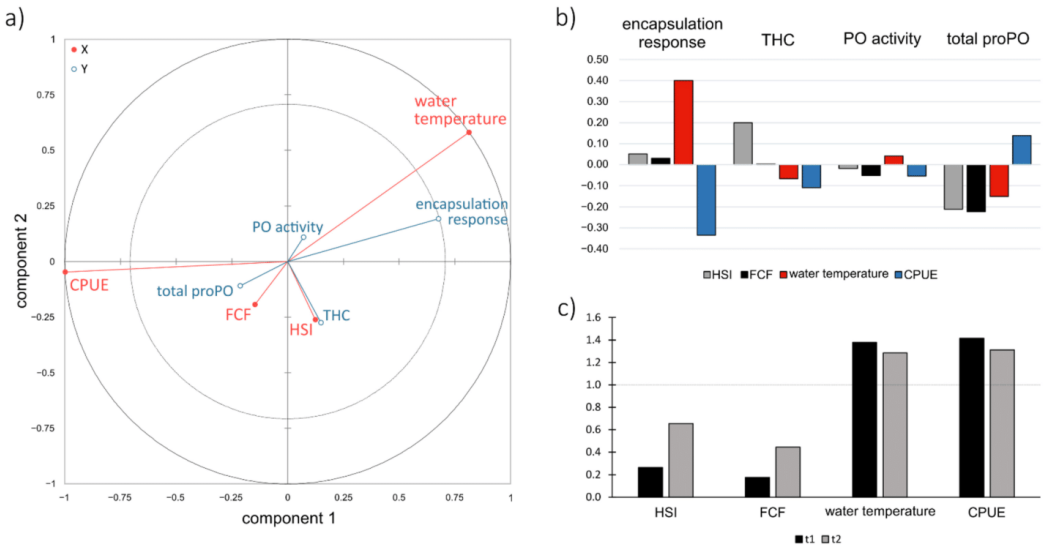

Figure 2. (**a**) Radar of correlation, illustrating the relationship between response variables (signal crayfish immune parameters, represented with blue lines) and predictors (represented with red lines). (**b**) Standardized coefficients of signal crayfish immune response. The closer to the absolute value of 1 the coefficient is, the stronger the effect of that predictor on the response variable (controlling for other variables in the equation). (**c**) Variable importance for the projection (VIP) for explanatory variables of first two components (t1 and t2). VIP > 1 indicate the explanatory variables that contribute the most to the PLS model. CPUE = catch per unit effort (relative crayfish abundance), FCF = Fulton's condition factor, HSI = hepatosomatic index, PO = phenoloxidaze, proPO = prophenoloxidaze, THC = total hemocyte count.

3.2. Comparisons of Immune Response between the Invasive Signal Crayfish and the Native Narrow-Clawed Crayfish

PCA of immune parameters between the two crayfish species revealed that the first two principal components explain 66% of the total variance, PC1 = 35.3% and PC2 = 30.7% (Figure 3). The biplot (Figure 3) shows the relationship between immune parameters. If the angle between the two variable vectors is zero, then it shows both variables are collinear. Here, results demonstrated that encapsulation response correlated the most with PO activity, while THC correlated with total proPO. The results of the generalized linear model (GLM; Table 3) showed significant separation (p = 0.006) between the two crayfish species according to immune response variables. No clustering was observed for sexes, showing no difference in immune response between males and females within each species. Further, PC loadings (Supplementary Table S4) on the first two PC's showed that all analyzed variables contributed in a very similar proportion to species separation (graphically presented by biplot obtained on the first two principal components, black arrows on Figure 3).

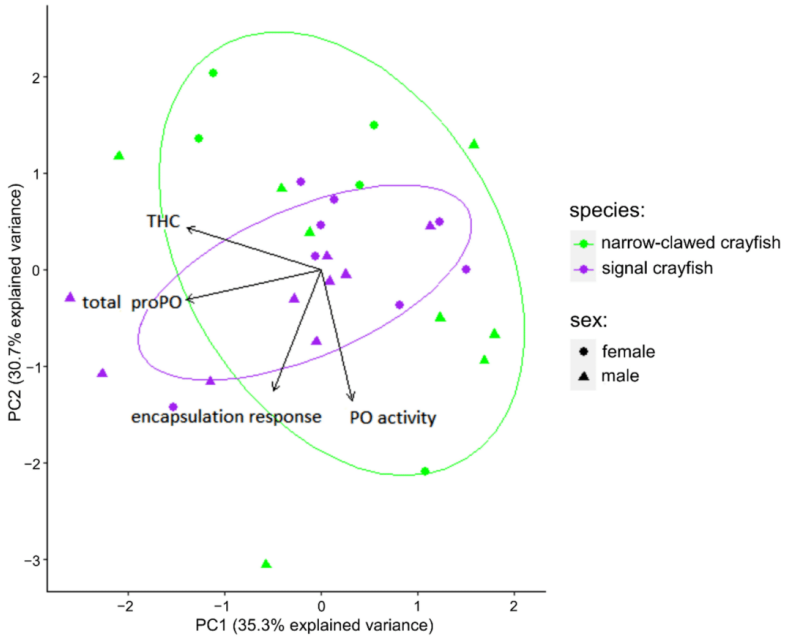

Figure 3. Principal component analysis (PCA) biplot on immune parameters of the signal crayfish and narrow-clawed crayfish from their mixed populations. Variables are indicated by black arrows, where their length represents the influence of a specific variable in shaping a model. 95% confidence ellipses are estimated around clusters. PO = phenoloxidaze, proPO = prophenoloxidaze, THC = total hemocyte count.

Table 3. GLM fitted with aov on PCA scores of immune parameters of two crayfish species. Significant differences ($p < 0.01$) are indicated with **. Df = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean squares.

MODEL	Df	Sum Sq	Mean Sq	F Value	*p* Value
species	1	23.6	23.59	9.91	0.006 **
sex	1	13.35	13.35	5.61	0.25
species:sex	1	0.1	0.1	0.04	0.83
residuals	26	61.91	2.38		

4. Discussion

Crayfish immune response is a result of complex interactions of multiple intrinsic (e.g., body condition, parasite load, diseases) and extrinsic (e.g., environmental conditions, population density, predation risk) factors [93–95]. In addition to these factors, the invasion process may also affect the immune response of both the crayfish invader and the native crayfish species due to potential trade-offs between immunity and the host's reproductive fitness [7,96,97] and potential spatial sorting of individuals with certain life-history traits during non-random dispersal (i.e., [98]). Here, we analyzed the differences in the immune response of the invasive signal crayfish along its invasion range. Additionally, we examined whether the immune response in crayfish is predominantly determined by intrinsic (body condition: hepatosomatic index (HSI), Fulton's condition factor (FCF)) or extrinsic (water temperature, relative crayfish abundance (CPUE)) factors. Finally, the immune response of the invasive signal crayfish was compared to that of the native narrow-clawed crayfish, a species negatively affected by the signal crayfish range expansion. As the immune system of IAS may affect their invasion success [21], our results contribute to better understanding of the factors determining the immune response during invasive species' range expansion.

4.1. Comparisons of Changes of the Signal Crayfish Immune Response along Its Invasion Range and Their Potential Drivers

Clear differences observed between all four signal crayfish populations (UF, UC, DC, DF) indicate that, based on the given predictors in PLS-R analysis, the immune response changes significantly along species' invasion range in the Korana River. Significant differences in the immune response were also established between upstream and downstream populations, but not between the invasion core and invasion front populations, suggesting that variation in the immune response resulting from range expansion may be outweighed by the effects of the local (abiotic) environmental factors, which may present a more prominent driver of changes in the immune response of crayfish. Our results are congruent with other studies [99,100] which examined the immune and glucocorticoid responses of invasive cane toads and found no differences in individuals from invasion core and invasion front populations, while multiple other studies report that immune response may show variation between the invasive species populations of different age and relative abundance along the invasion range [12,101–103].

The observed significant differences in immune response between the upstream and downstream signal crayfish populations might be due to differences in microhabitat characteristics. The approximately 33 km-long invasion range of the signal crayfish in the Korana River consists of microhabitats which differ in temperature, surrounding vegetation, water depth, sediment type, and anthropogenic pressure, with the upstream part of the Korana River flowing through the sparsely populated rural region, and the downstream part of the invasion range located in the industrial zone of the Karlovac City [104]. Additionally, it has been previously reported that different environmental parameters (e.g., temperature, pollution, oxygen levels, pH, and salinity) may affect crayfish immune system/health status [93,105–110]. In the case of the Korana River, similar water quality status in terms of general physical and chemical conditions and specific pollutants has been previously recorded across all seven water body monitoring sites monitored according to Water Frame-

work Directive [111], which also cover the whole signal crayfish invasion range within the river (Water Body Register 2016–2021; Croatian Waters). However, we recorded differences between upstream and downstream river segments in water temperature, which was 5.6 °C higher at downstream compared with upstream river segments. Temperature has been shown to exert variable and opposing effects on crustacean immune response in different studies [61,105,106,108,112,113] and has been suggested to have a prominent role in arthropod immune response along with population density [114–122]. This was corroborated by our study, where out of the predictor variables, water temperature and the relative crayfish abundance (CPUE) had much higher explanatory power of immune response in comparison with body condition parameters, and were considered the most relevant to the immune response construction.

4.2. Relationships between the Predictors and Immune Parameters

Stronger encapsulation response, i.e., higher level of melanization, is a result of an enhanced PO activity [49]. Since PO is released from the semigranulocytes and granulocytes by degranulation, this process leads to a drop in the number of hemocytes [53]. However, after the initial reaction to infection in the form of dramatic hemocyte depletion, the hematopoietic tissue is stimulated to rapidly synthesize and release new hemocytes [123,124]. Here, we found that total hemocyte count (THC) was the only immune response parameter which showed a negative correlation with both water temperature and relative crayfish abundance, suggesting that the exposure to environmental stress may lower the number of hemocytes [53,125,126], since hemocytes represent the first line of defense in the crayfish immune system and participate in the processes of immediate immune reactions (i.e., clotting, melanization, phagocytosis, encapsulation) and act as suppliers of antimicrobial peptides, lectins, proteinase inhibitors, and opsonins [51].

The encapsulation response strength and phenoloxidaze (PO) activity were positively correlated with water temperature, indicating that a higher level of melanization occurs as a stress response to higher environmental temperatures. Another study [118] reports similar findings in exploring the effects of temperature and population density on the immune response of the arthropod velvetbean caterpillar, suggesting that temperature is the main environmental factor affecting the host immune defense. Total protein content in the hemolymph (including PO) is dependent upon multiple factors, such as species, molting, reproduction, nutritional state, infection, stress response, salinity, season, light period length, temperature, and the level of dissolved oxygen [127,128]. The latter is directly related to water temperature (i.e., level of dissolved oxygen decreases with increasing water temperature). Therefore, changes in water temperature, which are manifested through changes in available oxygen, can directly or indirectly affect PO-specific activity (i.e., measured immune parameters). Furthermore, similarly to our results, [121] reported a temperature-dependent increase in capsule melanization (i.e., encapsulation response). However, mounting a strong encapsulation response may also negatively affect the crayfish due to self-reactivity costs of a strong immune defense [57,129] since the process of melanin synthesis during the encapsulation response also involves the release of cytotoxic byproducts, such as quinones and phenols, which damage the tissue [130].

Both encapsulation response strength and PO activity were negatively correlated with the relative crayfish abundance, which suggests that the encapsulation response strength (and PO activity) is higher in individuals from populations of low relative crayfish abundance (i.e., invasion front). Obtained results are further corroborated by the pattern of correlation observed for total prophenoloxidaze (proPO), which showed an inverse relationship compared with the above parameters (negative correlation with water temperature, and positive correlation with the relative crayfish abundance). This is consistent with our expectations that the decrease (activation) of proPO is followed by the increase in PO activity, and increase in the level of melanization (i.e., encapsulation response strength). Thus, while an overall immune response seemed not to be affected by the dispersal process (i.e., showed no significant differences between invasion core and invasion front popula-

tions), specific immune parameters showed density-dependent variation corresponding to increased investment in them during range expansion. Such conflicting effects have already been previously reported [100], and are not surprising since: i) density influences both intraspecific competition and pathogen transmission rates, and ii) the animal immune system is multifaceted and complex, and its different components may exhibit different patterns of change [101].

Despite an overall low explanatory power, both THC and total proPO were affected by crayfish condition (FCF: total proPO and HSI: total proPO and THC). However, the relationship between crayfish condition parameters and immune parameters is still largely unexplored. The hepatopancreas is a central organ of crustacean immunity and metabolism, as well as the main energy storage that supports key functions such as reproduction and growth [131,132], and organosomatic indices are considered as viable proxy measures of fitness [85,133]. Immunity is also an important fitness component, since it enables the organism to fight its pathogens, survive, and reproduce (i.e., [134]). Thus, the relationship between energy status of hepatopancreas (measured by organosomatic indices) and additional fitness determinants (such as, for example, reproductive success) and immune parameters should be further examined. However, this is a complex task since quantification of different fitness components/determinants needs to take into account the effects of the season, year cycle characteristics related to molting and mating, presence of an acute or chronic infection, etc. [135,136]. Finally, in order to elucidate the role of immunocompetence in invasion success, further studies involving examination of additional parameters related to both crayfish status (i.e., fecundity, individual health status), as well as environmental conditions (i.e., detailed ecological and chemical status), should be conducted. Additionally, future studies should also be performed at multiple locations (i.e., rivers, lakes) with recorded signal crayfish presence and over a longer time period.

4.3. Comparisons of Immune Response between the Invasive Signal Crayfish and the Native Narrow-Clawed Crayfish

Significant differences in the immune response were observed between the invasive and the native crayfish species in the Korana River, even though they inhabit the same local environment (i.e., co-occur at the same sites with same water temperature and relative crayfish abundance). This clearly shows the species-specific differences related to the measured immune parameters. Previous studies have shown that signal crayfish, the original host of the pathogen *Aphanomyces astaci* Schikora, 1906, has adapted to its presence in the body and is able to carry the latent infection by keeping the immune defense at a constantly high level [137,138]. The resistant invasive signal crayfish had continuously elevated levels of proPO expression, which could not be additionally increased by immunostimulants like in a susceptible native crayfish species [138], such as the narrow-clawed crayfish. The prevalence of crayfish plague in the signal crayfish population in the Korana River was very low (6% of individuals, distributed equally along the invasion range) [139], while qPCR quantification of *A. astaci* performed in parallel with this study identified very low agent levels (A0–A3, with the majority of samples from individuals of both species classified as A0; Bielen et al., in preparation). Therefore, neither native nor invasive crayfish populations show signs of recent crayfish plague outbreaks, which could potentially have a high impact on their immune response. Even though all immune parameters contributed similarly to the separation between species in PCA analysis, differences in proPO expression may represent the main driver of the observed differences in the immune response between the two species in this study, since they consequently affect all immune parameters measured here (as elaborated in the Introduction). However, immune response to *A. astaci* infection is much more complex than the activation of the proPO cascade, which makes only a small portion of crayfish humoral response [140]. Therefore, further studies are required to elucidate the mechanisms and energetic costs of mounting an immune response in invasive and native species differing in the proPO expression. Comparisons of energetic costs of immune response between invasive and native crayfish in pathogen-free and infected populations are required in order to clarify this question.

5. Conclusions

In conclusion, we observed differences in the immune response along the invasion range, with the environmental and population characteristics (water temperature and population density) being the more prominent drivers of changes in the immune response compared with the invasion process, whose impact on the immune system was not evident in this study (i.e., no significant differences in immune response were observed between invasion core and invasion front populations). While the overall immune response seemed not to be affected by the dispersal process, specific immune parameters showed density-dependent variation corresponding to an increased investment in them during range expansion. Furthermore, since the relationships between immune parameters were not as distinct as expected, further research is required to clarify the cause-and-effect relationship with animal condition and/or environmental factors (such as the season and year cycle characteristics related to molting and mating, temperature, presence of an acute or chronic infection, etc.). Finally, we confirmed that the immune response is species-specific, exhibiting significant differences between the co-occurring native narrow-clawed crayfish and invasive alien signal crayfish. The obtained results represent the first step in investigating the role of immunocompetence in the invasion success of an invertebrate freshwater invader, required for elucidating the costs of immunity and its links to individuals' reproductive success and overall fitness.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology10111102/s1, Figure S1: Model quality by number of components, Table S1: Number of signal crayfish per site included in the analyses of changes in the immune response along the species' invasion range and their potential drivers, Table S2: Number of narrow-clawed (A) and signal crayfish (B) per front site included in the comparative analyses of immune response between the invasive and native species, Table S3: Correlation matrix of the analyzed variables, Table S4: Principal component loadings (PC1 and PC2) on immune parameters of the invasive signal crayfish and the native narrow-clawed crayfish.

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Supplementary materials

Table S1. Number of signal crayfish per site included in the analyses of changes in the immune response along the species' invasion range and their potential drivers.

signal crayfish (all individuals)					
Location	Total individuals	Males	Females	Average postorbital carapace length (mm)	Average body weight (g)
upstream front	30	15	15	46.59 (±5.69)	55.13 (±19.36)
upstream core	32	18	14	46.91 (±6.13)	46.74 (±19.41)
downstream core	32	11	21	46.33 (±5.67)	41.78 (±13.84)
downstream front	32	9	23	42.11 (±6.02)	32.8 (±15.27)

Table S2. Number of narrow-clawed (A) and signal crayfish (B) per front site included in the comparative analyses of immune response between the invasive and native species.

A	narrow-clawed crayfish				
Location	Total individuals	Males	Females	Average postorbital carapace length (mm)	Average body weight (g)
upstream front	3	0	3	41.84 (±4.46)	30.94 (±10.59)
downstream front	10	8	2	45.73 (±5.33)	37.31 (±12.66)
B	signal crayfish (individuals chosen for comparative analyses with narrow-clawed crayfish)				
Location	Total individuals	Males	Females	Average postorbital carapace length (mm)	Average body weight (g)
upstream front	6	0	6	40.80 (±3.58)	36.69 (±5.89)
downstream front	12	7	5	40.98 (±5.02)	30.69 (±12.67)

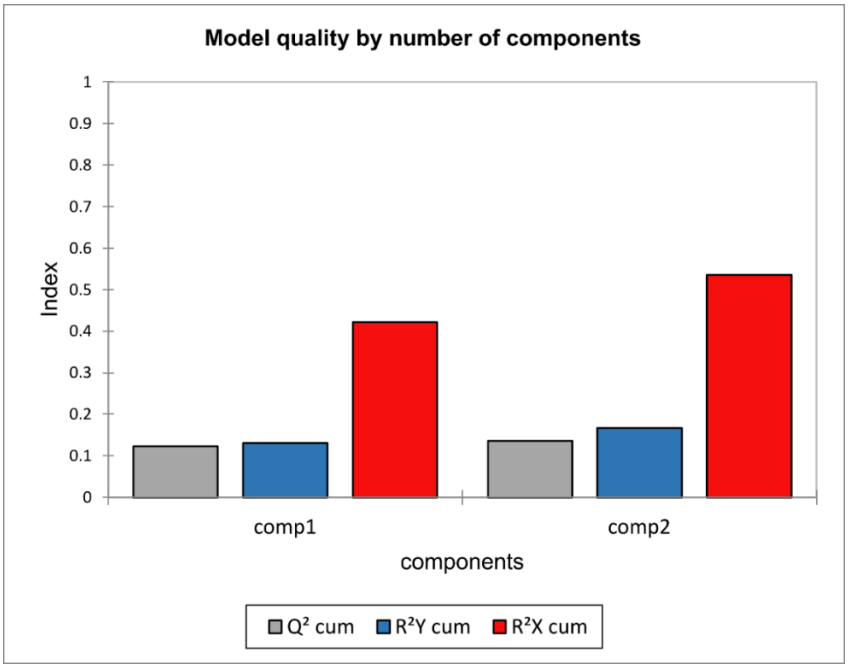

Figure S1. Model quality by number of components. Components were calculated using the Q^2(cum), R^2Y(cum), and R^2X(cum) parameters.

Table S3. Correlation matrix of the analyzed variables. CPUE = catch per unit effort, FCF = Fulton's condition factor, HSI = hepatosomatic index, PO = phenoloxidaze, proPO = prophenoloxidaze, THC = total hemocyte count.

VARIABLES	HSI	FCF	water temperature	CPUE
HSI	1	-0.207	-0.051	-0.113
FCF	-0.207	1	-0.232	0.154
water temperature	-0.051	-0.232	1	-0.835
CPUE	-0.113	0.154	-0.835	1
encapsulation response	0.054	-0.118	0.662	-0.680
THC	0.161	0.005	-0.038	-0.141
PO activity	0.018	-0.083	0.120	-0.071
total proPO	-0.207	-0.095	-0.237	0.215

Table S4. Principal component loadings (PC1 and PC2) on immune parameters of the invasive signal crayfish and the native narrow-clawed crayfish.

	PC1	**PC2**
Standard deviation	1.189	1.109
Proportion of Variance	0.353	0.307
Cumulative Proportion	0.353	0.661
encapsulation response	-0.240	-0.651
THC	-0.676	0.227
PO activity	0.159	-0.706
total proPO	-0.679	-0.162

3. RASPRAVA

Ovim istraživanjem ispitane su promjene u sastavu mikrobnih zajednica i imunosnog odgovora prilikom širenja vrlo uspješne invazivne vrste slatkovodnih ekosustava, signalnoga raka, s ciljem boljeg razumijevanja njihovih uloga u invazivnom uspjehu. Razumijevanje odrednica invazivnog uspjeha od velike je važnosti za predviđanje uspješnih invazivnih stranih vrsta i upravljanje njihovim širenjem. Ovo istraživanje provedeno je na populaciji signalnoga raka u rijeci Korani, čemu je prethodilo određivanje novih invazijskih fronti (Znanstveni rad br. 1).

3.1. Identifikacija mikrobnih patogena signalnoga raka u rijeci Korani

3.1.1. Klasifikacija mikroba slatkovodnih deseteronožnih rakova s obzirom na njihovu patogenost

Ispitivanje hipoteza o mogućim interakcijama invazivne strane vrste s (patogenim) mikrobima pri širenju u novi okoliš (navedene u uvodu, poglavlje 1.1.1.) zahtijevalo je sistematični pregled postojeće literature o mikrobima u slatkovodnim deseteronožnim rakovima te njihovu klasifikaciju na četiri kategorije: patogene, oportunističke/potencijalne patogene, nepatogene i one mikrobe za koje nema dovoljno podataka za određivanje njihove patogenosti (Znanstveni rad br. 2). Međutim, već se prilikom pregleda literature pojavio problem definiranja kategorije oportunističkog patogena. Prvenstveno, ne postoji jasna granica i/ili razlika u virulentnosti oportunističkog patogena naspram „pravog“ patogena te oportunistički patogen može izazvati puno teže posljedice za zaraženu jedinku nego „pravi“ patogen (npr. Hunter i Bean, 2013). Nadalje, oportunistički patogeni najčešće inficiraju imunosuprimirane jedinke (Martínez, 2014; Derome i sur., 2016) no često je kod inficirane jedinke nemoguće razlučiti radi li se o oportunističkom patogenu u imunosuprimiranoj jedinci, ili o pojavi novog „pravog“ patogena u populaciji. U stresnim uvjetima okoliša (npr. visoka temperatura vode ili zagađenje), nepatogeni mikrob bi se mogao ponašati kao oportunistički patogen (Derome i sur., 2016) i zaraziti većinu jedinki u populaciji te je nejasno u kojem se trenutku navedeni oportunist utvrđuje kao „pravi“ patogen u populaciji. Nadalje, oportunistički patogen može izazvati sekundarnu infekciju u jedinki prethodno zaraženoj nekim drugim patogenom (Martínez, 2014), pri čemu simptomi sekundarne infekcije mogu biti pogrešno pripisani prvotnoj infekciji/patogenu, čime oportunistički patogen ostaje nedetektiran (npr. Fairfax i sur., 2014). Stoga je kategorija „oportunističkog patogena“ izbačena iz daljnje analize zbog nejasnih kriterija za određivanje oportunističkog patogena.

Mikrobi (virusi, bakterije, mikrogljive i gljivama slični organizmi) u slatkovodnim deseteronožnim rakovima pronađeni u dostupnoj literaturi kategorizirani su u preglednom radu (Znanstveni rad br. 2) na patogene, nepatogene i mikrobe za koje nema dovoljno podataka za određivanje njihove patogenosti (*engl.* data deficient). Rezultati ovog istraživanja ukazali su na znatnu neistraženost mikroba rakova te na nesustavnost istraživanja njihove patogenosti. Od ukupno 117 pregledanih publikacija (objavljenih u rasponu od 1940. to 2020. godine) o mikrobima u slatkovodnim deseteronožnim rakovima, čak 59,4 % publikacija ne sadrži dovoljno podataka za određivanje patogenosti navedenih mikroba. Među svim skupinama patogena uključenih u ovo literaturno istraživanje (Znanstveni rad br. 2), mikrogljive i gljivama slični organizmi najzastupljenija su skupina u publikacijama (45,7 %), nakon čega slijede bakterije (27,5 %). Međutim, iako su mikrogljive i gljivama slični organizmi najčešće istraživana skupina patogena rakova, za 79,9 % svih mikroba u ovoj skupini ne postoji dovoljno podataka za određivanje njihove patogenosti, a slično je vidljivo i u slučaju bakterija (tj. 82,4 % zabilježenih bakterija u publikacijama je nepoznate patogenosti). Iako je najviše potvrđenih patogena/nepatogena slatkovodnih deseteronožnih rakova među virusima (52,4 %), oni su također najslabije zastupljena skupina u publikacijama o patogenima rakova (26,8 %). Uzroci praznina u znanju uočenih tijekom ovog opsežnog istraživanja mogu se podijeliti u dvije glavne skupine:

i. Nedosljednost u ispitivanju infektivnosti i potvrđivanju patogenosti/nepatogenosti

Prilikom sveobuhvatnog pregleda literature, uočeno je da su korištene raznolike metode u određivanju patogenosti te da opisivanje virulentnosti, kao i konačni zaključci o patogenosti, znatno variraju između publikacija. Većina publikacija temelji se na sljedećem protokolu: (1) pronalazak jedinke/jedinki koje pokazuju simptome zaraze, (2) sekcija zaraženih jedinki, (3) analiza tkiva (histološka analiza ili sekvenciranje) u potrazi za uzročnikom simptoma/zaraze te (4) pronalazak (potencijalnog) uzročnika. Eksperimentalni dizajn najčešće nije uključivao ispitivanje infektivnosti, tj. nije provođeno eksperimentalno zaražavanje zdravih jedinki rakova pronađenim (navodnim) uzročnikom kako bi se u konačnici potvrdila njegova patogenost. Bez ispitivanja infektivnosti, ne može se odrediti patogenost ili nepatogenost pronađenog (navodnog) uzročnika, budući da je nemoguće odrediti je li upravo on uzrokovao zabilježene simptome. Stoga su mikrobi, koji su proglašeni patogenima ili nepatogenima bez ispitivanja infektivnosti, razvrstani u kategoriju mikroba za koje nema dovoljno podataka za određivanje njihove patogenosti. Budući da većina publikacija nije uključivala ispitivanje infektivnosti,

potvrđeni patogeni i nepatogeni u istraživanju (Znanstveni rad br. 2) izdvojeni su iz relativno malog broja preostalih publikacija u kojima je ispitana infektivnost.

ii. Slaba istraženost patogena signalnoga raka

U jedinkama signalnoga raka u rijeci Korani detektirano je 7041 taksona bakterija i 9382 taksona mikrogljiva (Znanstveni rad br. 3; Prilog 1: neobjavljeni podaci o mikobiomu). Pretragom literature uočeno je da se značajan postotak ovih detektiranih mikroba ne spominje u kontekstu mikrobioma slatkovodnih deseteronožnih rakova, već nekih drugih organizama i/ili okoliša, kao što su ribe, biljke, sediment ili ljudi (npr. Yang i sur., 2011; Sandoval-Denis i sur., 2015; McAllister i sur., 2016; Berrios, 2022). Mnogi detektirani mikrobi prvi put su zabilježeni u mikrobiomu vrste slatkovodnog deseteronožnog raka upravo ovim istraživanjem (Znanstveni rad br. 3; Prilog 1). Zbog slabe istraženosti mikrobioma rakova, vrlo je malo literaturnih izvora s kojima je moguće usporediti mikrobiom signalnoga raka identificiran ovim istraživanjem. Nadalje, čak i u slučajevima gdje se određeni detektirani mikrob navodi kao dio mikrobioma slatkovodnog deseteronožnog raka, on najčešće nije istraživan u kontekstu patogena (npr. Skelton i sur., 2017; Hoverson, 2020). Stoga, za većinu mikroba detektiranih u ovom istraživanju ne postoji informacija o njihovoj patogenosti ili nepatogenosti u domaćinu signalnom raku.

Ovo istraživanje predstavlja prvi korak prema popunjavanju praznina u znanju o patogenima slatkovodnih deseteronožnih rakova te, s obzirom na navedenu neusklađenost istraživačkih metoda i nedovoljnu istraženost patogena, u poglavlju 3.3. navodi prijedloge i smjernice za daljnja istraživanja u ovom području.

3.1.2. Zastupljenost patogena u populaciji signalnoga raka u rijeci Korani

S obzirom na prethodno opisanu slabu istraženost patogena rakova, za terenska istraživanja odabran je potvrđeni patogen signalnoga raka – vodena plijesan *A. astaci* – čija je prisutnost ispitana u jedinkama signalnoga raka u rijeci Korani metodom qPCR (*engl.* quantitative polymerase chain reaction), tj. ciljanom detekcijom odabranog patogena. Zastupljenost *A. astaci* u populaciji signalnoga raka u rijeci Korani vrlo je mala: samo 6 % populacije (tj. 7 od 110 jedinki) pokazalo je vrlo nisku razinu zaraze navedenim patogenom (Pavić i sur., 2020). S obzirom na mali broj (slabo) zaraženih jedinki, nisu bile vidljive razlike u zastupljenosti patogena *A. astaci* duž invazivnog areala signalnoga raka u rijeci Korani, tj. nije moguće odrediti je li više zaraženih jedinki u invazijskom središtu ili fronti. Osim ove ciljane detekcije, mikrobiomi signalnoga raka dodatno su istraženi kako bi se u tkivima rakova

detektirali patogeni koji su potencijalno različito zastupljeni duž invazivnog areala. Za nekoliko detektiranih bakterijskih rodova (*Acinetobacter*, *Macrococcus*, *Phormidium*, *Arthronema*, *Aerococcus*, *Paulinella*, *Caulobacter*, *Psychrobacter* i *Salmonella*) i za dva roda mikrogljiva (*Ascochyta* i *Leptosphaeria*) zabilježene su značajne razlike u zastupljenosti u jedinkama signalnoga raka duž invazivnog areala (Znanstveni rad br. 3; Prilog 1). Međutim, ni jedan od navedenih rodova nije potvrđeni patogen signalnoga raka (Znanstveni rad br. 2). Iako je nekoliko vrsta roda *Acinetobacter* pronađeno u hemolimfi slatkovodnih deseteronožnih rakova, ni za jednu od pronađenih vrsta nije ispitana niti potvrđena patogenost (Znanstveni rad br. 2). Osim što nisu pronađeni potvrđeni patogeni u bakteriomu i mikobiomu, niti analizom viroma signalnoga raka iz rijeke Korane nije pronađen ni jedan poznati virusni patogen ove vrste (Bačnik i sur., 2021).

Unatoč tome što analize mikrobioma nisu rezultirale detekcijom potvrđenih patogena u populaciji signalnoga raka u rijeci Korani, kod dijela jedinki uočene su histopatološke promjene na hepatopankreasu, što ukazuje na postojanje neidentificiranog patogena koji negativno utječe na ovu populaciju (Bekavac i sur., 2022). Ovo, kao i činjenica da se virom jedinki signalnoga raka sastojao većinom od neklasificiranih virusa (Bačnik i sur., 2021), još jednom govori u prilog vrlo slabe istraženosti patogena slatkovodnih deseteronožnih rakova. Iako zbog navedene slabe istraženosti nisu mogle biti utvrđene razlike u zastupljenosti patogena u rakovima duž invazivnog areala u ovom istraživanju, očekuje se da razlike postoje s obzirom na spomenute hipoteze o mogućim interakcijama invazivne strane vrste s (patogenim) mikrobima pri širenju u novi okoliš (opisane u uvodu, poglavlje 1.1.1.). Za detekciju navedenih razlika, neophodno je popunjavanje praznina u znanju identificiranih u prethodnom poglavlju (3.1.1.).

3.2. Razlike u mikrobiomu i imunosnom odgovoru signalnoga raka duž invazivnog areala u rijeci Korani

Signalni rak već više od deset godina uspješno širi svoj invazivni areal u rijeci Korani uzvodno i nizvodno, čime ugrožava zavičajne vrste rakova (Hudina i sur., 2013; Hudina i sur., 2017; Znanstveni rad br. 1). Na trenutnoj uzvodnoj i nizvodnoj invazijskoj fronti signalnoga raka nalaze se populacije zavičajne vrste, uskoškaroga raka (Maguire i sur., 2011; Znanstveni rad br. 1). Kompeticijski pritisak zbog prisutnosti populacija uskoškaroga raka, kao i uvjeti lokalnog okoliša, mogu utjecati na mikrobiom i imunosni odgovor signalnoga raka te u konačnici, na njegovu brzinu širenja i invazivni uspjeh (Znanstveni rad br. 1; Znanstveni rad br. 3; Znanstveni rad br. 4).

Istraživanja provedena u sklopu ove doktorske disertacije pokazala su da se mikrobiomi (tj. mikrobiom egzoskeleta, hemolimfe, hepatopankreasa i crijeva) i imunosni odgovor signalnoga raka razlikuju duž invazivnog areala ove vrste u rijeci Korani (Znanstveni rad br. 3; Znanstveni rad br. 4). Ovakvi rezultati ukazuju na jedan od sljedećih scenarija:

a) Razlike u mikrobiomu i imunosnom odgovoru duž invazivnog areala potvrđuju postojanje početnih razlika u karakteristikama jedinki u invazivnoj populaciji. Drugim riječima, ovo govori u prilog hipotezi o nenasumičnom širenju jedinki i ukazuje na to da jedinke koje se prve šire imaju drugačije karakteristike (drugačiji mikrobiom i imunosni odgovor) od onih koje ostaju u invazijskom središtu (slično kao Hudina i sur., 2015; Rebrina i sur., 2015).

b) Mikrobiomi i imunosni odgovor jedinki u invazivnoj populaciji izvorno se nisu razlikovali, nego je do promjena u karakteristikama jedinki koje se šire došlo zbog samog procesa invazije, tj. zbog novog okoliša i/ili zbog ekoloških procesa proizašlih iz invazije (slično kao Brown i sur., 2007).

Međutim, moguć je i treći scenarij koji je kombinacija navedena dva scenarija: jedinke koje se šire imaju specifične/drugačije karakteristike (od onih koje ostaju u invazijskom središtu), a na njih također utječe i proces širenja u novi okoliš. Pritom je teško razlučiti utjecaj procesa širenja od utjecaja samog okoliša kod prirodnih populacija tijekom biološke invazije, no ovakav (treći) scenarij smatra se najvjerojatnijim s obzirom na rezultate ovog istraživanja, što je objašnjeno u narednim poglavljima (3.2.1. i 3.2.2.).

3.2.1. Što oblikuje mikrobiom i imunosni odgovor signalnoga raka tijekom širenja invazivnog areala?

U ovom su istraživanju uspoređeni mikrobiomi i imunosni odgovor rakova između invazijskih fronti i središta (tj. različitih gustoća populacije) te uzvodnog i nizvodnog dijela rijeke (tj. različitih okoliša). Invazijska fronta razlikuje se od invazijskog središta u biotičkim i abiotičkim karakteristikama (npr. u gustoći i strukturi populacije, značajkama životnog ciklusa jedinki, količini dostupne hrane i zaklona, stopi prijenosa patogena, ponašanju jedinki, temperaturi, količini otopljenog kisika itd.; Phillips i sur., 2010a; Seebacher i Franklin, 2011; Brandner i sur., 2013; Brown i sur., 2013; Churchill i sur., 2017; Gruber i sur., 2017). Najznačajnija biotička razlika između invazijske fronte i invazijskog središta je gustoća populacije koja utječe na mnoge aspekte života jedinke, kao što su brzina rasta i spolnog sazrijevanja, učestalost kompetitivnih interakcija, dostupnost hrane i zaklona te količina

patogena (Arneberg i sur., 1998; Steneck, 2006; Gutowsky i Fox, 2012). Usto, može utjecati i na sastav mikrobioma (npr. Li i sur., 2016; Mogouong i sur., 2020), što je potvrđeno i u ovom istraživanju (Znanstveni rad br. 3). Osim gustoće populacije, i okolišne karakteristike mogu utjecati na sastav mikrobioma (Karl i sur., 2018; Sze i sur., 2020). Uzvodna lokacija uzorkovanja jedinki rakova na rijeci Korani nalazi se u rijetko naseljenom, ruralnom području, dok je nizvodna lokacija uzorkovanja smještena u gušće naseljenoj, industrijskoj zoni pred gradom Karlovcem (Znanstveni rad br. 3; Znanstveni rad br. 4). Karakteristike lokalnog okoliša rijeke (npr. ispust otpadnih voda, eksploatacija rijeke ili količina obalne vegetacije) mijenjaju fizikalno-kemijske parametre vode (npr. Dosskey i sur., 2010; Camara i sur., 2019) što potencijalno utječe na sastav mikrobioma okoliša, ali i jedinki koje u njemu žive. K tome, izmjerena temperatura vode na uzvodnoj lokaciji bila je 5,6 °C niža u odnosu na nizvodnu lokaciju uzorkovanja (Znanstveni rad br. 4), a utjecaj različitih temperatura na sastav mikrobioma rakova (Ooi i sur., 2019) i na imunosni odgovor rakova (Gizem Korkut i sur., 2018) već je prethodno potvrđen.

Rezultati ovog istraživanja pokazali su da su neke karakteristike signalnoga raka bile više pod utjecajem procesa širenja (npr. mikrobiom crijeva, koji je pokazao razlike između invazijskih fronti i središta, ali ne i između uzvodnog i nizvodnog okoliša rijeke), dok su neke bile više pod utjecajem okoliša (npr. mikrobiom hemolimfe, koji je pokazao razlike između uzvodnog i nizvodnog okoliša rijeke, ali ne i između invazijskih fronti i središta; Znanstveni rad br. 3).

3.2.1.1. Mikrobiom signalnoga raka

Dijeljenje i prijenos mikroba među jedinkama češći su u populacijama s većim brojem interakcija jedinki (Antwis i sur., 2018), tj. u gustim populacijama (Li i sur., 2016). Također, zbog učestalih agonističkih interakcija među rakovima u gustim populacijama (Steneck, 2006), češće dolazi do ozljeda jedinki, pri čemu okolišni mikrobi koloniziraju mikroozljede kutikule i mijenjaju sastav mikrobioma egzoskeleta (Ribet i Cossart, 2015). K tome, zbog povećanog stresa u gustoj populaciji mogu se očekivati promjene i u mikrobiomima drugih tkiva (npr. Zha i sur., 2018; Liu i sur., 2020b).

Ovo istraživanje pokazalo je da se bakteriom egzoskeleta razlikovao između populacija invazijskih fronti i središta te populacija uzvodnog i nizvodnog dijela rijeke, što upućuje na to da je oblikovan djelomično karakteristikama jedinke (koje proizlaze iz gustoće populacije u kojoj živi), a djelomično i okolišem jedinke (Znanstveni rad br. 3). Egzoskelet dijeli brojne mikrobe s okolišem budući da je istovremeno dodirna točka i barijera između organizma i

njegovog okoliša. S obzirom na to da je signalni rak bentička vrsta, mikrobiom egzoskeleta po sastavu je najsličniji mikrobiomu sedimenta, ali pokazuje i značajne razlike u odnosu na njega, kao što je vidljivo na primjeru bakterioma (Skelton i sur., 2017; Znanstveni rad br. 3) i mikobioma (Prilog 1) signalnoga raka. Suprotno bakteriomu egzoskeleta, mikobiom egzoskeleta signalnoga raka razlikuje se između uzvodnog i nizvodnog okoliša rijeke Korane, ali ne i između jedinki invazijskih fronti i središta (Prilog 1). Međutim, iako se mikobiom egzoskeleta ne razlikuje s obzirom na gustoću populacije, uočene su značajne razlike između mikobioma sedimenta i egzoskeleta s iste lokacije (Prilog 1), što ukazuje na to da i drugi čimbenici, osim okoliša, oblikuju mikobiom signalnoga raka. Dakle, mikrobiom (bakteriom i mikobiom) egzoskeleta signalnoga raka oblikovan je kombinacijom okolišnih karakteristika i karakteristika same jedinke, tj. populacije.

Bakteriomi hepatopankreasa i crijeva, važnih organa probavnog sustava, određeni su primarno gustoćom populacije (Znanstveni rad br. 3). Gustoća populacije utječe na režim i učestalost hranjenja, a posljedično i na kondiciju rakova (Ghedini i sur., 2017). Međutim, s obzirom na funkciju ovih organa i njihov kontinuirani kontakt s hranom (tj. tvarima iz okoliša), ne smije se zanemariti utjecaj okolišnih karakteristika (npr. tip i količina dostupne hrane u staništu; Hildebrandt i sur., 2009; Xia i sur., 2014; Carrier i sur., 2018) u oblikovanju bakterioma hepatopankreasa i crijeva. Stoga su bakteriomi hepatopankreasa i crijeva oblikovani utjecajem dva glavna čimbenika: primarno gustoćom populacije, a sekundarno i okolišnim karakteristikama (Znanstveni rad br. 3).

Nasuprot tome, bakteriom hemolimfe više je određen okolišnim uvjetima, budući da se razlikuje među jedinkama uzvodnog i nizvodnog okoliša rijeke te ne pokazuje značajne razlike s obzirom na gustoću populacije (Znanstveni rad br. 3). Mnoga istraživanja (npr. Lokmer i Wegner, 2015; Auguste i sur., 2019; Ooi i sur., 2019) potvrdila su da različite karakteristike okoliša (npr. temperaturni stres, zagađenje) mogu uzrokovati promjene u sastavu mikrobioma hemolimfe. Stoga su pronađene razlike u sastavu bakterioma hemolimfe potencijalno posljedica različitih karakteristika uzvodnog i nizvodnog okoliša rijeke Korane.

3.2.1.2. Imunosni odgovor signalnoga raka

Imunosni odgovor signalnoga raka u rijeci Korani primarno je određen okolišnim uvjetima budući da pokazuje značajne razlike između jedinki uzvodnog i nizvodnog okoliša rijeke, među kojima postoji razlika u temperaturi vode (Znanstveni rad br. 4). Temperatura je jedan od najvažnijih abiotičkih čimbenika koji određuju raspodjelu jedinki u prostoru, budući

da utječe na biokemijske i stanične procese u organizmu te posljedično i na funkcioniranje organizma (Kelley, 2014). Optimalna okolišna temperatura od velike je važnosti za poikilotermne organizme kao što su slatkovodni deseteronožni rakovi: povišena temperatura okoliša u ovim organizmima dovodi do ubrzanja biokemijskih i fizioloških procesa (Woods i sur., 2003). Osim primarno temperaturom, imunosni odgovor signalnoga raka sekundarno je određen i populacijskim karakteristikama, tj. gustoćom populacije (Znanstveni rad br. 4). Brojna istraživanja ističu ulogu gustoće populacije u oblikovanju imunosnog odgovora člankonožaca (npr. Wilson i Reeson, 1998; Kong i sur., 2013; Silva i sur., 2013; Kong i sur., 2020), budući da stopa prijenosa patogena (i potreba za odgovarajućim imunosnim odgovorom) raste s povećanjem gustoće populacije domaćina (Anderson i May, 1981). Rezultati ovog istraživanja slični su rezultatima rada Silva i Elliot (2016), koji također potvrđuju da (primarno) temperatura okoliša i (sekundarno) gustoća populacije utječu na imunosni odgovor člankonošca, ličinke vrste *Anticarsia gemmatalis* Hübner.

Slično imunosnom odgovoru, i bakteriom hemolimfe primarno je određen lokalnim okolišnim uvjetima (opisano u poglavlju 3.2.1.1.). Jedna od glavnih funkcija hemolimfe je aktivno sudjelovanje u imunosnom odgovoru, budući da sadrži brojne antimikrobne peptide i hemocite na čijoj se aktivnosti temelje reakcije imunosnog sustava (opisano u uvodu, poglavlje 1.2.2.). Iako hemolimfa sadrži mnoge bakterije (Znanstveni rad br. 3), one koegzistiraju s antimikrobnim peptidima i hemocitima te ne izazivaju imunosni odgovor sve dok postoji homeostaza u organizmu (Wang i Wang, 2015). Iz ovoga je vidljiva poveznica imunosnog odgovora i mikrobioma hemolimfe, koji pokazuju isti obrazac odgovora na vanjski čimbenik – temperaturu vode.

3.2.2. Je li ulaganje u imunosni odgovor jedinki na invazijskim frontama smanjeno ili povećano?

Jedinke signalnoga raka pokazuju varijacije u imunosnom odgovoru duž cijelog invazivnog areala (Znanstveni rad br. 4). Međutim, nisu zabilježene razlike između invazijskih fronti i središta, što ukazuje na to da imunosni odgovor nije primarno oblikovan procesom širenja populacije, tj. procesom invazije, već kombinacijom specifičnih uvjeta pojedinog staništa (temperaturom vode i gustoćom populacije; opisano u poglavlju 3.2.1.). Stoga, promatranjem cjelokupnog imunosnog odgovora, nije vidljivo je li on na invazijskim frontama povišen ili smanjen u odnosu na invazijsko središte. No, gledajući svaki mjereni standardni imunosni parametar pojedinačno, vidljivo je da neki od njih pokazuju negativnu korelaciju s

gustoćom populacije (enzimska aktivnost PO i snaga reakcije inkapsulacije), dok drugi pokazuju pozitivnu (ukupna koncentracija proPO u hemolimfi). Ovi rezultati ukazuju na to da su pojedini standardni imunosni parametri oblikovani gustoćom populacije. Nadalje, navedene korelacije ukazuju na povećano ulaganje u određene imunosne parametre u populacijama manje gustoće, tj. na invazijskim frontama. Specifično, vidljivo je povećano ulaganje u enzimsku aktivnost PO i snagu reakcije inkapsulacije koji su, među svim mjerenim standardnim imunosnim parametrima, najvažniji segmenti mehanizma obrane od patogena (Znanstveni rad br. 4). S obzirom na to, moguće je pretpostaviti da jedinke na invazijskim frontama svoje energetske resurse ulažu više u imunosni sustav, a manje u procese koji potiču širenje populacije.

Povećano ulaganje u određene imunosne parametre na invazijskim frontama signalnoga raka u Korani potencijalno je rezultat kombinacije nekoliko čimbenika. Jedinke na invazijskim frontama u kompeticiji su s jedinkama uskoškaroga raka (Hudina i sur., 2017; Znanstveni rad br. 1). Osim što već sama kompeticija predstavlja stres za signalnoga raka (Jones i sur., 2011), on se na području rijeke Korane vjerojatno susreće s novim nepoznatim patogenima s kojima je uskoškari rak, kao zavičajna vrsta, potencijalno koevoluirao (Hilker i sur., 2005). Stoga, zbog postojanja kompetitora i potencijalne prilagodbe njegovim patogenima, u jedinkama signalnoga raka javlja se potreba za jačim imunosnim odgovorom.

3.3. Smjernice za daljnja istraživanja

S obzirom na brojne negativne učinke signalnoga raka u ulozi invazivne vrste (opisani u uvodu, poglavlje 1.2.3.1.), od iznimne je važnosti nastaviti pratiti širenje ove vrste u rijeci Korani. Donošenje ažurnih smjernica za sustavno praćenje mikrobnih zajednica, patogena i bolesti signalnoga raka doprinijelo bi učinkovitijem upravljanju ovom invazivnom populacijom te bi omogućilo učinkovitije praćenje potencijalnih prijenosa patogena između populacija zavičajnih i invazivnih stranih vrsta rakova. Trenutni plan upravljanja signalnim rakom (https://esavjetovanja.gov.hr/ECon/MainScreen?entityId=16914) ne uključuje sustavno praćenje mikrobnih zajednica, patogena ili bolesti signalnoga raka te bi ove stavke trebale biti uključene u buduće verzije plana.

Pregled literature o patogenima slatkovodnih deseteronožnih rakova dao je uvid u značajnu neistraženost ove teme. Mikrobi signalnoga raka detektirani u ovom istraživanju uglavnom su nepoznati i slabo zastupljeni u literaturi, što također potvrđuje nedostatnu istraženost patogena i, općenito, mikrobnih zajednica signalnoga raka i slatkovodnih deseteronožnih rakova kao

skupine. Usto, eksperimentalni dizajn i zaključci o patogenima rakova u većini pregledanih znanstvenih radova znatno variraju i vrlo su neusklađeni, pri čemu je najveći problem neprovođenje ispitivanja infektivnosti – zbog čega se, u konačnici, ne može utvrditi, već samo nagađati, patogenost ili nepatogenost mikroba. Stoga je potrebno standardizirati metode određivanja patogenosti tako da se obavezno provodi ispitivanje infektivnosti te uskladiti metode mjerenja virulentnosti patogena. Također, potrebno je uložiti dodatne napore u daljnja istraživanja patogena slatkovodnih deseteronožnih rakova, budući da se rakovi često prenose preko granica u svrhu trgovine kućnim ljubimcima i/ili, kao neželjeni akvarijski ljubimci, puštaju u lokalna vodna tijela (Peay, 2009), čime svojim potencijalnim patogenima ugrožavaju zavičajne vrste. Stoga, patogeni slatkovodnih deseteronožnih rakova predstavljaju veliki biosigurnosni rizik zbog brojnih nepoznanica u ovom području (npr. neistraženost viroma, većinom nepoznati patogeni osim nekoliko najznačajnijih i najčešćih u akvakulturi) i trgovine rakovima kao kućnim ljubimcima (kojom se nenamjerno unose različiti račji patogeni koje, na temelju trenutnoga znanja, nije moguće predvidjeti). Ako se izuzme nekoliko najpoznatijih račjih patogena, ovim istraživanjem utvrđen je vrlo nizak stupanj znanja o navedenoj problematici.

Prethodno predloženo sustavno praćenje mikrobnih zajednica, patogena i bolesti signalnoga raka moglo bi doprinijeti pronalasku i/ili definiranju zdravog mikrobioma (ili njegovih varijacija; opisano u uvodu, poglavlje 1.1.1.2.) jedinki signalnoga raka u rijeci Korani. Definiranje zdravog mikrobioma signalnoga raka omogućilo bi praćenje njegovih promjena s obzirom na biotičke i abiotičke čimbenike/stresore u okolišu tijekom biološke invazije. Nadalje, definiranje zdravog mikrobioma predstavlja polazišnu točku za buduća istraživanja o njegovim devijacijama (tj. patobiomu) i predviđanja pojave bolesti u jedinki. Mikrobiomi zdravih jedinki mogli bi biti uspoređeni s mikrobiomima jedinki sa simptomima bolesti kako bi se razabrali patogeni, tj. uzročnici bolesti, u mikrobiomu. Detektiranje patogena u mikrobiomu omogućilo bi praćenje njihovih negativnih učinaka na razini stanica, tkiva i organa, što bi, u konačnici, doprinijelo procjeni utjecaja patogena na kondiciju i mortalitet jedinki.

Odnosi mjerenih standardnih imunosnih parametara (opisani u uvodu, poglavlje 1.2.2.) teoretski su jasno definirani: ulazak stranog tijela u organizam uzrokuje aktivaciju proenzima proPO u enzim PO, tj. uzrokuje smanjenje koncentracije proPO u hemolimfi i povećanje enzimske aktivnosti PO. Povišena enzimska aktivnost PO vodi do pojačane sinteze melanina i reakcije inkapsulacije. Istovremeno, pada broj hemocita u hemolimfi, budući da se nakupljaju oko stranog tijela i budući da dolazi do njihove degranulacije s ciljem otpuštanja proPO u

hemolimfu. Unatoč ovom mnogo puta opisanom mehanizmu, u ovom istraživanju nisu se svi imunosni parametri ponašali prema očekivanom obrascu, niti su svi bili pod utjecajem istih čimbenika (Znanstveni rad br. 4). Stoga je u budućim istraživanjima potrebno dodatno razjasniti odnose između imunosnih parametara i njihove uzročno-posljedične veze, kao i međusobni utjecaj okolišnih čimbenika, zdravstvenog stanja životinje i imunosnih parametara. Primjerice, sustavno mjerenje okolišnih čimbenika (tj. fizikalno-kemijskih parametara) i prikupljanje aktualnih bioloških podataka o staništu, doprinijelo bi određivanju ekološkog statusa staništa te bi se ove podatke moglo integrirati u istraživanja imunosnog statusa i zdravstvenog stanja organizama. U konačnici, bolje razumijevanje imunosnih parametara, njihovih međusobnih odnosa i čimbenika koji ih oblikuju temelj su za precizniju procjenu troška ulaganja u imunosni odgovor i određivanje njegove uloge u invazivnom uspjehu jedinke.

Zaključno, daljnja istraživanja trebala bi uključiti usporedo uzorkovanje jedinki za analizu mikrobioma, histopatološke analize i ispitivanje imunosnog odgovora te detaljno simultano mjerenje biotičkih (gustoća populacije, gustoća kompetitora, prisutnost predatora…) i abiotičkih čimbenika (temperatura, pH, zasićenost kisikom...). Nadalje, u svrhu ispitivanja hipoteza o mogućim interakcijama invazivne strane vrste s (patogenim) mikrobima pri širenju u novi okoliš (navedene u uvodu, poglavlje 1.1.1.), trebalo bi istražiti mikrobiom zavičajne vrste i invazivne vrste u populacijama koje su u kontaktu te ih usporediti s konspecifičnim populacijama signalnoga ili uskoškaroga raka. Rezultati ovih istraživanja doprinijeli bi utvrđivanju potencijalnih učinaka mikroba pri širenju invazivne strane vrste u novi okoliš i razumijevanju u kolikoj je mjeri mikrobiom specifičan za određenu vrstu, a koliko se mijenja u doticaju s jedinkama druge vrste.

4. ZAKLJUČAK

U ovom istraživanju prvi put su ispitane razlike u mikrobnim zajednicama i imunosnom odgovoru jedinki na invazijskoj fronti i invazijskom središtu u kontekstu ekologije invazivnih stranih vrsta. Dobiveni rezultati predstavljaju temelj za daljnje procjene i identifikaciju mikrobioma zdravih jedinki, koje mogu poslužiti kao pokazatelj fiziološkog statusa i vijabilnosti populacija, kako invazivnih stranih vrsta, tako i ugroženih i zaštićenih populacija zavičajnih vrsta rakova. Nadalje, rezultati ovog istraživanja omogućili su sustavno povezivanje empirijskih rezultata s postojećim hipotezama o promjenama imunosnog odgovora tijekom širenja areala invazivnih stranih vrsta. Konačno, spoznaje dobivene ovim istraživanjem direktno doprinose razumijevanju odrednica invazivnog uspjeha. Osnovni zaključci ovog istraživanja su:

- Potrebna je standardizacija protokola i metoda prilikom ispitivanja infektivnosti mikroba slatkovodnih deseteronožnih rakova, kako bi se na temelju dobivenih ujednačenih rezultata mogli donijeti zaključci o virulentnosti i patogenosti ispitanih mikroba u podložnom domaćinu.
- Mikrobiom i imunosni odgovor signalnoga raka mijenjaju se tijekom širenja invazivnog areala u rijeci Korani.
- Okolišni čimbenici i populacijske karakteristike, tj. gustoća populacije, u različitoj mjeri oblikuju mikrobiom i imunosni odgovor signalnoga raka tijekom širenja invazivnog areala.
- Jedinke signalnoga raka na invazijskim frontama pokazuju povišeno ulaganje u neke standardne imunosne parametre.

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6. PRILOZI

Prilog 1: neobjavljeni podaci o analizama mikobioma

1. Materials and methods

Study area

Fieldwork has been conducted in lower reaches of the Korana River, located in continental Croatia, where signal crayfish is spreading in both upstream and downstream directions (Dragičević et al., 2020). Korana is a 134 km long karstic river belonging to the Sava River basin, with multiple natural and man-made cascades present along the whole length of its watercourse (Hudina et al., 2017). The study area included 33 km of the Korana lower watercourse, which cover the whole length of signal crayfish invasion range in this river. The upstream section of the studied river segment is passing through a sparsely populated rural region, while the downstream section is flowing through the industrial zone of the Karlovac City. Additionally, differences in water temperature have been recorded along this part of the watercourse: water temperature at upstream river segment was 5.6 °C lower in comparison to the downstream segment (Dragičević et al., 2021). Except for differing environmental conditions, the study area also includes sites which differ in crayfish community composition: dense intraspecific populations of signal crayfish (located in the centre of the studied area, U2 and D1; Table 1), and less abundant heterospecific populations of signal crayfish and narrow-clawed crayfish (located at the edges of the studied area; U1 and D2; Table 1) (Dragičević et al., 2020).

Sampling procedure

Crayfish sampling was performed in the early autumn of 2018, during the period of increased crayfish activity of both sexes (i.e. before the mating period; Souty-Grosset et al., 2006). The signal crayfish individuals were captured at four sites along the aforementioned 33 km of the Korana lower watercourse, with two sites previously categorized as upstream (U1 and U2), and the other two sites belonging to downstream river segment (D1 and D2; Table 1; Dragičević et al., 2020). Crayfish individuals were captured using baited LiNi traps (Westman et al., 1978) which were left in the water overnight, and identified to species level by visual inspection upon capture. Captured native narrow-clawed crayfish were returned to the river. A total of 110 signal crayfish individuals of both sexes (Table 1) were placed in individual containers on ice and taken to the laboratory for tissue sampling. In addition to crayfish, environmental samples (water and sediment) were collected at all four sites. Water sampling was performed using 1000 mL sterile bottles (a total of 6 bottles; Table 1), while sediment was taken as composite sample

(4-5 samples at each of four sites which were collected approximately 1-2 m apart, from the surface of the sediment: 0-5 cm; Table 1) using a sterile sampling spoon and immediately transported to the laboratory on ice.

Table 1. Number of collected crayfish individuals, water and sediment samples, and geographic coordinates of sampling sites along the invasion range of the signal crayfish in the Korana River in 2018.

Site	Number of captured crayfish individuals	Number of collected water samples	Number of collected sediment samples	X (WGS84)	Y (WGS84)
upstream 1 (U1)	27	2	5	45.320915	15.518373
upstream 2 (U2)	30	1	4	45.371918	15.521505
downstream 1 (D1)	30	1	4	45.411808	15.609231
downstream 2 (D2)	23	2	5	45.451355	15.567030

In the laboratory, collected water samples were vacuum-filtered through 0.22 μm pore-size membrane (MCE) filters, which were then stored at –20 °C along with the collected sediment samples until DNA extraction. Four types of crayfish samples were collected from each individual crayfish: exoskeletal biofilm, hemolymph, hepatopancreas, and intestine (i.e. midgut and hindgut). Exoskeletal biofilm was sampled by taking cuticle swabs as described by Pavić et al. (2020). In brief, after manual removal of loosely adhered debris (e.g. vegetation, mud or sediment) from the crayfish, the individuals were thoroughly scrubbed with a sterile brush wetted with the 0.1% NaCl, 0.15 M Tween 20 solution. The suspension was centrifuged at 10,000×g for 15 min at 4 °C, supernatant was discarded, and the pellet of epibiotic cells was frozen at −20 °C. Further, using a sterile needle as described by Ooi et al. (2019), we collected 400 μL of hemolymph in 200 μL of anticoagulant solution (0.49 M NaCl, 30 mM trisodium citrate, 10 mM EDTA) from the base of the individual's walking leg, previously rinsed by 70% ethanol. The collected hemolymph was centrifuged at 10,000×g for 10 min at 4 °C, supernatant was discarded, and the pellet was frozen at −20 °C until DNA extraction. After hemolymph sampling, each individual was killed according to available guidelines for humane killing of crayfish (rapid cut of nerve cord from thorax to the end of abdomen; Conte et al., 2021). Each animal was then dissected and the same sampling procedure was used to obtain both

hepatopancreas and intestine: complete organ was removed from the body, placed in a sterile Petri dish and carefully chopped into small pieces using a sterile scalpel, and frozen at −20 °C. Non-disposable dissecting equipment was alcohol flame sterilized between each individual sample. For subsequent analyses, samples from two upstream sites (U1 and U2), as well as samples from two downstream sites (D1 and D2), were pooled together (and will be referred to) as "upstream river segment" and "downstream river segment" groups.

DNA extraction

Genomic DNA was extracted from four types of crayfish samples (exoskeletal biofilm, hemolymph, hepatopancreas and intestine) using the NucleoSpin™ Microbial DNA kit (Macherey-Nagel, Germany) as per manufacturer's protocol, with modifications regarding sample lysis by agitation as described by Pavić et al. (2020). Genomic DNA from sediment and water samples was extracted using DNeasy PowerSoil Pro Kit (Qiagen, Germany). A total of nine replicates of each composite sediment sample were isolated from upstream river segment samples, and nine from downstream river segment samples. DNA quantity was analyzed in all samples using the QuantiFluor™ ONE dsDNA System and the Quantus™ Fluorometer (Promega, USA). Finally, based on satisfactory DNA concentration, we have chosen 192 samples from all six sample groups for amplicon sequencing of variable region ITS2 of the ITS region of fungal ribosomal DNA (Table 2A).

Table 2. Number of sequenced and analyzed samples per site and sample type.

	samples chosen for amplicon sequencing of variable region ITS2 of the ITS region of fungal ribosomal DNA (192 samples)					
A	**water**	**sediment**	**exoskeletal biofilm**	**hemolymph**	**hepatopancreas**	**intestine**
upstream 1	2	6	8	12	10	13
upstream 2	1	3	8	11	10	12
downstream 1	1	3	12	11	12	13
downstream 2	2	6	11	7	6	11
TOTAL	6	18	39	41	38	49

Table 2. (continued)

B	samples included in alpha and beta diversity analyses (56 samples)					
	water	**sediment**	**exoskeletal biofilm**	**hemolymph**	**hepatopancreas**	**intestine**
upstream 1	2	5	3	excluded from these analyses	excluded from these analyses	5
upstream 2	1	3	2			0
downstream 1	1	2	11			5
downstream 2	2	4	7			3
TOTAL	6	14	23	0	0	13

Library preparation, sequencing and bioinformatics analysis

Amplification and sequencing of the variable ITS2 region of the ITS region was performed by Microsynth, Switzerland. Illumina library was prepared using ITS Nextera two-step PCR using forward ITS3 (5′- GCATCGATGAAGAACGCAGC -3′) and reverse ITS4 (5′- TCCTCCGCTTATTGATATGC -3′) primers, and sequenced on an Illumina MiSeq using the MiSeq Reagent Kit v2 (2x250 bp paired-end). The analyses of Illumina raw paired-end sequences were conducted using the 'Quantitative Insights Into Microbial Ecology 2' (QIIME2) software (Bolyen et al., 2019), release 2021.2. After importing raw demultiplexed paired-end fastq files into QIIME2 using a manifest file, they were quality filtered, trimmed, dereplicated, denoised, merged and assessed for chimaeras to produce amplicon sequence variants (ASVs) using the DADA2 plugin (Callahan et al., 2016). The DADA2 generated feature table was filtered to remove singletons. Upon obtaining the final feature table, hemolymph and hepatopancreas samples were excluded from further analyses due to small number of features.

Taxonomy was assigned to ASVs using a pre-trained Naïve Bayes classifier. Classifier was trained on the UNITE version 8.3 database of reference sequences clustered at 99% sequence similarity (Abarenkov et al., 2021) using the QIIME2 feature-classifier plugin (Bokulich et al., 2018). A phylogenetic tree was generated using fasttree2 based on mafft alignment of ASVs as implemented in the q2-phylogeny plugin. The mycobiome diversity and richness of all samples were estimated using alpha (observed features) and beta (Jaccard index and Bray-Curtis dissimilarity) diversity metrics using the diversity plugin within QIIME2. For these analyses,

the samples were subsampled to 6500 reads per sample. This threshold was chosen to prevent excessive loss of samples (leaving a total of 56 samples for the diversity analyses, Table 2B), even though the generated rarefaction curve was not saturated (Figure 1). Alpha and beta diversity metrics were calculated for: 1) four remaining groups of samples (water, sediment, exoskeletal swab and intestine) with the samples of the same type pooled together across sites (i.e. river segments) in order to analyze the mycobiome diversity of crayfish samples and environment, and 2) each group of samples separately between upstream and downstream river segments in order to analyze differences in composition of mycobiome along the invasion range of signal crayfish. Since no significant differences between sexes were recorded for any of the crayfish sample groups (exoskeletal biofilm, intestine) in both alpha and beta diversity analyses, sexes were pooled together. Differences between all four sample groups and between upstream and downstream river segments were tested with Benjamini–Hochberg corrected Kruskal–Wallis and permutational multivariate analysis of variance (PERMANOVA) tests (Anderson, 2001) for alpha and beta diversity, respectively. Visualization of beta diversity metrics was made by generating principal coordinates analysis (PCoA) plot using Emperor (Vázquez-Baeza et al., 2013). Additionally, analysis of compositions of microbiomes (ANCOM) tests (Mandal et al., 2015) were used to identify features that are differentially abundant between upstream and downstream river segments using the composition plugin within QIIME2.

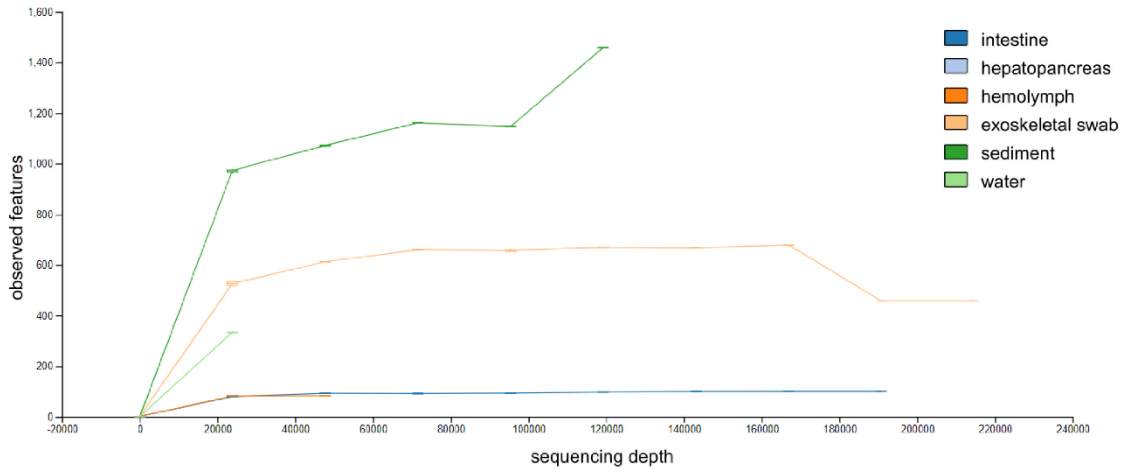

Figure 1. Alpha rarefaction plot of observed features against sampling depth for each sample group.

2. Results

After processing the reads with DADA2 plugin (Callahan et al., 2016), and filtering of the resulting feature table (i.e. removing singletons, hemolymph and hepatopanceas samples), 3,658,377 merged reads from 113 samples were obtained, and a total of 9,382 ASVs were identified.

Diversity and composition of environmental and crayfish mycobiomes

Alpha and beta diversity

Overall, alpha diversity, presented as the number of observed features, differed significantly (Kruskal-Wallis test: P = 7.09E–07, H = 31.37) between all samples. However, pairwise comparisons showed that crayfish intestine samples have significantly lower number of observed features (i.e. lower richness) compared to other three sample groups (Kruskal-Wallis test: P = 5.00E–06 for exoskeleton, P = 3.00E–05 for sediment, and P = 1.24E–03 for water). No significant differences in the number of observed features were recorded between exoskeleton, sediment and water (Figure 2).

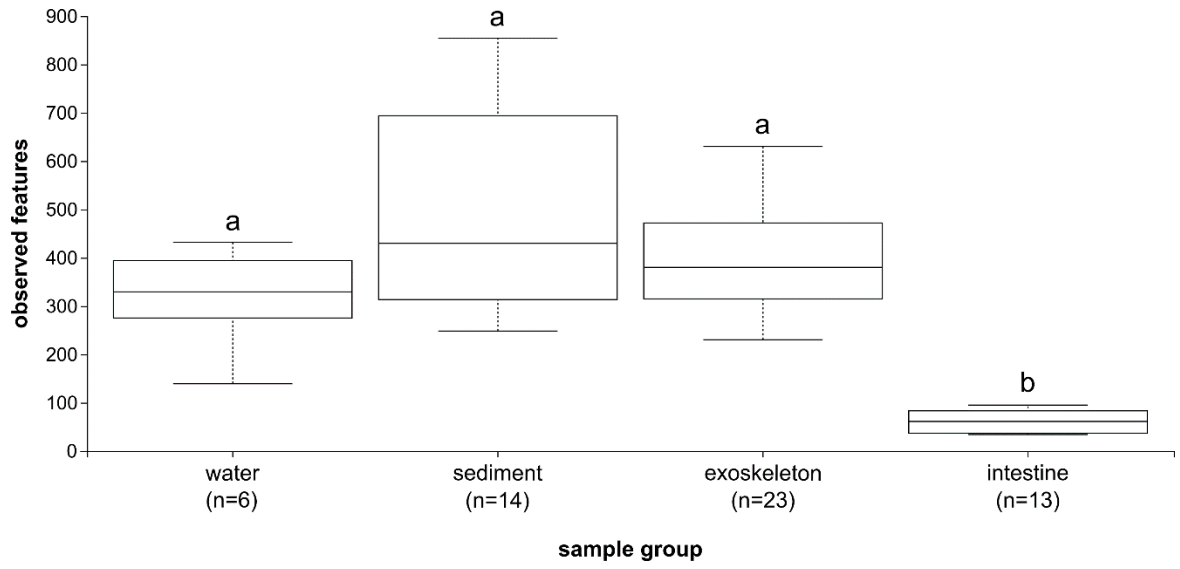

Figure 2. Alpha diversity analysis showing number of observed features in mycobiomes of different sample groups. Significant differences are marked with different letters.

Both beta diversity analyses (i.e. Jaccard index and Bray-Curtis dissimilarity) showed overall significant differences (PERMANOVA test: P = 0.001, pseudo-F = 4.0 and P = 0.001, pseudo-

F = 7.0, respectively) between mycobiomes of all four sample groups. Further, beta diversity pairwise tests exhibited significant differences between all pairs of sample groups (P = 0.001). PCoAs based on Jaccard and Bray-Curtis distance matrices show clear separation of all four sample groups (Figure 3A&B).

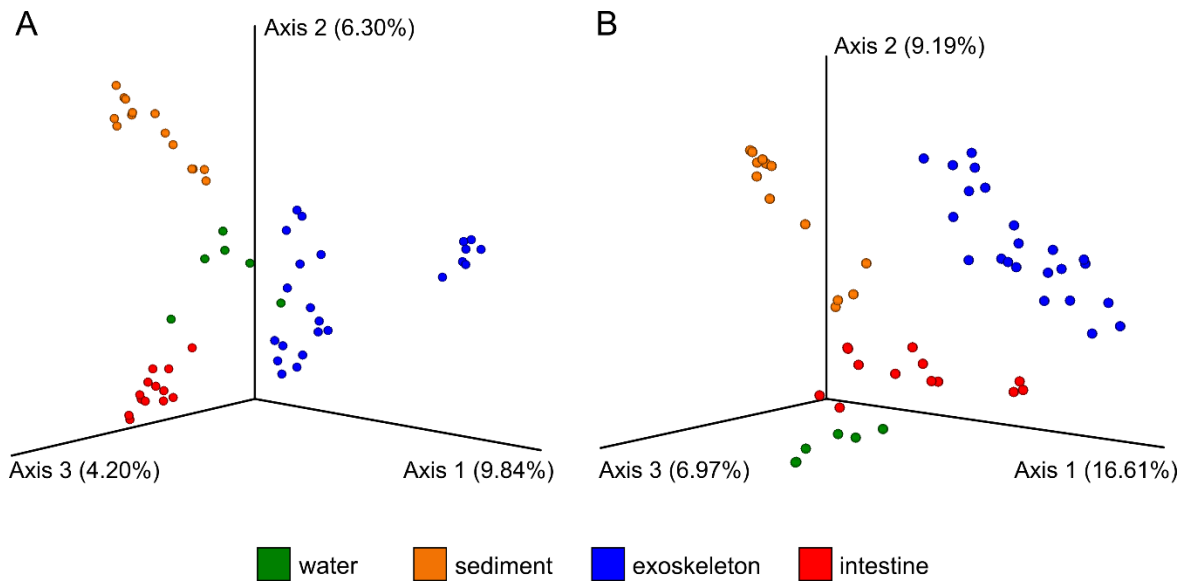

Figure 3. Beta diversity analyses of mycobiomes between different sample groups. PcoAs are based on Jaccard (A) and Bray-Curtis distance matrices (B).

Mycobiome composition

At examined taxonomic level of genus, a total of 860 taxa were detected in all four sample groups. Genus *Ciliophora* was the most abundant genus in environmental samples (13.92% of water and 15.06% of sediment mycobiome) and crayfish exoskeleton (33.98%), while the intestinal samples were dominated by genus *Hanseniaspora* (24.48%). Additionally, genus *Hanseniaspora* constituted only a small percentage (<0.02%) of mycobiomes of other three sample gropus. Further, genus *Pyrenochaetopsis* represented 8.19% of water and 12.67% of exoskeletal mycobiome, but <3% of intestinal and sediment mycobiomes. Undetermined genus from family Didymellaceae showed notable abundance of 12.12% of intestinal mycobiome, but composed <4.2% of other sample groups' mycobiomes. The category „other“, which included a total of 834 taxa with <1% abundance, constituted between 11.84% and 17.19% of all sample groups' mycobiomes. However, a relatively high percentage of fungal taxa in each sample

group, especially in environmental samples, could not be identified past the kingdom level (water 37.61%, sediment 61.89%, exoskeleton 6.58% and intestine 16.44%, Figure 4).

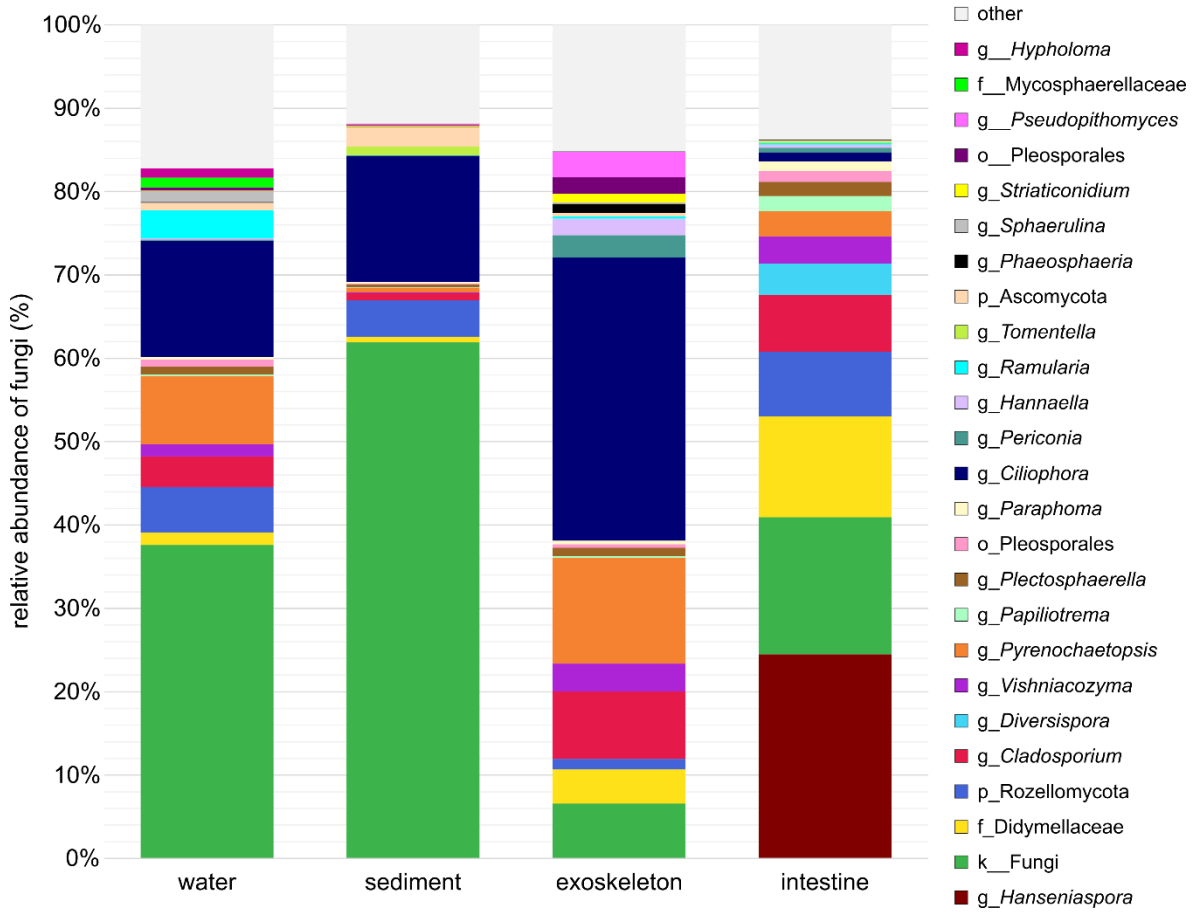

Figure 4. Relative abundance (%) of the overall most prevalent genera of all four sample groups. Features that could not be identified to genus (g) level are marked with the letter corresponding to the last known taxonomic level (k = kingdom, p = phylum, o = order, f = family). Fungal taxa with an overall abundance of >1% are shown, while the remaining taxa were pooled and marked as "other."

Comparison of environmental and crayfish mycobiomes between upstream and downstream river segments

Alpha and beta diversity analyses were used to examine potential differences between mycobiomes of upstream and downstream river segments in all four sample groups. Significant differences in both alpha and beta diversity were found between sediment and exoskeletal samples. Sediment showed significantly higher number of observed features at downstream

river segment (Kruskal-Wallis test: P = 4.51E–03, H = 8.07; Figure 5A), while the opposite pattern was observed for exoskeleton, which showed significantly higher number of observed features at upstream river segment (Kruskal-Wallis test: P = 2.30E–02, H = 5.17; Figure 5B).

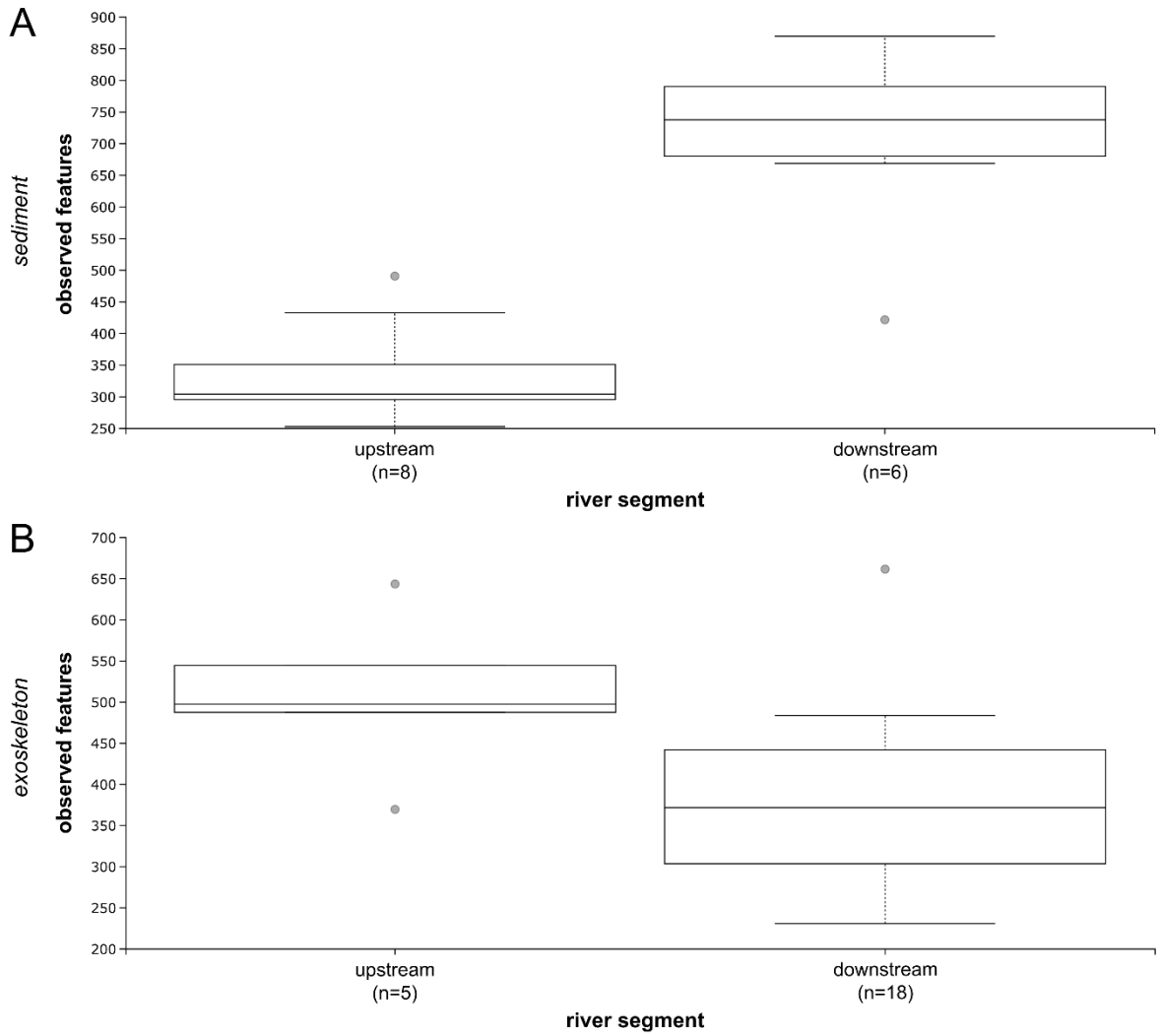

Figure 5. Alpha diversity analysis showing number of observed features in mycobiomes of upstream and downstream river segments. Significant differences between two river segments were recorded for sediment (A) and exoskeletal mycobiomes (B).

Furthermore, significant differences between upstream and downstream mycobiomes for sediment (Figure 6A&C) and exoskeleton (Figure 6B&D) were recorded in both beta diversity analyses used: Jaccard index (PERMANOVA test: P = 0.004, pseudo-F = 2.1 for sediment and P = 0.001, pseudo-F = 2.2 for exoskeleton; Figure 6A&B) and Bray-Curtis dissimilarity

(PERMANOVA test: P = 0.001, pseudo-F = 4.1 for sediment and P = 0.006, pseudo-F = 2.6 for exoskeleton; Figure 6C&D). Finally, neither alpha nor beta diversity analyses showed any significant differences between upstream and downstream river segments in case of water and intestinal mycobiomes.

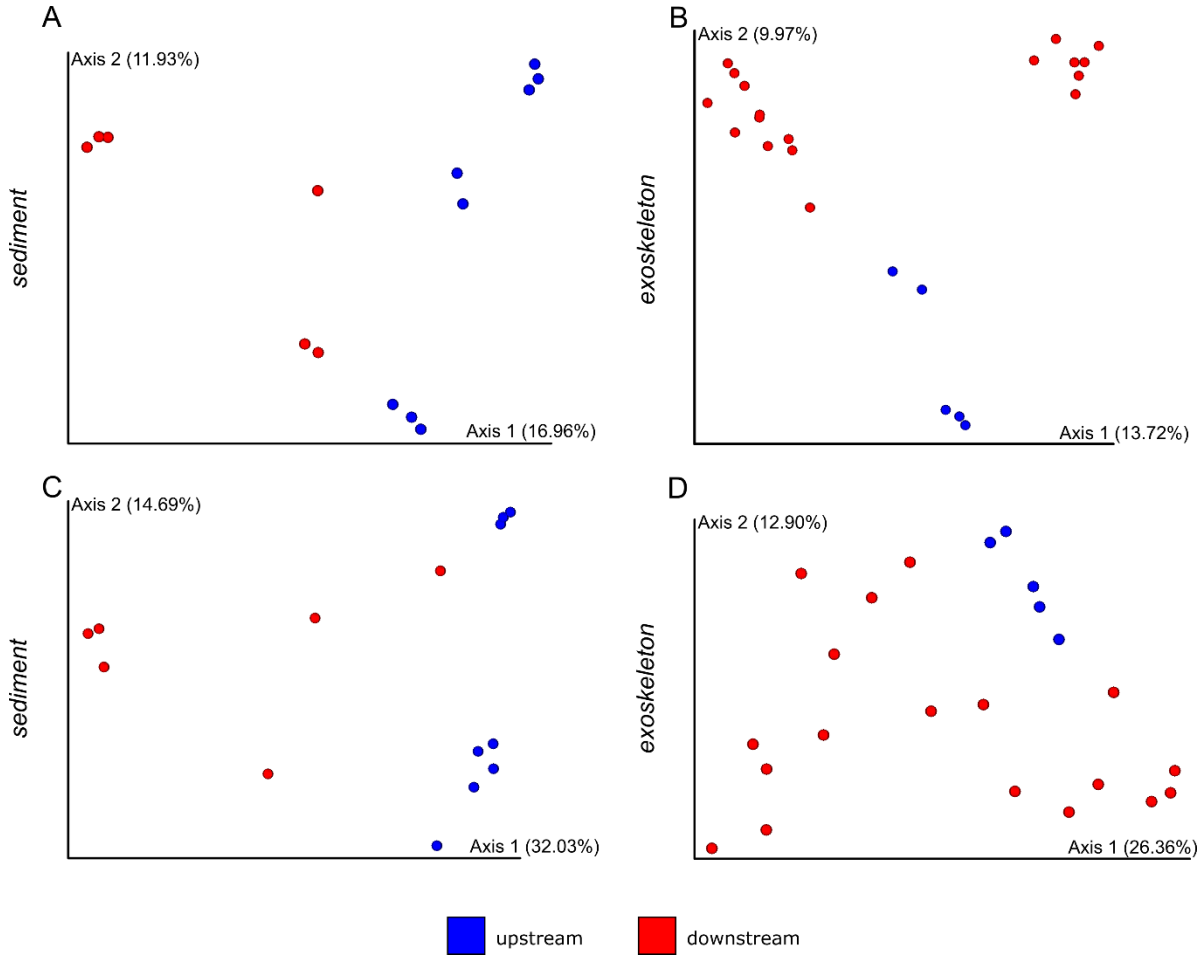

Figure 6. Beta diversity analyses of sediment (A,C) and exoskeletal (B,D) mycobiomes between upstream and downstream river segments. PcoAs are based on Jaccard (A,B) and Bray-Curtis (C,D) distance matrices.

Significant differences in differential abundance of features between upstream and downstream river segments were recorded for sediment and exoskeleton, but not for water and intestinal samples (Table 3). Genus *Gorgonomyces* showed higher abundance in sediment of upstream river segment. In the case of exoskeleton, genera *Ascochyta* and *Leptosphaeria* exhibited

significantly higher abundances in individuals at downstream river segments in comparison to upstream.

Table 3. List of features showing significantly different abundances between upstream and downstream river segments. The analysis (ANCOM) was performed at genus level. Locations with highest feature abundances were determined based on the median number of features.

	Genus	W-statistic value	clr mean difference	highest feature abundance
water	no significant differences in abundance			
sediment	k__Fungi;p__Chytridiomycota;c__Rhizophydiomycetes;o__Rhizophydiales;f__Gorgonomycetaceae;g__Gorgonomyces	210	3.086	upstream
exoskeleton	k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Ascochyta	546	3.045	downstream
	k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Leptosphaeriaceae;g__Leptosphaeria	541	2.378	downstream
intestine	no significant differences in abundance			

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7. ŽIVOTOPIS

Paula Dragičević rođena je 13.7.1993. u Zagrebu, gdje je završila Osnovnu školu Dobriše Cesarića i II. gimnaziju. Nakon završetka gimnazije 2012., upisala je preddiplomski sveučilišni studij Biologije na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Pri istoj obrazovnoj ustanovi, 2015. godine upisala je diplomski sveučilišni studij Eksperimentalne biologije, modul Zoologija, kojeg je završila 2018. godine obranom diplomskog rada na temu „Razine molekularnih biomarkera i akumulacija metala u probavnoj žlijezdi školjkaša *Anodonta exulcerata* Porro, 1838 iz Visovačkog jezera“, čime je stekla titulu magistre eksperimentalne biologije (magna cum laude). Iste godine zaposlena je na radnom mjestu asistenta na projektu Hrvatske zaklade za znanost pod naslovom “Promjene sastava patogena i imunološkog odgovora tijekom širenja areala uspješnih invazivnih vrsta slatkovodnih rakova (STRIVE)“, voditeljice doc. dr. sc. Sandre Hudine pri Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu. U jesen 2018. godine na istome fakultetu upisuje doktorski studij Biologije (s punim radnim vremenom). Za vrijeme doktorskog studija, nekoliko je puta bila na znanstvenom usavršavanju i radionicama unutar i izvan Hrvatske: učenje metode inducirane reakcije inkapsulacije (Finska, jesen 2019.), tečaj za osposobljavanje osoba koje rade s pokusnim životinjama i životinjama za proizvodnju bioloških pripravaka (Hrvatska, 2019.), radionica metagenomike (Švicarska, 2019.) i stručna praksa s ciljem provođenja znanstvenog istraživanja (Češka, 2021.). Dobitnica je stipendije za mlade znanstvenike FEMS (Congress Attendance Grant). Prva je autorica na četiri znanstvena rada, a koautorica na dva znanstvena rada, od kojih su svi objavljeni u časopisima indeksiranima u WoS bazi. Aktivno je izlagala na jednom simpoziju izvan Hrvatske i na pet simpozija u Hrvatskoj. Tijekom akademskih godina 2018./2019. i 2019./2020. vodila je praktikumsku nastavu iz kolegija *Opća Zoologija* i *Ekotoksikologija* za studente preddiplomskih i diplomskih studija na PMF-u te je 2018./2019. sudjelovala u održavanju terenske nastave za studente iz University Queen Mary of London. Tijekom svog preddiplomskog, diplomskog i doktorskog studija sudjelovala je u popularizaciji znanosti kroz „Noć Biologije“ u sklopu manifestacije „Dan i noć na PMF-u“.

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- **Aktivno sudjelovanje**

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