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Original Research Article

Vpliv simptomatske bakterijske okužbe na izražanje genov, povezanih z imunostjo v hemocitih kopenskega raka *Porcellio scaber*

Andraž Dolar [1,*], Jernej Ogorevc [2], Anita Jemec Kokalj [1]

Izvleček

Kopenski enakonožci vrste *Porcellio scaber* so v naravnem okolju izpostavljeni številnim patogenom in parazitom, ki lahko pri gostitelju povzročijo poškodbe tkiv ter vplivajo na imunokompetenco in fitnes organizma. Bakterijska okužba povzroči aktivacijo mehanizmov prirojene imunosti, kot so fagocitoza, tvorba reaktivnih kisikovih in dušikovih zvrsti, aktivnost antioksidativnih encimov, nodulacija ter proces melanizacije. Molekularni vzorci patogenov oziroma mikrobov ter s patogenezo povezane poškodbe pri gostitelju sprožijo prepisovanje genov v celicah hemolimfe, tj. hemocitih, ki opravljajo pomembno funkcijo mediatorjev imunskega odgovora. V aktualni raziskavi smo preučevali spremembe v izražanju genov ob simptomatski bakterijski okužbi z *Rhabochlamydia porcellionis* ter jih primerjali z asimptomatskimi oziroma zdravimi *P. scaber*. Iz hemolimfe (hemocitov) asimptomatskih in simptomatskih živali smo izolirali celokupno RNA, jo prepisali v cDNA ter z metodo RT-qPCR določili relativno izražanje izbranih genov, povezanih z imunostjo (*Toll4, Dscam, MyD88, Cat, MnSod, CypG, A2m, Atg5* in *Nos*). Ugotovili smo značilne spremembe v izražanju izbranih genov, kar kaže na njihovo vlogo v imunskem odgovoru *P. scaber* v primeru bakterijske okužbe, dodatno pa smo z biokemijskimi metodami dokazali povečano aktivnost encima alfa-2-makroglobulin ter mejno značilno povišanje encima katalaza. Na podlagi rezultatov lahko zaključimo, da preučevani geni predstavljajo molekularne označevalce za imunski odziv, ki jih je moč uporabiti v različnih okoljskih raziskavah.

Ključne besede

bakterijska okužba, imunski odziv, izražanje genov, kopenski enakonožec *Porcellio scaber*, *Rhabdochlamydia porcellionis*

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Effect of symptomatic bacterial infection on the expression of immune-related genes in haemocytes of the terrestrial crustacean *Porcellio scaber*

Abstract

Terrestrial isopods *Porcellio scaber* are exposed to many pathogens and parasites in their natural environment, which can cause tissue damage in the host and affect the immunocompetence and fitness of the organism. Bacterial infection leads to activation of innate immunity mechanisms, such as phagocytosis, formation of reactive oxygen and nitrogen species, activity of antioxidant enzymes, nodule formation, and the process of melanization. Molecular patterns of pathogens or microbes and pathogenesis-induced injury in the host trigger the transcription of genes in haemolymph cells, i.e., haemocytes, which have an important function as mediators of the immune response. In the current study, we examined changes in gene expression during symptomatic bacterial infection with *Rhabochlamydia porcellionis* and compared them with asymptomatic or healthy *P. scaber*. We isolated total RNA from the haemolymph (haemocytes) of asymptomatic and symptomatic animals, transcribed it into cDNA, and determined the relative expression of selected immune-related genes (*Toll4, Dscam, MyD88, Ppae2a, Cat, MnSod, CypG, A2m, Atg5,* and *Nos*). We found characteristic changes in the expression of selected genes confirming their role in the immune response of *P. scaber* in case of bacterial infection, and, in addition, biochemical methods showed increased activity of the enzyme alpha-2-macroglobulin and a borderline characteristic increase in the enzyme catalase. Based on the results, we can conclude that the studied genes represent molecular markers of immune response that can be used in various environmental studies.

Keywords

bacterial infection, gene expression, immune response, terrestrial isopod *Porcellio scaber*, *Rhabdochlamydia porcellionis*

Uvod

Navadni prašiček *Porcellio scaber* (Latreille, 1804) je predstavnik enakonožnih rakov, ki poseljujejo naravne habitate centralne in zahodne Evrope, hkrati pa so izrazito sinantropna vrsta, kar pomeni, da so tesno povezani s človeškimi bivališči in njegovo dejavnostjo. Znotraj skupine rakov so enakonožci najuspešnejši kolonizatorji kopenskega okolja, ki so tekom prilagajanja na kopensko življenje razvili številne vedenjske, fiziološke in strukturne lastnosti (Hornung, 2011). V ekosistemu opravljajo pomembno vlogo razkrojevalcev odmrlega organskega materiala, s čimer prispevajo h kroženju snovi v naravi in so tako nepogrešljiv člen talnega ekosistema (Hornung in sod., 1998; van Gestel in sod., 2018). V okolju so kopenski enakonožci, podobno kot tudi ostali organizmi, podvrženi različnim nevarnostim, kot so patogeni in paraziti. Znano je, da virusne in bakterijske okužbe pogosto prizadenejo naravne populacije višjih rakov (Wang, 2011). V naravni populaciji kopenskih rakov *P. scaber* pogosto zaznamo Iridovirusno IIV-31 okužbo, ki je izražena pri 15-20 % osebkov naravne populacije (Cole in Morris, 1980), medtem ko je prevalenca okužbe z bakterijo *Rhabdochlamydia porcellionis* še višja, in sicer dosega 27 % (Kostanjšek in Pirc Marolt, 2015). Bakterijo *R. porcellionis* (družina Rabdoklamidij) uvrščamo v skupino patogenih, obligatno znotrajceličnih bakterij, imenovanih klamidije, s širokim naborom gostiteljev, tako med vretenčarskimi kot tudi nevretenčarskimi organizmi (Kostanjšek in sod., 2004; Halter in sod., 2022). Pri *P. scaber* je okužba z *R. porcellionis* primarno omejena na celice prebavne žleze hepatopankreas, na površini katere v simptomatski fazi okužbe opazimo bele lise, od tod pa se okužba lahko razširi tudi na druga tkiva, med drugim tudi v hematopoetsko tkivo in pa celice hemolimfe, tj. hemocite (Kostanjšek in Pirc Marolt, 2015). V hemolimfi *P. scaber* s simptomatsko *R. porcellionis* okužbo pride do oblikovanja izrazitega imunskega odgovora, kar se kaže v drastični spremembi vrednosti imunskih parametrov hemolimfe, v primerjavi z asimptomatskimi živalmi (Kostanjšek in Pirc Marolt, 2015; Dolar in sod., 2020). Gostiteljski organizmi se

na vdor patogenov, parazitov in na poškodbo odzovejo z aktivacijo nabora mehanizmov prirojene imunosti, katerih glavni namen je ponovna vzpostavitev stabilnega notranjega ravnovesja (tj. homeostaza) ter preprečitev nadaljnjih poškodb oziroma smrti organizma (Wang in Wang, 2013; Mengal in sod., 2023). V tem pogledu simptomatska bakterijska okužba, ki je prisotna v naravni populaciji *P. scaber* predstavlja dostopen in enostaven model za študije posameznih komponent in mehanizmov prirojene imunosti v luči odkrivanja novih bioloških označevalcev, ki jih je moč prenesti v okoljske raziskave z namenom ocene stanja organizma po izpostavitvi različnim okoljskim onesnažilom, kot so recimo kemikalije in umetnimi delci, npr. nano- in mikroplastika (Dolar in sod., 2021, 2022b,c; Jemec Kokalj in sod., 2021, 2022).

Prirojen imunski sistem kopenskega enakonožca *P. scaber* je odgovoren za prepoznavanje in odzivanje na raznovrstne zunanje in notranje »izzivalce« (npr. patogene, parazite, poškodbe, okoljske spremembe), ki lahko resno ogrozijo zdravje in v skrajnem primeru povzročijo tudi smrt organizma (Dolar in sod., 2020, 2022a,b,c). Imunski odgovor gostitelja na mikrobno okužbo temelji na evolucijsko ohranjenih efektorskih mehanizmih celične in humoralne komponente prirojene imunosti, kot so fagocitoza, nodulacija in enkapsulacija, proces melanizacije in produkcija ter sproščanje drugih humoralnih molekul, npr. reaktivnih kisikovih (ROS) in dušikovih spojin (RNS) ter antimikrobnih peptidov (Jiravanichpaisal in sod., 2006; Söderhäll, 2016). V hemolimfi *P. scaber* so prisotni trije glavni tipi celic hemolimfe oziroma hemociti, tj. semigranularni, granularni in hialini, ki opravljajo specifične naloge tekom imunskega odgovora, pri čemer so hialinociti v splošnem odgovorni za fagocitozo, delno tudi semigranulociti, medtem ko pa so semigranulociti in večinsko granulociti odgovorni za produkcijo in sekrecijo različnih humoralnih molekul v hemolimfo in oblikovanje humoralnega imunskega odgovora (Tassanakajon in sod., 2013, 2018). Bakterijska okužba pri rakih izzove značilne spremembe v celokupnem (angl. total haemocyte count) kot tudi diferencialnem številu (angl. differential haemocyte count) hemocitov (Dolar in sod., 2020). Hkrati lahko bakterijski toksini, kot je endotoksin lipopolisaharid, poškodujejo hemocite in povzročijo njihov propad, kar se odrazi v zmanjšani viabilnosti hemocitov (Dolar in sod., 2022a). Slednji pojav je povezan tudi s povečano fagocitotsko aktivnostjo hemocitov (hialinih in semigranularnih) in tvorbo ROS ter RNS (npr. dušikov oksid; NO) z namenom razgradnje fagocitiranih tujkov (Raman in sod., 2008; Sánchez-Salgado in sod., 2019). Povečana proizvodnja reaktivnih spojin v primeru mikrobne okužbe v hemolimfi rakov posledično izzove tudi povečano aktivnost encimov superoksid dismutaze in katalaze, ki sta odgovorna za odstranjevanje presežka reaktivnih spojin (Gopalakrishnan in sod., 2011; Liu in sod., 2013). Histopatološke poškodbe, ki jih povzročajo patogene bakterije, lahko prizadenejo tudi ostala tkiva in organe gostitelja, med drugim tudi eksoskelet, kar še dodatno poveča tveganje za vdor novih patogenov v organizem (Esteve in Herrera, 2000; Chevalier in sod., 2011; Wang, 2011; Kostanjšek in Pirc Marolt, 2015). Poškodba tkiva sproži aktivacijo sistema profenoloksidaze (proPO), kaskado dogodkov in komponent, med katerimi ima terminalno vlogo encim fenoloksidaza (PO), ki katalizira proces sinteze rdeče-rjavega pigmenta melanina, ki skupaj s hemociti fizično omeji tujke v telesu gostitelja (t. i., nodulacija ali enkapsulacija), medtem ko stranski produkti melanizacije (kinoni, ROS, RNS) delujejo toksično in povzročijo lizo mikrobnih celic (Amparyup in sod., 2013). Pomembna komponenta proPO sistema je tudi serinski proteinazni inhibitor alfa-2 makroglobulin, ki se aktivira ob prisotnosti mikroorganizmov ter regulira fenoloksidazno aktivnost, poleg tega pa je vpleten tudi v proces strjevanja hemolimfe ter fagocitoze (Ponprateep in sod., 2017).

Vdor patogena v telo oziroma poškodba gostitelja v prvi vrsti povzroči aktivacijo receptorskih molekul, ki se nahajajo v plazmi hemolimfe oziroma na površini hemocitov, sledi signalna transdukcija, tj. kaskada dogodkov, ki privede do sprememb v profilu izražanja genov, ki so posredno ali neposredno povezani z imunostjo in drugimi procesi, odgovornimi za uravnavanje homeostaze (Sánchez-Salgado in sod., 2021; Liu in sod., 2022). V fizioloških in funkcionalnih raziskavah genov in molekul, ki so odgovorni za imunske mehanizme v primeru mikrobne okužbe gostitelja se uporablja napredne omske metode, te metode pa v okoljskih raziskavah odpirajo povsem novo ero v raziskovanju vplivov različnih onesnažil in okoljskih sprememb na poskusne organizme (Lou in sod., 2022; Sun in sod., 2022; Mengal in sod., 2023).

Namen študije je bil preučiti izražanje izbranih genov, povezanih z imunostjo, v hemocitih *P. scaber* s simptomatsko okužbo z *Rhabdochlamydia porcellionis*. Izražanje genov smo primerjali z asimptomatskimi osebki *P. scaber*. Poleg tega nas je zanimala tudi razlika v aktivnosti encimov alfa-2-makroglobulin ter katalaza v primeru simptomatskih in asimptomatskih živali.

Material in metode

Poskusni organizem

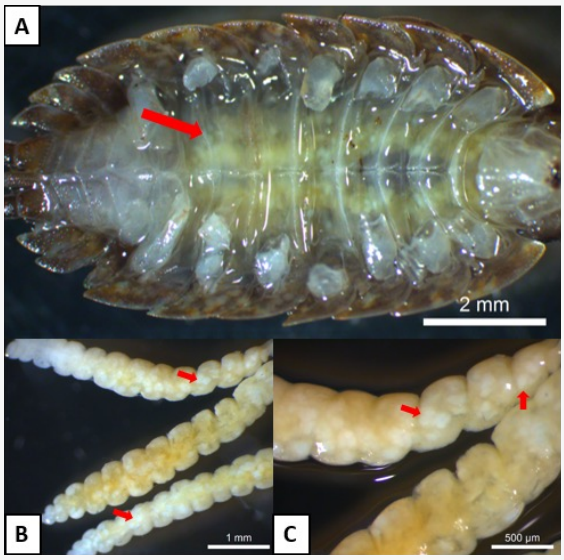
Poskusni organizmi *P. scaber* so bili izbrani iz laboratorijske kulture, ki smo jo gojili v steklenih terarijih pri konstantnih pogojih, tj. temperaturi 20 ± 2 °C, visoki vlažnosti in dnevno nočnem režimu (16/8 ur : svetloba/tema). Stekleni terarij je bil napolnjen z ne-kontaminirano zemljo in debelo plastjo posušenih listov navadne leske (*Corylus avellana*), ki so bili predhodno sterilizirani. Za analizo izražanja genov so bili odbrani poskusni organizmi z izraženimi simptomatskimi belimi lisami na prebavnih žlezah, ki jih je mogoče opazovati neposredno skozi ventralno stran telesa, kot je opisano v Kostanjšek in Pirc Marolt (2015) ter Dolar in sod. (2020). Odbrane živali so imele prisotne značilne simptome bakterijske okužbe z *Rhabdochlamydia porcellionis* (Sl. 1). Živali brez izraženih simptomov okužbe z *R. porcellionis* (asimptomatske živali) smo uporabili kot kontrolo, kot je opisano v Dolar in sod. (2020).

Izolacija hemolimfe

Hemolimfo smo izolirali iz posameznih *P. scaber* v skladu s protokolom opisanim v Dolar in sod. (2020). Na kratko, s sterilno siringo smo prebodli integument med 5. in 6. dorzalnim segmentom *P. scaber* in z rahlim stiskanjem živali pridobili kapljico hemolimfe, ki smo jo posesali s stekleno mikrokapilarno pipeto (Brand). Hemolimfo za meritev aktivnosti encimov katalaze in alfa-2-makroglobulina smo pridobili iz posamezne živali in jo nemudoma razredčili v fosfatnem pufru Dulbecco (DPBS; pH 7,1-7,5), medtem ko smo vzorec hemolimfe za izolacijo RNA pridobili z združevanjem hemolimfe iz 30 živali na skupino, tj. asimptomatskih in simptomatskih *P. scaber*. Vzorce hemolimfe smo do analize hranili na ledu.

Aktivnost encima katalaza

Aktivnost encima katalaza (CAT) smo v hemolimfi *P. scaber* določili fotometrično z meritvijo razgradnje vodikovega peroksida (H_2O_2). Izolirano hemolimfo (5 µL) smo nemudoma razredčili v 65 µL 50 mM kalij-fosfatnega pufra (KP, pH= 7) z dodano 5 mM EDTA. Za meritev smo uporabili UV mikrotiterske plošče s 96 vdolbinicami. V vdolbinico na plošči smo odpipetirali 20 µL redčene hemolimfe, 30 µL KP pufra s 5 mM EDTA in nemudoma pred meritvijo dodali še 100 µL 15,18 mM H_2O_2, pripravljenega v 50 mM KP pufru (pH = 7) z dodano 5 mM EDTA. Reakcijski volumen smo pred meritvijo 5-krat premešali s pipeto. Absorbanco smo merili pri 240 nm in 25 °C, 3 minute s 30-sekundnim intervalom med zaporednimi meritvami, na mikročitalcu Cytation 3 imaging reader (Biotek, ZDA). Kot negativno kontrolo smo uporabili 50 mM KP pufer (pH= 7) s 5 mM EDTA. Aktivnost CAT smo izračunali kot spremembo absorbance, merjene pri 240 nm na minuto na mg proteinov (ΔA_{240nm} min^{-1} mg $proteinov^{-1}$).



Slika 1. (A) Simptomatska bakterijska okužba z *R. porcellionis* v prebavni žlezi *P. scaber*, razvidna z ventralne strani skozi sternite organizma. (B, C) Simptomi okužbe, tj. značilne drobne bele pike (rdeča puščica) v celicah prebavne žleze hepatopankreas.

Figure 1. (A) Symptomatic bacterial *R. porcellionis* infection in the digestive gland of *P. scaber* seen from the ventral side through the sternite of the organism. (B, C) Symptoms of infection, i.e., characteristic small white dots (red arrow) in the cells of the digestive gland hepatopancreas.

Aktivnost encima alfa-2-makroglobulin

Aktivnost encima alfa-2-makroglobulin (A2M) smo v vzorcih hemolimfe *P. scaber* določili fotometrično z meritvijo absorbance pri 405 nm. Izolirano hemolimfo (5 µL) smo redčili v razmerju 1 : 15 (v/v) s pufrom DPBS (pH 7,1–7,5), dobro premešali in do uporabe shranili na ledu. K 50 µL razredčenega vzorca hemolimfe smo dodali 50 µL tripsina (1 mg/mL DPBS, Sigma) in inkubiral 10 min pri 37 °C na toplotnem mešalu Thermomixer compact (Eppendorf). K reakcijski mešanici smo dodali 20 µL sojinega tripsinskega inhibitorja (2 mg/mL DPBS, Sigma) in znova inkubirali 15 min pri 37 °C. Nato smo dodali še 250 µL N-benzoil-DL-arginin-pnitroanilida (BAPNA; 0,5 mg/L DPBS, Sigma), ki smo ga pripravili s 100-kratnim redčenjem založne raztopine BAPNA (50 mg/mL dimetil sulfokida; DMSO) v pufru DPBS. Sto µL reakcijske mešanice smo v treh ponovitvah prenesli na mikrotitersko ploščo s 96 vdolbinicami in 30 min merili absorbanco z mikročitalcem Cytation 3 imaging reader (Biotek, ZDA) pri 405 nm in 37 °C. Aktivnost A2M v vzorcu hemolimfe smo merili posredno preko detekcije aktivnosti tripsina. Aktivnost A2M smo izrazili kot spremembo absorbance pri 405 nm na minuto na mg proteinov (ΔA_{405nm} min^{-1} mg $proteinov^{-1}$).

Koncentracija proteinov v hemolimfi

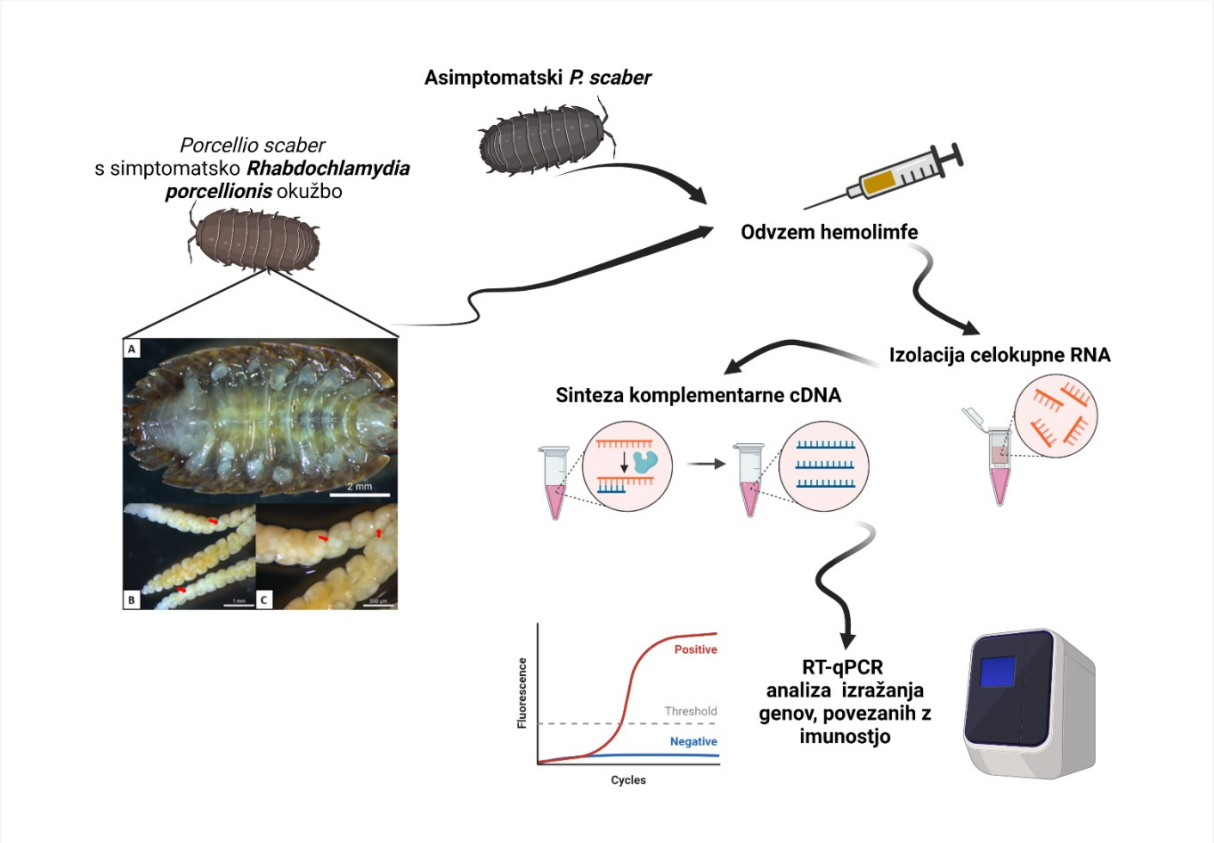
Vsebnost proteinov v hemolimfi smo določili z uporabo komercialnega kita BCA™ (Pierce, Rockford, IL, ZDA). Reagenta A in B smo pripravili v razmerju 50 : 1 (v/v) in 190 µL odpipetirali k 10 µL vzorca hemolimfe, kar je povzročilo barvno reakcijo, sorazmerno količini proteinov v vzorcu. Reakcijsko mešanico smo v treh ponovitvah prenesli na mikrotitersko ploščo s 96 vdolbinicami, inkubirali 30 min pri 37 °C, nato pa absorbanco pri 562 nm z mikročitalcem Cytation 3 imaging reader (Biotek, USA). Koncentracijo proteinov v vzorcu smo izračunali glede na umeritveno krivuljo za goveji serumski albumin (BSA; 25–2000 µM). Za negativno kontrolo smo uporabili pufer DPBS (pH= 7,1–7,5).

Izolacija RNA in prepis v cDNA

Celokupno RNA smo izolirali iz hemocitov v skladu z modificiranim protokolom z uporabo kita RNeasy Plus Micro (Qiagen, Nemčija). Za simptomatsko in asimptomatsko skupino smo izolirali skupno 3 neodvisne vzorce RNA, pri čemer je vsak vzorec predstavljal biološko ponovitev. Združeni vzorec hemolimfe smo homogenizirali v 600 µL pufra RTL Plus in 30 sekund močno stresali, nato pa ga prenesli na gDNA odstranjevalno kolono, ki smo jo postavili v 2 mL zbiralno plastično epruveto, in centrifugirali 15 sekund pri 12.000 obratih na minuto (Eppendorf Minispin centrifuga). Eluatu smo dodali enak volumen 70 % etilnega alkohola (EtOH) in vse skupaj prenesli v RNeasy MinElute kolono, nameščeno v 2 mL zbiralno plastično epruveto, ter centrifugirali 15 sekund pri 12.000 obratih na minuto. Vsi nadaljnji koraki centrifugiranja, vključno s prejšnjimi, so bili izvedeni pri sobni temperaturi (20–25 °C) in pri 12.000 obratih na minuto. Eluat smo zavrgli in nadaljevali s čiščenjem vzorca z dodajanjem pufrov RW1 in RPE ter 15 sekundnim centrifugiranjem, oziroma dodatkom 80-odstotnega EtOH in 2 minutnim centrifugiranjem. V nadaljevanju smo zbiralno plastično epruveto zamenjali z novo in kolono ponovno centrifugirali, tokrat 5 minut. Celokupno RNA smo izolirali iz RNeasy MinElute kolone z dodajanjem 14 µL vode brez RNaz in centrifugiranjem 1 minuto v novo sterilno mikrocentrifugirko. Izolirano RNA smo do uporabe hranili (na hladnem) pri -70 °C. Količino in kakovost izolirane RNA smo preverili s fluorometrom Qubit 2.0 (Thermo Fisher Scientific) z uporabo kita RNA HS Assay (Qiagen, Nemčija) in Tape Station 4100 (Agilent technologies, ZDA). Iz 35 ng izolirane RNA smo pripravili komplementarno cDNA z uporabo kompleta reagentov za reverzno transkripcijo (A3500; Promega, Wisconsin, ZDA), v skladu z navodili proizvajalca (Sl. 2). Skupni reakcijski volumen sintetizirane cDNA je bil 20 µL. Do uporabe smo cDNA hranili (na hladnem) pri -20 °C.

Izbor kandidatnih genov, povezanih z imunostjo ter oblikovanje začetnih oligonukleotidov

Kandidatni geni, ki igrajo vlogo v imunskem sistemu rakov (*Cat*, katalaza; *MnSod*, mangan superoksid dismutaza; *Nos*, sintaza dušikovega oksida; *CypG*, ciklofilin G; *Dscam*, celična adhezijska molekula Downovega sindroma; *Toll4*, toll-u podobni receptor 4; *MyD88*, mieloidni diferenciacijski faktor 88; *Ppae2a*, profenoloksidaza aktivacijski encim 2a; *A2m*, alpha-2-makroglobulin; *Atg5*, avtofagni protein 5) so bili izbrani zaradi njihove nepogrešljive vloge pri delovanju prirojenega imunskega sistema rakov oziroma drugih nevretenčarjev in so pogosto preučevani



Slika 2. Shema analize izražanja genov, povezanih z imunostjo, v hemocitih *P. scaber*.

Figure 2. Scheme of analysis of immune-related gene expression in *P. scaber* haemocytes.

in diferenčno izraženi v primeru izpostavitve različnim stresorjem (Chevalier in sod., 2012; Tassanakajon in sod., 2013; Clark in Greenwood, 2016; Sun in sod., 2020). Poleg genov povezanih z imunostjo smo uporabili tudi dva referenčna gena, tj. faktor raztezka 2; *Ef2* in beta aktin; *β-Act*. Zaporedja genov (kandidatnih in referenčnih) smo pridobili iz podatkovne zbirke NCBI za druge skupine rakov. Z uporabo orodja BLAST (Discontiguous Megablast in BLAST; E-vrednost < 1e-5) za prepoznavanje podobnih zaporedij smo jih primerjali s transkriptomom *P. scaber*. V ta namen smo uporabili transkriptom kopenskega raka *P. scaber* (Dolar in sod., 2022d), kot tudi dodatni transkriptom, ki je prosto dostopen v podatkovni zbirki NCBI (referenčna številka: SRX2600493; Becking in sod., 2017). Zaporedja začetnih oligonukleotidov za referenčne gene in gene, povezane z imunostjo, so bila zasnovana s programsko opremo Geneious Prime 2022.0.1 (Biomatters, Nova Zelandija) in sintetizirana pri komercialnem ponudniku IDT (Coralville, ZDA) (Tab. 1).

Analiza izražanja genov

Reakcija RT-qPCR je bila izvedena v volumnu 10 µL z uporabo GoTaq qPCR Master Mix (Promega, ZDA). V reakcijski volumen smo dodali 5 µL reagenta GoTaq® qPCR Master Mix, 1,25 µL F začetnega oligonukleotida (5 µM) in 1,25 µL R začetnega oligonukleotida (5 µM) in 2,5 µL vzorca cDNA (5-krat razredčenega v vodi brez nukleaz). Reakcije RT-qPCR so bile izvedene na plošči s 384 vdolbinicami (MicroAmp™ Optical 384-Well Reaction Plate, Applied Biosystems™), z uporabo sistema PCR v realnem času ViiA 7 (Applied Biosystems, ZDA) z naslednjim profilom cikla: 3 minute pri 95 °C in 40 ciklov (15 s pri 95 °C , 25 s pri 60 °C in 35 s pri 72 °C), čemur je sledila določitev (talilne) krivulje. Vse reakcije kot tudi negativna kontrola brez dodane matrice (cDNA) so bile izvedene v treh tehničnih ponovitvah. Izražanje genov smo normalizirali z uporabo dveh referenčnih genov, tj. *Ef2* in *β-Act*, ki imata stabilno izražanje in sta bila uporabljena v podobnih

Tabela 1. Podatki o začetnih oligonukleotidih, ki smo jih uporabili v analizi RT-qPCR.

Table 1. Data on primers used in RT-qPCR analysis.

Gen	Oligonukleotidni začetnik (5′ –> 3′)	Velikost produkta (bp)	Referenčna št.
β-Act	**F:** CGGACGTACCACTGGTATCG **R:** GAGGAGGCTGCAGTTGTCAT	264	KY780298.1
Ef2	**F:** CGACAAAGGAAGGTGTTCTC **R:** ACCACCTCCACGATGAATA	101	FQ896398
Cat	**F:** ATT GGA GAG CGA GGT CCT CT **R:** TGT TCC CGA CCA AAT CCC AG	316	KC668411.1
MnSod	**F:** TCACCCAATGGTGGAGAA **R:** TGATCCTTGAACAGCAACAG	117	MF289344.1
Nos	**F:** CCGTCAGCACTAGGTTTATC **R:** GGTCCACCTACTTGCATTT	102	GQ865598
CypG	**F:** GAGATGGTACTGGAGGAAGA **R:** CAGCATTAGCCATTGAAAGC	102	EU216759.1
Dscam	**F:** GTCCTTGCGTTCACTTCT **R:** GTTGGAGCCTCTGGAATATC	86	JX679085
Toll4	**F:** GAGATCCGAAGTATAGGTTATGC **R:** AGTCCTCCTGCTGTTGT	101	MF124331
MyD88	**F:** TGATTCTCTTCGCTGACAAA **R:** CTCAGACCACCAACCATATC	115	FQ906745.1
Ppae2a	**F:** ACTACCCTAAGCCAGTGAA **R:** CTCCAAATTGAGTCTGTGTTATG	114	FJ620685.1
A2m	**F:** AAATGACGAATCGGGATCTAC **R:** CAACCATTCCCTCGTTATGT	115	KJ540280
Atg5	**F:** AGC TTT GGA CAG GCT TGT GT **R:** ACG GTG GTT CAA TGC CTT GA	261	KP317125.1

študijah pri drugih rakih (Chevalier in sod., 2012; Xu in sod., 2020). Učinkovitost pomnoževanja začetnih oligonukleotidov kandidatnih genov smo določili s pripravo redčitvene vrste cDNA in validacijske krivulje. Relativno izražanje kandidatnih genov je bilo izračunano po metodi $2^{-\Delta\Delta Ct}$, ki temelji na qPCR učinkovitosti za izbrane začetne oligonukleotide, ki je znašala med 90 % in 110 % (Pfaffl, 2001). Rezultati, ki prikazujejo stopnjo izražanja genov, povezanih z imunostjo, v asimptomatski in simptomatski skupini so podani kot relativne vrednosti.

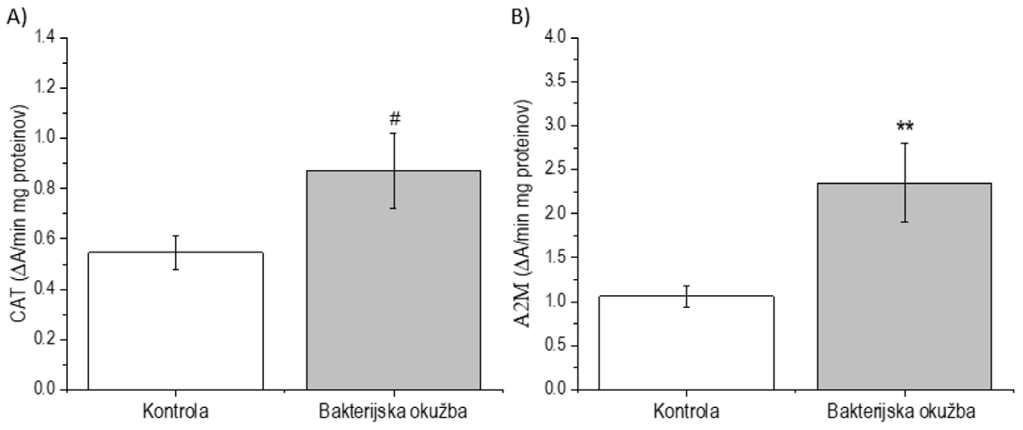
Statistična analiza

Statistično obdelavo in prikaz podatkov smo izvedli v programu OriginPro 2022v (Origin Lab). Porazdelitev podatkov smo preverili s Kolmogorov-Smirnovim testom. V primeru simetrične porazdelitve podatkov smo uporabili T-test za neodvisne vzorce, pred tem pa preverili enakost varianc. V primeru asimetrične porazdelitve ali neenakosti varianc podatkov smo aplicirali Mann-Whitney test. Z zvezdicami nad stolpci na grafu smo označili statistično značilne razlike med testiranimi skupinami (*, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$), medtem ko lojtra (#) označuje mejno značilne spremembe ($0,05 < p < 0,1$).

Rezultati

Spremembe aktivnosti encimov katalaza in alfa-2-makrogobulin v hemolimfi *P. scaber* z bakterijsko okužbo

V hemolimfi *P. scaber* s simptomatsko bakterijsko okužbo smo opazili mejno značilno povečanje aktivnosti encima katalaza (Sl. 3A), v primerjavi s kontrolo. Nasprotno pa je bila aktivnost encima alfa-2-makroglobulin značilno povečana ($p < 0,01$; Sl. 3B), v primerjavi z asimptomatskimi organizmi.

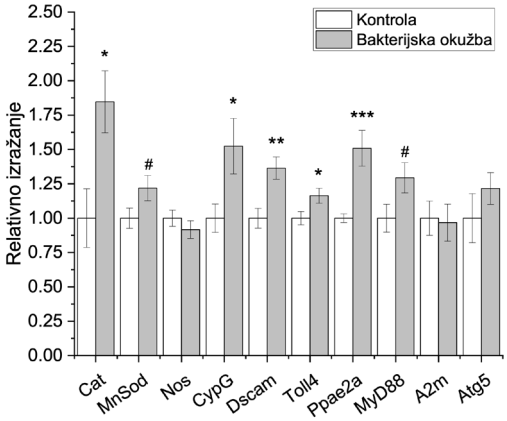


Slika 3. Aktivnost encimov (A) katalaza (CAT) in (B) alfa-2-makroglobulin (A2M) v hemolimfi asimptomatskih *P. scaber* in osebkih s simptomatsko okužbo z *R. porcellionis*. Zvezdica nad stolpci na grafu prikazuje statistično značilno razliko v primerjavi z asimptomatskimi (kontrolnimi) živalmi (**; $p < 0,01$), medtem ko lojtra (#) označuje mejno značilne razlike ($0,05 < p < 0,1$).

Figure 3. Activity of the enzymes (A) catalase (CAT) and (B) alpha-2-macroglobulin (A2M) in the haemolymph of asymptomatic *P. scaber* and individuals with symptomatic *R. porcellionis* infection. An asterisk above the graph bars indicates a statistically significant difference compared to asymptomatic (control) animals (**, $p < 0.01$), while a hash mark (#) indicates a borderline significant difference ($0.05 < p < 0.1$).

Spremembe v izražanju genov povezanih z imunostjo pri *P. scaber* s simptomatsko bakterijsko okužbo

Okužba P. scaber z bakterijo Rhabdochlamydia porcellionis je izzvala spremenjeno izražanje genov, povezanih z imunostjo, v primerjavi z asimptomatskimi, tj. zdravimi organizmi (Sl. 4). V hemocitih okuženih živali smo opazili statistično značilno povečano izražanje genov Cat ($p < 0,05$), CypG ($p < 0,05$), Dscam ($p < 0,01$), Toll4 ($p < 0,05$) in pa Ppae2a ($p < 0,001$), medtem ko za gene Nos, A2m in Atg5 nismo zaznali sprememb v izražanju. V primeru genov MnSod ($p= 0,082$) in MyD88 ($p= 0,067$) pa smo opazili mejno značilno povečano izražanje.



Slika 4. Izražanje genov povezanih z imunostjo v hemocitih *P. scaber* s simptomatsko bakterijsko (*R. porcellionis*) okužbo. Kontrolo predstavljajo asimptomatske živali, tj. živali brez znakov okužbe. Podatki so predstavljeni kot relativna sprememba v izražanju genov v primerjavi s kontrolo, povprečna vrednost ± standardna napaka je izračunana iz treh neodvisnih vzorcev RNA. Zvezdica nad stolpci na grafu prikazuje statistično značilno razliko v primerjavi z asimptomatskimi (kontrolnimi) živalmi (*, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$), medtem ko lojtra (#) označuje mejno značilne razlike ($0,05 < p < 0,1$).

Figure 4. Expression of immune-related genes in haemocytes of *P. scaber* with symptomatic bacterial (*R. porcellionis*) infection. Control is represented by asymptomatic animals, i.e., animals without signs of infection. Data are presented as relative expression change compared with control, mean ± standard error calculated from three independent RNA samples. An asterisk above the bars in the graph indicates a statistically significant difference compared to asymptomatic (control) animals (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), while a number sign (#) indicates a marginally significant difference ($0.05 < p < 0.1$).

Diskusija

V raziskavi smo določili spremembe v izražanju genov, povezanih z imunostjo, v hemocitih kopenskih rakov *P. scaber* z izraženo (tj. simptomatsko) bakterijsko okužbo z *R. porcellionis*. V primerjavi z asimptomatskimi *P. scaber* smo v okuženih živalih opazili statistično značilne spremembe v izražanju imunskih genov *Toll4, Dscam, Ppae2a, CypG* in *Cat*, medtem ko je bilo izražanje genov *MnSod* in *MyD88* zgolj mejno značilno spremenjeno ($0,05 < p < 0,1$). Ti rezultati pomembno prispevajo k razumevanju dogajanja na nivoju transkriptoma v primeru mikrobne okužbe kopenskega enakonožca *P. scaber*.

V okuženih živalih je bilo izražanje genov *Toll4* in *Dscam* pričakovano značilno povečano. Gena namreč kodirata evolucijsko ohranjena receptorska proteina (tj. imunski receptor; PRR), odgovorna za prepoznavo molekularnih vzorcev patogenov oziroma mikroorganizmov (P/MAMP) (Tran in sod., 2020; Sánchez-Salgado in sod., 2021). O podobnem povečanem izražanju *Toll* in *Dscam* genov poročajo tudi Li in sod. (2019) v hemocitih rakov *Eriocheir sinensis*, okuženih z bakterijo *Staphylococcus aureus* in pa Pan in sod. (2019), v primeru bakterijske *Vibrio sp.* okužbe raka *Macrobrachium nipponense*. Znano je, da aktivacija Toll-u podobnih receptorjev sproži prepisovanje genov, ki sodelujejo v protibakterijski obrambi rakov, tj. sintezi antimikrobnih peptidov (Pan in sod., 2019), nasprotno pa aktivacija gena *Dscam* promovira fagocitozo bakterij, medtem ko novejše raziskave dokazujejo udeležbo produktov gena *Dscam* tudi v regulaciji signalne poti Toll (Li in sod., 2019). Poleg povečanega izražanja omenjenih genov smo opazili tudi mejno značilno povečanje izražanja *MyD88*, ki predstavlja pomemben adaptorski protein udeležen v signalni kaskadi Toll (Habib in Zhang, 2020; Gao in sod., 2021), kar sovpada s povečanim izražanjem *Toll4*. Vezava P/MAMP na plazemske oziroma PRR, vezane v membrano hemocitov, sproži evolucijsko ohranjen proces fagocitoze, tj. požiranje tujih delcev, npr. bakterij, ki so znotraj hemocitov uničeni in razgrajeni s strani ROS in RNS (Raman in sod., 2008; Rodríguez-Ramos in sod., 2010). Povečano produkcijo RNS v hemolimfi enakonožcev v primeru mikrobne okužbe smo dokazali že v raziskavi Dolar in sod. (2020), v kateri poročamo o značilno povečani koncentraciji dušikovega oksida (NO) v hemolimfi *P. scaber* s simptomatsko *R. porcellionis* okužbo. Nasprotno pa v aktualni raziskavi v okuženih *P. scaber* nismo zaznali sprememb v izražanju gena *Nos*, ki kodira encim sintaza dušikovega oksida, odgovornega za produkcijo NO. Koncentracija reaktivnih zvrsti (ROS in RNS) mora biti v celicah in tkivih strogo regulirana, saj lahko povečane koncentracije trajno poškodujejo celice gostitelja. Za uravnavanje nivoja ROS in RNS so odgovorni antioksidativni encimi (npr. CAT in SOD), ki predstavljajo pomembno komponento prirojene imunosti rakov (Gopalakrishnan in sod., 2011; Liu in sod., 2013). V povezavi s tem smo v hemolimfi bakterijsko okuženih *P. scaber* opazili značilno povečano izražanje gena *Cat*, medtem ko je bilo izražanje gena *MnSod* zgolj mejno značilno spremenjeno. Za primerjavo, v raziskavi Dolar in sod. (2020) smo v hemolimfi bakterijsko okuženih *P. scaber* izmerili značilno povečano aktivnost encima SOD, v primerjavi z asimptomatskimi živalmi, medtem ko smo v aktualni raziskavi dodatno analizirali tudi aktivnost encima katalaza in dokazali mejno značilno povečanje, kar sovpada z rezultati izražanja gena *Cat* v hemocitih *P. scaber.*

Simptomatska *R. porcellionis* okužba pri *P. scaber* izzove aktivacijo celične komponente prirojene imunosti, kar je jasno razvidno iz zmanjšanega deleža semigranularnih (SGC) in povečanega deleža granularnih hemocitov (GC) v hemolimfi okuženih *P. scaber* (Dolar in sod., 2020). Spremenjen delež SGC lahko razložimo z migracijo le-teh na mesto okužbe in tvorbe nodulov, o čemer poročata že Kostanjšek in Pirc Marolt (2015), medtem ko je razlog za neznačilno povečanje deleža GC moč iskati v proizvodnji ključnih humoralnih molekul, vključno s komponentami sistema proPO (Herbinière in sod., 2005; Tassanakajon in sod., 2018). To sovpada z opaženim značilno povečanim izražanjem gena *CypG*, ki kodira regulatorni protein, za katerega se domneva, da v granularnih hemocitih sodeluje pri ohranjanju konformacijske celovitosti shranjenih granularnih beljakovin, s čimer je pomembno udeležen v regulacijo sekrecije vsebine citoplazemskih granul, tj. humoralnih molekul (Takaki in sod., 1997; Herbinière in sod., 2008). Domnevamo, da aktivacija signalne poti Toll vodi v prepisovanje protibakterijskih genov in sintezo humoralnih obrambnih molekul v granularnih in semigranularnih hemocitih. Dodatno smo v simptomatskih *P. scaber* opazili tudi značilno povečano izražanje gena *Ppae2a*, odgovornega za aktivacijo proPO sistema oziroma procesa melanizacije (Charoensapsri in sod., 2011). Proces je namreč aktivno udeležen pri tvorbi nodulov in se aktivira ob prisotnosti P/MAMP oziroma molekularnih vzorcev poškodovanih celic in tkiv (t.j. DAMP) (Cerenius in Söderhäl, 2021). Opažena sprememba

v izražanju gena Ppae2a je lahko neposredno povezana s poškodbo organizma, zaradi vdora bakterij, ali pa gre za posledico aktivacije Toll signalne poti, s katero si proPO sistem deli skupne regulatorne proteine (Cerenius in Söderhäll, 2021). S tem rezultatom sovpada tudi zaznana povečana aktivnost encima alfa-2-makroglobulin, ki predstavlja pomembno serinsko proteazo, odgovorno za regulacijo proPO sistema in procesa melanizacije (Ponprateep in sod., 2017).

Zaključki

Na podlagi rezultatov lahko nedvomno zaključimo, da simptomatska bakterijska okužba *P. scaber* z *R. porcellionis* izzove značilne spremembe v izražanju izbranih kandidatnih genov. Rezultati potrjujejo pomembno vlogo analiziranih genov v imunskem odgovoru v primeru mikrobne okužbe. Hkrati pa preučevani geni, povezani z imunostjo, predstavljajo potencialne biološke označevalce v okoljskih raziskavah.

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Research Article

Contribution of neutral processes to the assembly of microbial communities on *Phragmites australis* leaf litter

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Abstract

Phragmites australis is a remarkable aquatic plant known for its adaptability, wide ecological range and extensive presence in natural wetlands. When combined with its microbiome, it holds unique potential to enhance the overall functionality of wetland ecosystems. To fully harness this potential in both natural and constructed wetlands, it becomes crucial to understand the dynamics of decomposition regarding the substantial biomass generated by *P. australis*. However, our understanding of the selective and neutral processes that shape the microbial communities responsible for decomposing *P. australis* litter remains somewhat limited. In this context, our research reveals that the majority of microbial taxa inhabiting *P. australis* leaves and litter follow neutral distribution patterns, indicating they are less likely to be specifically adapted to the host plant or habitat. Their presence in the community primarily results from their prevalence in the broader metacommunity and source pool. Nonetheless, this should not be interpreted as these taxa being functionally unimportant or lacking close interactions with their host. Instead, the host environment does not differentially select them, and as a consequence, their distributions are shaped predominantly by neutral processes of dispersal and drift.

Keywords

Bacterial communities, fungal communities, decomposition, freshwater ecosystem, ephemeral lake

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Pomen nevtralnih procesov pri oblikovanju mikrobnih skupnosti na listnem opadu vrste *Phragmites australis*

Izvleček

Phragmites australis je izjemna vodna rastlina, znana po svoji prilagodljivosti, širokem ekološkem razponu in obsežni prisotnosti v naravnih mokriščih. V kombinaciji s svojim mikrobiomom ima edinstven potencial za izboljšanje splošne funkcionalnosti mokriščnih ekosistemov. Za izkoriščanje njenega potenciala v naravnih in zgrajenih mokriščih pa je ključnega pomena razumevanje dinamike razgradnje velike količine biomase, ki jo ustvari *P. australis*. Naše razumevanje selektivnih in nevtralnih procesov, ki oblikujejo mikrobne združbe, odgovorne za razgradnjo listnega opada *P. australis*, je še vedno nekoliko omejeno. V tem kontekstu naša raziskava razkriva, da večina mikrobnih taksonov, ki naseljujejo liste in listni opad *P. australis*, sledi nevtralnim vzorcem porazdelitve, kar kaže, da je manj verjetno, da so posebej prilagojeni gostiteljski rastlini ali habitatu. Njihova prisotnost v združbi je predvsem posledica njihove razširjenosti v širši metaskupnosti in izvornem naboru vrst. Kljub temu to ne pomeni, da so ti taksoni funkcionalno nepomembni ali da nimajo tesnih interakcij z gostiteljem. Le njihovo okolje ne vrši močnega selektivnega pritiska nanje, zaradi česar njihovo porazdelitev oblikujejo pretežno nevtralni procesi razširjanja in ekološkega zdrsa.

Ključne besede

Bakterijske združbe, glivne združbe, razgradnja, sladkovodni ekosistem, presihajoče jezero

Introduction

Phragmites australis is a perennial grass-like aquatic plant known for their remarkable adaptability and wide ecological range. It has the capacity to establish dense, dominant communities within aquatic ecosystems and is among the most prevalent plant species found in wetlands (Kowalski et al., 2015; Stottmeister et al., 2003). Given their extensive presence in natural wetlands and widespread use in constructed wetlands, *Phragmites* and its microbiome possess distinctive potential for enhancing the overall functionality of wetland ecosystems. Nevertheless, in order to harness its potential in natural and constructed wetlands, it is important to understand also the decomposition dynamics of the vast biomass produced by *P. australis*.

The decomposition process within wetlands can be influenced by a range of factors, with its hydrology and temperatures playing pivotal roles (Dolinar et al., 2016; Serna et al., 2013) in the formation of microbial communities decomposing the plant litter. Apart from climatic conditions, the composition of litter and site elements, specifically the nutrient content within plant litter, may also substantially influence decomposition rates (Alfredsson et al., 2016; Sistla et al., 2012). This is because plants in nutrient-deficient environments often produce litter of lower quality, which consequently slows the decomposition processes (Rejmánková & Houdková, 2006).

Although the list of potential factors influencing microbial communities is extensive, they can be categorized into two primary groups: selective processes, where microbes thrive within an environment due to differences in their ecological fitness, and neutral processes, which encompass passive dispersal dynamics and the impacts of ecological drift (Chase & Myers, 2011). Although much research is focused on interactions between microbes and their environment, the relative contributions of neutral processes in shaping microbial communities associated with hosts have been mainly overlooked. In contrast, these processes have been subjects of study within the broader field of ecology for many decades, experiencing a renewed surge of interest in recent years (Burns et al., 2016; Cao et al., 2019; Heys et al., 2020).

The neutral theory derives its name from its core assumption of species having equivalent per-capita growth, death, and dispersal rates, assuming species are ecologically 'neutral' in their fitness. Without differences in these factors, community assembly is governed by stochastic processes involving dispersal and drift. Within this framework, organisms within a community are randomly lost over time and are replaced by individuals randomly

either from within the same community or through the dispersal of individuals from an outside community.

Despite simple assumptions of ecological equivalence at the base of neutral models, they have proven remarkably successful in predicting the structures of communities, including microbes (Burns et al., 2016; Cao et al., 2019; Heys et al., 2020). These models find utility in modelling microbial systems where the vast diversity of communities makes it challenging to characterize the specific ecological traits of each taxon. Moreover, they enable researchers to quantify the significance of processes that are challenging to observe directly, such as dispersal, despite their potentially substantial impacts on microbial communities (Kerr et al., 2002; Shen et al., 2018).

This study aimed to build on the previous analysis of fungal communities on decomposing *P. australis* leaves and to evaluate the relative impact the ecological factors and neutral model processes have on the microbial communities on these leaves. Our previous study Likar et al. (2022) showed that a complex network of fungi forms already in the senescent leaves of *P. australis* and persists to the decomposition phase. Furthermore, it seems that habitat has a lower impact on the formation of the community during the early decomposition phase than on the interaction between its members.

In the present study, we assess the ability of neutral models to explain the distribution of microorganisms among a population of decomposing *P. australis* leaves and then determine the conditions leading to departures from neutral behavior. If a reduction in the fit of the neutral model reflects heightened selection pressures, we assumed that deviations from the neutral predictions should show distinct compositional changes in the communities, particularly in cases where ecological traits are conserved. By investigating these hypotheses, we aim to establish a framework for identifying communities and taxa that might be of particular interest based on the extent to which they deviate from the expectations set forth by neutral theory.

Materials and Methods

Study location and experimental conditions

The lake Cerknica experiences cyclical inundation, submerging its surroundings for approximately nine months each year. Within this dynamic ecosystem, *P. australis*, a robust plant species, thrives in diverse aquatic environments, encompassing both the inner lake region and the periphery bordering the lake's tributaries (Longhi et al., 2008).

The experimental conditions followed the procedure described in (Grašič et al., 2022). In brief, we gathered both the upper and lower leaves of *P. australis* during the conclusion of the vegetation period when the plants retained their active. We collected leaves from different parts of the plant to cover the possible microbial diversity. Subsequent to collection, the plant material underwent air-drying at room temperature until a consistent weight was achieved. For the decomposition experiment we used litter bags (1 mm × 1 mm plastic mesh) containing 4 g of the plant material. Water level data were monitored from the nearby hydrological station at Gorenje Jezero-Stržen. In order to minimize direct contact with the substrate, the litter bags were affixed to wooden poles. The decomposition phase of our experiment spanned a duration of 45 days, after which the samples were collected and subjected to air-drying until a consistent weight was reached. Following the drying process, we separated the plant material from non-plant material and processed them for metagenomic analysis.

Metagenomics

In the present dataset, we analysed the sequences obtained by shotgun sequencing (see Likar et al., 2022 for details), deposited at MG-Rast depository under project mgp97071, libraries mgm4915122.3-mgm4915169.3. In short, whole community DNA from common reed leaves was used for shotgun sequencing (Illumina HiSeqX, 2 x 150 nt pair-end, TruSeq Nano kit). The analysis and annotations were executed on the Metagenomics Rapid Annotation (MG-RAST) online server [22], with the default parameters. Taxonomic identification in this analysis utilized an E-value threshold of $1e^{-5}$. All sequencing data are openly available on the MG-Rast server.

Plant litter elemental analysis

Plant litter was analysed using X-ray fluorescence spectrometry (XRF). Five samples of each treatment were used for multielemental analysis as described in (Grašič et al., 2022). In short: From 100–500 mg of dried and powdered plant material was pressed into pellets using a pellet die and a hydraulic press. ^{55}Fe (25 mCi; Isotope

Products Laboratories, Valencia, PA, USA) was used as the primary excitation source for the analysis. The analysis of the X-ray spectra was carried out using an iterative least-squares programme, as included in the quantitative X-ray analysis system software package (Vekemans et al., 1994). The quality assurance for the element analysis was determined using standard reference materials: NIST SRM 1573a (tomato leaves as a homogenised powder), in the form of pressed pellets.

Statistics

All analyses were performed in R (v4.3.1).

To estimate the effect of environment and geo-location on the composition of fungal and bacterial communities, were performed variation partitioning using vegan v2.6-4 library. Geographical distances were transformed to rectangular data using principal coordinates of neighbourhood matrix (PCNM) before the analysis. Prior to analysis, the environmental factors and PCNM vectors were subjected to forward stepwise redundancy analysis to reduce the number of variables used in the variation partitioning.

Null models are an essential tool for assessing the issue of multiple assembly processes by mimicking the consequences of random processes, therefore we calculated a modified stochasticy index as describe in (Liang et al., 2020) using NST v3.1.10 library. The null communities are generated by randomizing the observed community structure 1000 times based on a null model algorithm described previously (Stegen et al., 2013).

Adherence to the Sloan neutral model was calculated using the R code published by (Burns et al., 2016). Accordingly, the OTUs were grouped into three partitions based on whether they occurred more frequently than ('above' partition), less frequently than ('below' partition), or within ('neutral' partition) the 95% confidence interval of the neutral model predictions. For meaningful comparisons among partitions, we rarefied each partition to an equal number of OTUs, matching the size of the smallest partition.

Furthermore, we compared the fit of the neutral model to that of a binomial distribution model to ascertain whether incorporating drift and dispersal limitations improved the model fit beyond random sampling from the source metacommunity (Sloan et al., 2007). The binomial distribution model represents the scenario where local communities are random subsets of the metacommunity in the absence of drift and dispersal limitations. To compare the fit of the neutral and binomial models, we examined the Akaike information criterion for each model. Computation of the Akaike information criterion was performed in R, and 95% confidence intervals for all fitting statistics were generated using bootstrapping with 1000 bootstrap replicates.

Results and Discussion

Step-wise RDA of explanatory datasets selected P concentration, PCNM1 and PCNM2 for fungal community dataset, whereas complete models with all environmental parameters and PCNMs were selected for the bacterial community dataset.

Variation partitioning for fungal communities explained 56% of the overall variation (Fig. 1). Measured environmental parameters explained only 1.5% alone and additional 10.7% in combination with geographical location. Geographical location alone explained the largest portion of the variability. Measured environmental parameters were a little better predictor for bacterial communities and explained 16% when not controlling for geographical location. In contrast to fungal communities, geographical location alone did not explain any variation in the bacterial communities, which showed a very high percentage of unexplained variation.

As measured and unmeasured environmental parameters seem to explain only around 15-55% of the total variation in the microbial communities, we examined the importance of neutral processes on the formation of these communities.

Comparing the number of OTUs shared between fresh and decomposing leaves showed that the largest number of OTUs was specific for all three treatments (Fig. 2). This shows the importance of the initial phyllosphere communities on the formation of microbial communities during the decomposition process.

The same was true for the enriched and under-enriched fungal OTUs, suggesting that these represent ubiquitous generalist that start as colonisers of fresh leaves and after leaf-fall proceed to decomposition of leaf litter. The second largest group of OTUs in the present study was characteristic for dryer habitats t.i. fresh leaves and leaves decomposing in the dry habitat. Similarly, identification of indicator species yielded a large portion of fungal taxa that were indicative for both the fresh leaves and leaves

decomposing in the dry habitat (Likar et al., 2022). Only a few fungal OTUs were characteristic for a single treatment: *Corynespora* was enriched, whereas *Marasmius* and *Hanseniaspora* were under-enriched on fresh leaves. In addition, *Lycoperdon* was under-enriched on decomposing leaves independently of habitat. Interestingly, none of the taxa that deviated from the neutral model were observed as indicator species for either fresh leaves or leaves decomposing in wet or dry habitat (Likar et al., 2022).

Modified Raup-Crick with Bray-Curtis dissimilarity showed that the local fungal communities on plant litter were more dissimilar than expected by random chance (Fig. 3a). In contrast, local communities on fresh leaves were as dissimilar as expected by the null model. All treatment showed values which indicate that the turnover in fungal communities was governed by drift alone (-0.95 < RC < 0.95). To evaluate the stochastic processes in the population, we calculated the modified ST (Fig. 3b). Community assembly was relatively more stochastic on fresh leaves (72% stochasticity ratio (ST) and in litter decomposing in the dry habitat (68% ST) than in the wet habitat (38% ST). These results suggest that deterministic processes became increasingly important during the decomposition and especially in the wet habitat, where the MST values are under 42%.

In contrast to fungal communities, bacterial communities in plant litter showed RC values above 0.95, which show a significant departures from the degree of turnover expected when drift acts alone (Chase et al., 2011). Values of RC > 0.95 indicate that dispersal limitation governs observed compositional differences. MST values under 0.5 that were calculated for bacterial communities well support this. Further more, it seems that selection was increased during the transition from fresh leaves to plant litter, as bacterial communities on fresh leaves showed dissimilarity that was well expected by random chance.

While phyllosphere microbial communities of different species exhibit significant dissimilarities (Bao et al., 2019), they all share a similar underlying structure (Wallace et al., 2018). As plants gradually undergo senescence, the variability in phyllosphere microbes tends to incrementally rise (Ferreira et al., 2016), with changes in microbial communities on leaves undergoing decomposition (Kembel et al., 2014; Whipps et al., 2008) increasingly influenced by the leaves' physicochemical properties and competition between the microbes.

Out of on average 244 bacterial families that were observed on plant material in our study, only one family (0.4% of all the families) did not fall into the neutral model and showed enrichment (Suppl. Table S1). None of the bacterial families showed under-enrichment against the neutral model. This would suggest greater importance

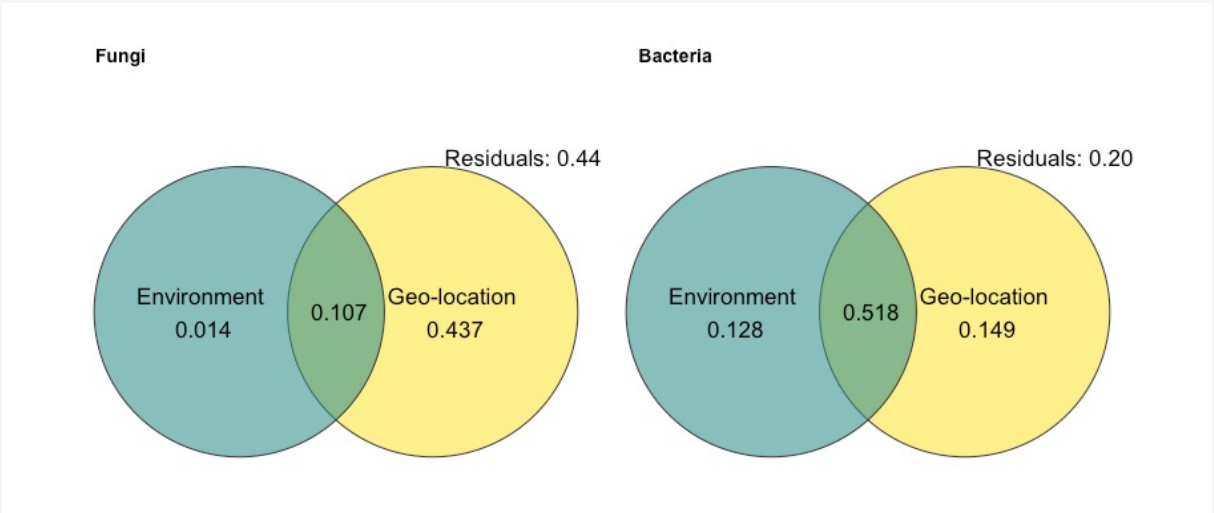

Figure 1. Variation partitioning for fungal and bacterial communities using measured environmental parameters and geo-location selected by step-wise redundancy analysis (RDA), as the explanatory datasets. Values < 0 are not displayed.

Slika 1. Pojasnitev variabilnosti za glivne in bakterijske združbe z uporabo izmerjenih okoljskih parametrov in geografske lokacije, izbrane s postopno redundančno analizo (RDA) kot razlagalni nizi podatkov. Vrednosti < 0 niso prikazane.

of dispersal, as selection would increase or decrease the frequency of bacterial taxa in comparison with neutral model. Nevertheless, unclassified - derived from Rhizobiales group showed frequencies that were above the neutral model. Alphaproteobacteria, which comprised of several symbionts of plants, such as *Rhizobium* as the most abundant group in the roots of *P. australis* He et al. (2022) and among the top generalists in the phyllospheres of nine perennial plants in a Mediterranean ecosystem (Vokou et al., 2019). Their presence could be beneficial for the plant and provide a nitrogen source for the growth of plants (Sawada et al., 2003) as well as other physiological benefits (Jaiswal et al., 2021), which could explain the departure from the neutral model.

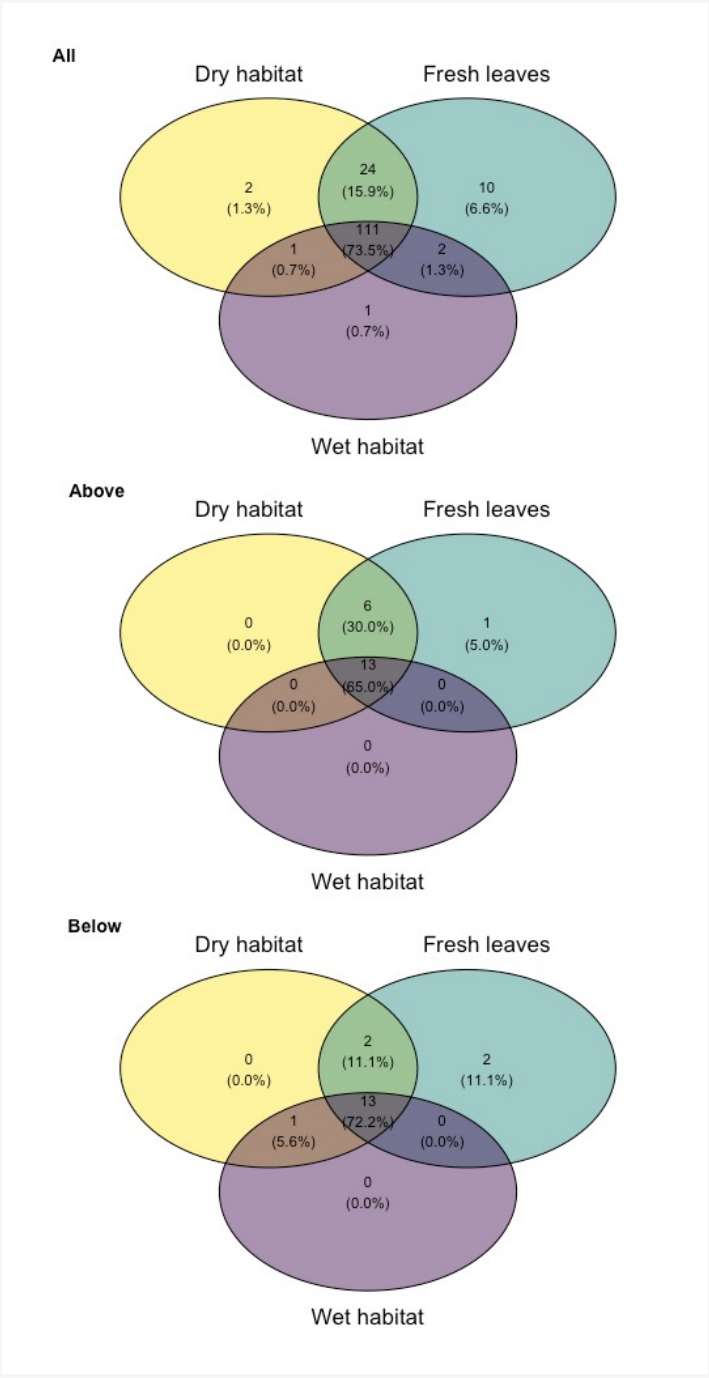

Figure 2. Venn diagrams for fungal communities (All), enriched fractions (Above) and under-enriched fractions (Below) according to Sloan's neutral model for fresh *Phragmites australis* leaves and leaf litter decomposing in either dry or wet habitat. Numbers represent the absolute number of OTUs and percentages represent their proportions regarding all OTUs in the individual representation.

Slika 2. Vennovi diagrami za glivne združbe (All), obogatene frakcije (Above) in podobogatene frakcije (Below) v skladu s Sloanovim nevtralnim modelom za sveže liste *Phragmites australis* in listni opad, ki se razkraja v suhem ali mokrem habitatu. Številke predstavljajo absolutno število OTU-jev, odstotki pa njihove deleže glede na vse OTU-je v posamezni predstavitvi.

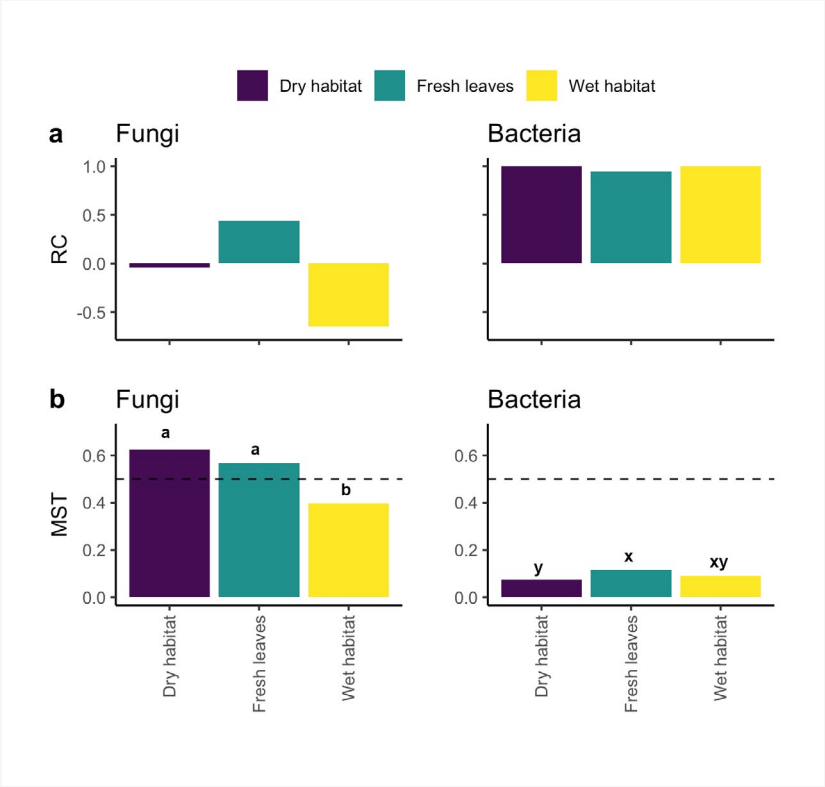

Figure 3. Raup-Crick a) and modified stochasticy b) indices for fungal and bacterial communities on the fresh leaves of *Phragmites australis* and its leaf litter decomposing in either dry or wet habitat. Dashed line represents the MST = 0.5 t.i. limitation between predominant stochasticity or determinism. Different letter depict statistically significant difference of PERMANOVA test at $p < 0.05$.

Slika 3. Raup-Crickov a) in modificirani stohastični b) indeksi za glivne in bakterijske združbe na svežih listih *Phragmites australis* in njenem listnem opadu, ki se razkraja v suhem ali mokrem habitatu. Črtkana črta predstavlja MST = 0,5 t.i. omejitev med prevladujočo stohastičnostjo ali determinizmom. Drugačna črka prikazuje statistično značilno razliko testa PERMANOVA pri $p < 0,05$

In contrast to bacterial communities, fungal communities showed higher taxa numbers that deviated from the neutral model. Overall, the frequency with which fungal taxa occurred in individual communities was well described by the neutral model (Suppl. Table S2, Fig. 4). The migration rate was not very variable and ranged from 0.73-0.89.

The fit of the neutral model was compared with the fit of a binomial distribution, which represents the absence of processes of drift and dispersal limitation using Akaike information criterion and Bayes information criterion (Table 1). We observed a much better fit of the model, if we used the neutral model compared to binomial distribution model. This suggest that the processes of passive dispersal and ecological drift have an impact on the fungal communities. This was observed for fungal communities in various biological systems (Gao et al., 2020; Liu et al., 2023; Zhang et al., 2023).

Table 1. Akaike and Bayes information criterions for neutral model and binomial distributions for fungal communities on fresh *Phragmites australis* leaves or leaf litter in either dry or wet habitat.

Tabela 1. Informacijsk kriterij Akaike in Bayes za nevtralni model in binomske porazdelitve za glivne skupnosti na svežih listih *Phragmites australis* ali listnem odpadku v suhem ali mokrem habitatu.

Plant material/ decomposition habitat	AIC neutral	BIC neutral	AIC binomial
Fresh leaves	-157.059	-151.219	-218.616
Dry habitat	-191.509	-185.655	-223.202
Wet habitat	-219.466	-213.976	7.746

AIC...Akaike information criterion; BIC...Bayes information criterion

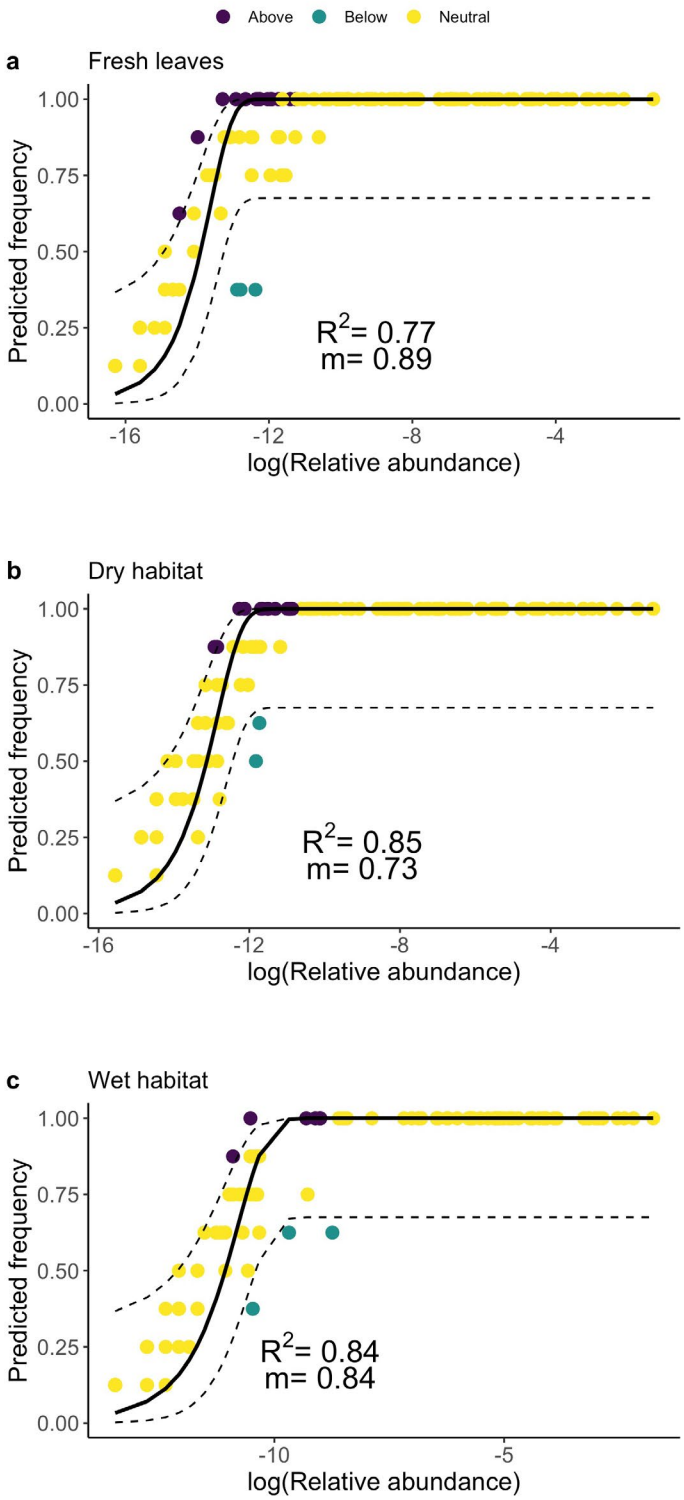

Figure 4. Fit of the neutral model. The predicted occurrence frequencies for a) fresh leaves and leaves decomposing in either b) dry or c) wet habitat. OTUs that occur more frequently than predicted by the model are shown in purple while those that occur less frequently than predicted are shown in green. Dashed lines represent 95% confidence intervals around the model prediction (solid line). The number represent the model fit (generalized R2) and migration rate (m) of the model.

Slika 4. Prileganje nevtralnega modela. Predvidene pogostnosti pojavljanja za a) sveže liste in liste, ki se razkrajajo v b) suhem ali c) mokrem habitatu. OTU-ji, ki se pojavljajo pogosteje, kot je napovedal model, so prikazani v vijolični barvi, tisti, ki se pojavljajo manj pogosto, kot je predvideno, pa so prikazani v zeleni barvi. Črtkane črte predstavljajo 95 % intervale zaupanja okoli napovedi modela (polna črta). Število predstavlja prileganje modela (posplošen R2) in stopnjo migracije (m) modela.

Conclusions

Our results suggest that the majority of microbial taxa in the studied ecosystem/plant host system are neutrally distributed and are less likely to be specifically adapted to the host. Therefore their presence in the community is largely the result of their abundance in the surrounding meta-community and source pool. Nevertheless, this does not mean that these taxa are functionally unimportant or even that they are not interacting intimately with their hosts. Rather the host environment is not differentially selecting them, and consequently their distributions are the result of neutral dispersal and drift.

Supplementary Materials

Suppl. Table S1. Observed frequencies for individual bacterial OTUs and their predicted frequencies under the Sloan neutral model. Upper and lower limits of 95% confidence interval for predicted frequencies are also displayed.

Suppl. Table S2. Observed frequencies for individual fungal OTUs and their predicted frequencies under the Sloan neutral model. Upper and lower limits of 95% confidence interval for predicted frequencies are also displayed.

Author Contributions

Conceptualization, M.L. and A.G.; methodology, M.L. and A.G.; formal analysis, M.G. and M.L.; writing—original draft preparation, M.L.; writing—review and editing, M.L., M.G., and A.G.; visualization, M.L.; funding acquisition, A.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All sequencing data are openly available on the MG-Rast depository under project mgp97071, libraries mgm4915122.3-mgm4915169.3.

Conflicts of Interest

The authors declare no conflict of interest.

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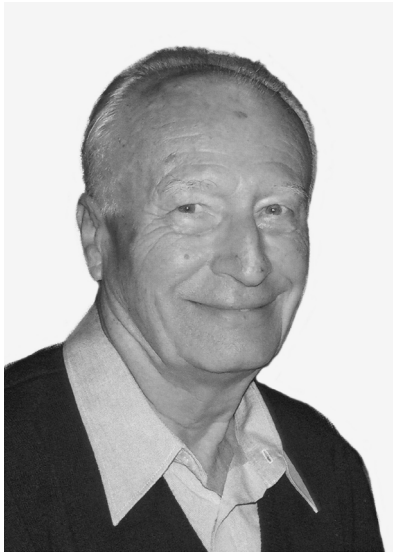
In memoriam:
prof. Dr. Dušan Zavodnik (1934–2023)



Spoštovane kolegice in kolegi,
obveščamo vas, da je v torek, 18. julija 2023, v 89. letu starosti preminil prof. dr. Dušan Zavodnik, izjemni hrvaški zoolog, sistematik, biogeograf in biocenolog.

Dr. Zavodnik se je rodil 30. decembra 1934 v Ljubljani, kjer je leta 1954 končal gimnazijo. Diplomiral je iz biologije na Naravoslovni fakulteti Univerze v Ljubljani leta 1985 pod vodstvom prof. J. Hadžija, leta 1964 pa je doktoriral na Biotehniški fakulteti Univerze v Ljubljani pod mentorstvom prof. M. Zeia.
Interes za znanstveno delo in življenje v morju je kazal že kot srednješolec, saj je med poletnimi počitnicami opravljal prakso v laboratorijih Inštituta v Rovinju in Splitu.
Leta 1960 se je zaposlil v novo ustanovljenem Inštitutu za biologijo morja Jugoslovanske akademije znanosti in umetnosti (JAZU) v Rovinju, leta 1964 pa je postal tudi direktor inštituta. Leta 1968 je ustanovil Laboratorij za ekologijo in sistematičnost, leto kasneje pa se je njegov laboratorij pridružil novo ustanovljenemu oddelku Centra za raziskovanje morja Rovinj - Zagreb (CIM Rovinj - Zagreb) v okviru Inštituta Ruđer Bošković (IRB). Po razdelitvi oddelka CIM Rovinj - Zagreb v samostojne organizacijske enote v okviru IRB je bil dr. Zavodnik v obdobju od 1981 do 1984 direktor rovinjskega dela.
Leta 1975 je postal znanstveni svetovalec. Kot redni profesor na Univerzi v Zagrebu je predaval na treh kolegijih podiplomskega študija biologije morja in bil mentor številnih diplomskih, magistrskih in doktorskih del. Leta 1999 se je upokojil, a je nadaljeval z delom vse do konca življenja.

Znanstveni opus dr. Zavodnika je obsežen in raznolik. Objavil je več kot 150 izvirnih znanstvenih člankov v domačih in tujih revijah ter več deset strokovnih člankov, predvsem o biologiji morja in zaščiti Jadrana. Organiziral je šest mednarodnih znanstvenih konferenc, objavljal je tudi v strokovnih revijah in dnevnem tisku. Bil je član več bioloških društev in je bil v enem mandatu predsednik Jugoslovanskega društva biosistematikov.
Osrednje področje njegovih raziskav so bili kompleksni procesi v obalnih in odprto-morskih ekosistemih vzhodnega dela Jadranskega morja. Posebno pozornost je namenil bodičarjem, školjkam in ribam Jadrana ter življenjskim skupnostim litoralnih stopnic. Aktivno se je ukvarjal tudi s zgodovino raziskav Jadrana, predvsem z zgodovino matične ustanove v Rovinju. Po upokojitvi se je posvetil biotski raznovrstnosti in biogeografiji favne Jadrana.
Med celotno svojo poklicno kariero je terensko delo štel za temelj svojega raziskovalnega in pedagoškega delovanja. Zgodaj se je zanimal za samostojno potapljanje, ki ga je štel za skorajda neizogibno metodo terenskega dela vsakega "morskega" biologa. Zato ga lahko smatramo za pionirja in začetnika znanstvenega potapljanja v Rovinju. Bogato znanje in izkušnje terenskega dela je nesebično delil s svojimi sodelavci in študenti.
Dr. Dušan Zavodnik je bil pokopan v četrtek, 18. julija 2023, v Rovinju.

dr. Andrej Jaklin

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