DJELOVANJE STROBILURINSKIH FUNGICIDA NA BIOKEMIJSKE, STANIČNE I POPULACIJSKE BIOMARKERE ENHITREJA VRSTA Enchytraeus crypticus i Enchytraeus albidus

Kovačević, Marija

Doctoral thesis / Disertacija

2023

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Science / Sveučilište u Zagrebu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:217:097712

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET BIOLOŠKI ODSJEK

Marija Kovačević

DJELOVANJE STROBILURINSKIH FUNGICIDA NA BIOKEMIJSKE, STANIČNE I POPULACIJSKE BIOMARKERE MALE ENHITREJE (*Enchytraeus crypticus*) i BIJELE ENHITREJE (*Enchytraeus albidus*)

DOKTORSKI RAD

Zagreb, 2022

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET BIOLOŠKI ODSJEK

Marija Kovačević

DJELOVANJE STROBILURINSKIH FUNGICIDA NA BIOKEMIJSKE, STANIČNE I POPULACIJSKE BIOMARKERE ENHITREJA VRSTA *Enchytraeus crypticus* i *Enchytraeus albidus*

DOKTORSKI RAD

Mentor: prof.dr.sc. Branimir K. Hackenberger

Zagreb, 2022

University of Zagreb

FACULTY OF SCIENCE DIVISION OF BIOLOGY

Marija Kovačević

EFFECT OF STROBILURIN FUNGICIDES ON BIOCHEMICAL, CELLULAR AND POPULATION BIOMARKERS OF *Enchytraeus crypticus* AND *Enchytraeus albidus*

DOCTORAL THESIS

Supervisor: Branimir K. Hackenberger, PhD, Professor

Zagreb, 2022

Ovaj doktorski rad izrađen je na Odjelu za biologiju Sveučilišta Josipa Jurja Strossmayera u Osijeku na Zavod za kvantitativnu ekologiju u Laboratoriju za analizu bioloških sustava, pod vodstvom prof.dr.sc. Branimira K. Hackenbergera, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Istraživanja su provedena u okviru projekta „Različiti učinci okolišno relevantnih mješavina metal temeljenih nanočestica i pesticida na faunu tla: Nove smjernice za procjenu rizika (IP-09-2014-4459, voditelj projekta: B.K. Hackenberger)“ i projekta „Razvoj karijera mladih istraživača – izobrazba novih doktora znanosti“ (DOK-2018-01-4257) financiranih sredstvima Hrvatske zaklade za znanost (HrZZ).

Informacije o mentoru

HACKENBERGER, Branimir K. (Osijek, 9. rujan 1965), redoviti profesor

Matični broj znanstvenika: 221360

Prof. dr. sc. Branimir Hackenberger Kutuzović redoviti je profesor na Odjelu za biologiju, Sveučilišta Josipa Jurja Strossmayera u Osijeku. Rođen je u Osijeku gdje je završio temeljno obrazovanje i studij biologije i kemije. Doktorsku disertaciju izradio je na Institutu Ruđer Bošković, a titulu doktora znanosti iz područja biologije stekao je 2001. godine na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu. Nositelj je mnogobrojnih kolegija na preddiplomskom i diplomskim studijima Odjela za biologiju u Osijeku, kao i na poslijediplomskom studiju Zaštita prirode i okoliša u Osijeku. Osnivač je Laboratorija za analizu bioloških sustava te predstojnik Zavoda za kvantitativnu ekologiju Odjela za biologiju Sveučilišta Josipa Jurja Strossmayera u Osijeku.

Znanstveni rad Branimira K. Hackenbergera usmjeren je na istraživanja kvantitativnih ekotoksikoloških i ekoloških procesa, njihovu karakterizaciju u ekološkim sustavima te istraživanja ranjivih sektora prirodnih ekoloških sustava i bioraznolikosti, ali i upravljanje rizicima. Nadalje, bavi se matematičkim modeliranjem, strojnim učenjem, prikupljanjem i analizom velikih skupova podataka (Big Data), te optimizacijom sustava primjenom statističkih metoda. Autor je više od 70 znanstvenih radova, od čega su 64 rada objavljena u časopisima koje referira baza Current Contents. Osim toga, autor je poglavlja u knjizi, te softverskog rješenja. Član je pet strukovnih udruga u Hrvatskoj i u inozemstvu, te recenzent u brojnim časopisima citiranim u Current Contents bazi.

Zahvale

….

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorski rad

DJELOVANJE STROBILURINSKIH FUNGICIDA NA BIOKEMIJSKE, STANIČNE I POPULACIJSKE BIOMARKERE ENHITREJA VRSTA *Enchytraeus crypticus* i *Enchytraeus albidus*

MARIJA KOVAČEVIĆ

Odjel za biologiju, Sveučilište Josipa Jurja Strossmayera u Osijeku

Enhitreje su bliski srodnici gujavica te modelni organizmi u ekotoksikološkim istraživnjima tla. Iako imaju važnu ulogu u ekološkim sustavima tla, djelovanje pojedinih zagađivala, posebice strobilurinskih fungicida nedovoljno je istraženo. Stoga je cilj ovog doktorskog rada nadopuniti postojeća istraživanja novim znanjima o utjecaju strobilurinskih fungicida na enhitreje vrsta *E. crypticus* i *E. albidus*. Razvijena je metoda za mjerenje promjena aktivnosti mehanizma multiksenobiotske rezistentnosti čime se dokazala prisutnost ovog mehanizma kod enhitreja i mogućnost njegova korištenja kao biomarkera zagađenja. Nadalje, uočeno je negativno djelovanje strobilurinskih fungicida na preživljavanje, reprodukciju i uspjeh izlijeganja enhitreja. Prikupljena inicijalna znanja o djelovanju aktivne tvari i različitih pripravaka na biokemijske i stanične biomarkere ukazuju na moguće mehanizme djelovanja strobilurinskih fungicida naglašavajući važnost sveobuhvatnog pristupa.

(121 stranica, 7 slika, 9 tablica, 213 literaturnih navoda, jezik izvornika: hrvatski)

Ključne riječi: enhitreje, strobilurinski fungicidi, reprodukcija, oksidativni stres, energetski status

Mentor: prof.dr.sc. Branimir K. Hackenberger

Ocjenjivači: prof. dr. sc. Goran Klobučar

prof. dr. sc. Ivana Maguire

prof. dr. sc. Maja Šegvić Klarić

University of Zagreb
Faculty of Science
Department of Biology

Doctoral thesis

EFFECT OF STROBILURIN FUNGICIDES ON BIOCHEMICAL, CELLULAR AND POPULATION BIOMARKERS OF *Enchytraeus crypticus* AND *Enchytraeus albidus*

MARIJA KOVAČEVIĆ

Department of Biology, Josip Juraj Strossmayer University in Osijek

Enchytraeids are close relatives of earthworms and model organisms of soil ecotoxicology. Although they play an important role in soil ecosystems, the effects of certain pollutants, especially strobilurin fungicides, have not been sufficiently investigated. Therefore, the main objective of this doctoral thesis is to supplement existing research with new knowledge about the influence of strobilurin fungicides on the enchytraeid species *E. crypticus* and *E. albidus*. The development of a method for measuring changes in the activity of the multixenobiotic resistance mechanism proved the presence of this mechanism in enchytraeids and the possibility of its use as a biomarker. Furthermore, a negative effect of strobilurin fungicides was observed on the survival, reproduction, and hatching success of enchytraeids. Initial knowledge collected on the effect of the active ingredient and different formulations on biochemical and cellular biomarkers indicates the possible mechanisms of action of strobilurin fungicides, emphasizing the importance of a comprehensive approach.

(121 pages, 7 figures, 9 tables, 213 references, original in Croatian)

Keywords: enchytraeid, strobilurin fungicides, reproduction, oxidative stress, energy status

Supervisor: Branimir K. Hackenberger, PhD, Professor

Reviewers: Goran Klobučar, PhD, Professor

Ivana Maguire, PhD, Professor

Maja Šegvić Klarić, PhD, Professor

SADRŽAJ

POPIS PUBLIKACIJA

Kovačević M, Hackenberger DK, Lončarić Ž, Hackenberger BK (2021) Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology. Chemosphere. 279:130549. https://doi.org/10.1016/j.chemosphere.2021.130549

Kovačević M, Hackenberger DK, Hackenberger BK (2021) Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifoxystrobin) on survival, reproduction and hatching success of Enchytraeus crypticus. Science of the Total Environment. 790:148143. https://doi.org/10.1016/j.scitotenv.2021.148143

Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Toxicity of fungicide azoxystrobin to Enchytraeus albidus: differences between the active ingredient and formulated product. Pesticide Biochemistry and Physiology. 187: 105198. https://doi.org/10.1016/j.pestbp.2022.105198

Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Comprehensive study of the effects of strobilurin-based fungicide formulations on *Enchytraeus albidus*. Ecotoxicology, 31:1554–1564 https://doi.org/10.1007/s10646-022-02609-4

1. UVOD

Funkcionalnost ekoloških sustava tla ključna je za kopnene sustave, a svaki rizik od narušavanja stabilnosti ekoloških sustava tla treba shvatiti ozbiljno. Informacije o utjecaju pojedinih stresora i zagađivala na organizme tla izrazito su važne, ali nedostatne. Naime, kompleksnost tla kao medija i ograničena dostupnost organizama koji se nalaze u tlu, čine istraživanja ekoloških sustava tla izrazito složenima. Razvoj znanosti i tehnologije doveo je do razvoja ekotoksikologije tla, grane znanosti koja omogućava prikupljanje novih znanja koja vode ka točnijem predviđanju utjecaja okolišnih stresora i zagađivala na organizme tla, ali i čitave ekološke sustave.

Razvoj ekotoksikologije tla je započeo 1960-ih godina prvim opažanjima negativnog utjecaja pesticida na beskralježnjake tla (van Gestel, 2012). Uz akvatičku, terestričku, te ekotoksikologiju sedimenata, ekotoksikologija tla čini jednu od glavnih grana ekotoksikologije (Zhou i sur., 2019). Ekotoksikologija je multidisciplinarna znanost, koja integrira toksikologiju i ekologiju. Njen primarni cilj je detektirati i predvidjeti učinke zagađenja u kontekstu svih ostalih okolišnih čimbenika, kako bi se spriječio negativan utjecaj na strukturu, funkciju i bioraznolikost ekoloških sustava tla (Tarazona i Ramos-Peralonso, 2014; Zhou i sur., 2019). Učinak različitih zagađivala proučava se na više razina složenosti, u rasponu od biomolekula, stanica, organa i organskih sustava sve do razine organizama, populacija, ekoloških sustava i ukupne biosfere. Osim toga, ekotoksikologija proučava kretanje zagađivala u okolišu, uključujući njihovu migraciju, transformaciju i degradaciju. Ekotoksikologija tla usredotočuje se na utjecaj zagađivala i stresora na organizme ili populacije organizama koje posljedično djeluju na cjelokupne zajednice. Nadalje, procjena utjecaja na nižim razinama organizacije često omogućuje predviđanje utjecaja na višim organizacijskim razinama. Stoga se utjecaj na jedinke koristi kao početna točka ekotoksikologije. Naime, na taj način određuju se krajnje točke koje su lako mjerljive, a mogu predvidjeti utjecaj na čitave populacije. Najčešće korištene krajnje točke su preživljavanje, reprodukcija i produkcija biomase koja se procjenjuje kao stopa rasta.

Krajnje točke na nižim organizacijskim razinama uglavnom se određuju tijekom testova u kontroliranim laboratorijskim uvjetima, dok se istraživanje i mjerenje krajnjih točaka na višim organizacijskim razinama najčešće provodi pomoću mikro i mezo kozmičkih sustava te istraživanjima u okolišu. Kako bi se odredile promjene različitih krajnjih točaka razvijen je čitav niz analitičkih metoda i posebnih protokola za dokumentiranje utjecaja. Biomarkeri su mjerljive molekularne, stanične, fiziološke ili biokemijske promjena kod jedinki. Upravo se pomoću biomarkera mogu kvantificirati učinci izloženosti prije pojave vidljivog štetnog utjecaja. Kako bi biomarkeri bili prikladni za korištenje u ekotoksikologiji moraju biti

učinkoviti (niska cijena i jednostavna primjena), precizni, odgovarajuće osjetljivosti i dosljednosti, te moraju imati sposobnost generiranja jasnih rezultata.

Kako bi se omogućila procjena utjecaja zagađivala na ekološke sustave tla, 80-ih godina prošlog stoljeća započeo je razvoj testova toksičnosti. Prvi protokol za provođenje kratkotrajnih (akutnih) testova toksičnosti koji je koristio gujavice kao modelne organizme publiciran je 1984. godine (OECD, 1984). U narednim godinama istraživana je moguća upotreba različitih organizama tla, te se pokazalo kako su gujavice, enhitreje, skokuni i grinje najpogodnije skupine. Iako je upotreba gujavica i danas najzastupljenija, sve važniju ulogu preuzimaju enhitreje. Naime, specifičnosti ove skupine organizama omogućuju provođenje ekotoksikoloških testova različitog dizajna, čime se povećava broj promatranih krajnjih točaka, ali i pruža mogućnost za korištenje novih biomarkera.

1.1. Enhitreje – modelni organizmi ekotoksikologije tla

Tlo predstavlja osnovu i temelj svakog kopnenog ekološkog sustava, neovisno o razini antropogenih promjena, te je najvažniji izvor bioraznolikosti koji odražava metabolizam cjelokupnog ekološkog sustava budući da se u njemu isprepliću svi biogeokemijski procesi različitih komponenti ekoloških sustava (Menta, 2012).

Zbog iznimne brojnosti, ali i izrazite funkcionalne i taksonomske raznolikosti beskralježnjaci su sastavni dio ekoloških sustava tla. Upravo su beskralježnjaci ključni dio hranidbenih mreža u tlu, te igraju neizostavnu ulogu u regulaciji funkcije ovog ekološkog sustava. Generalno, organizme tla s obzirom na veličinu dijelimo na mikrofaunu, mezofaunu, makrofaunu i megafaunu. Mikrofaunu čine organizmi čija je veličina tijela između 20 μm i 200 μm (Wallwork, 1970) kao što su sitne grinje (Acarina), oblići (Nematoda), kolnjaci (Rotifera), dugoživci (Tardigrada), te veslonošci (Copepoda). U kategoriju mezofaune ubrajamo sve organizme čija je veličina tijela između 200 μm i 2 mm (Wallwork, 1970). Enhitreje (Enchytraeus), te mikročlankonošci poput grinja i skokuna (Collembola) glavni su predstavnici ove skupine. Uz njih u skupinu mezofaune ubrajamo oblića, kolnjake, dugožicve, manje paučnjake (Arachnida), pseudoškorpione (Pseudoscorpiones), ličinke kukaca (Insecta), male jednakonožce (Isopoda) i stonoge (Myriapoda). Makrofaunu čine organizmi veličine od 2 mm do 20 mm (Wallwork, 1970). Ovoj kategoriji pripadaju neke gujavice (Lumbricidae), puževi (Gastropoda), jednakonožci, stonoge, paučnjaci i većina kukaca. Najveću skupinu čine organizmi čija veličina prelazi 20 mm, a nazivamo ih megafauna tla.

Uz skokune i grinje, enhitreje su najvažniji pripadnici mezofaune tla. Pripadaju koljenu kolutićavaca (Annelida), razredu kolutićavaca (Clitellata) i porodici Enchytraeidae (Tablica 1).

Tablica 1 - Klasifikacija enhitreja

	Klasifikacija
Carstvo	Životinje (Animalia)
Koljeno	Kolutićavci (Annelida)
Razred	Pojasnici (Clitellata)
Red	Haplotaxida
Porodica	Enchytraeidae

Ovu slabo poznatu, ali izrazito važnu skupinu beskralježnjaka tla odlikuje bilaterno simetrično tijelo bez pigmentacije ili blijedo žute boje, duljine nekoliko mm do nekoliko cm. Poput gujavica, enhitreje imaju razvijene četine koje služe za oslanjanje pri kretanju, a nalazimo ih na svim kolutićima osim prvog i posljednjeg. Enhitreje su saprofazi koji igraju izrazito važnu ulogu u razgradnji organske tvari, održavanju poroziteta i strukture tla, ali i kruženju nutrijenata i energije unutar tla (Briones, 2014). Prisutne su u ekološkim sustavima diljem svijeta, od tropskih do polarnih regija. Preferiraju šumska i livadna tla bogata organskom tvari, ali ih se može pronaći i u krškim zajednicama, unutar raspadajućih stabala, močvara, duž riječnih obala i obala mora, kao i na dnu jezera i oceana. Svojom aktivnošću enhitreje stvaraju pogodna mikrostaništa za rast i razvoj različitih skupina mikroorganizama (Bardgett i sur., 2005). Posebnu važnost enhitreje imaju u područjima poput Arktika i subarktičkih tundri, močvara, planinskih travnjaka, crnogoričnih šuma i poljoprivrednih površina u zoni tajgi gdje su gujavice odsutne (Swift i sur., 1998). Osim toga, dokazano je da pod pritiskom intenzivnog oranja i obrade tla dolazi do smanjenja brojnosti gujavica, dok se brojnost i aktivnost enhitreja povećava (Topoliantz i sur., 2000).

Danas je poznato više od 700 vrsta enhitreja koje su podijeljene u 33 roda (Schmelz i Collado 2010). Enhitreje su uglavnom hermafroditi, međutim neke se vrste mogu razmnožavati fragmentacijom, partenogenezom ili samooplodnjom (Amorim i Scott-Fordsmand, 2021). Glavnina vrsta se razmnožava spolnim putem tako što proizvode spermije i jajašca, međusobno se oplođuju i polažu kokone.

Vrste roda *Enchytraeus* zasigurno su najpoznatije vrste enhitreja koje se zbog svojih osobina učestalo koriste u ekotoksikološkim istraživanja. Međutim, prije uvrštavanja na popis indikatorskih organizama, odabrane vrste enhitreja morale su ispuniti propisane uvijete. Naime,

indikatorski organizmi koji se koriste u ekotoksikološkim istraživanjima moraju biti prisutni u različitim tipovima tla gdje se njihova pojavnost i brojnost mogu povezati s kvalitetom i očuvanosti ekosustava. Osim toga njihovo izlaganje u kontroliranim laboratorijskim uvjetima treba rezultirati informacijama o mogućim utjecajima zagađivala koje su primjenjive na cjelokupne ekološke sustave tla. Stoga indikatorski organizmi moraju ispunjavati sljedeće uvijete:

imaju ključnu ulogu u funkciji ekoloških sustava tla, a njihov odgovor na promjene u ekološkim sustavima i prisutnost zagađivala relevantan je za zaključke koji se odnose na nivo cijelog ekološkog sustava;
prisutni su u čitavom nizu ekoloških sustava tla diljem svijeta, što omogućuje usporedbu pojava između različitih ekoloških sustava;
iznimno su brojni u ekološkim sustavima tla, što omogućava lako opažanje utjecaja zagađivala i okolišnih stresora;
jednostavni su za rukovanje, što omogućava lak uzgoj u laboratorijskim uvjetima i jednostavno prikupljanje na terenu;
dolaze u dodir sa zagađivalima koja se nalaze u različitim fazama tla (tekuća, čvrsta i plinovita faza u tlu);
dovoljno su osjetljivi kako bi bila moguća detekcija negativnih utjecaja zagađivala i okolišnih stresora, ali ne pretjerano osjetljivi kako bi se izbjeglo ugibanje tijekom laboratorijskih testova ili izumiranje u okolišnim uvjetima prilikom izloženosti zagađivalima ili okolišnim stresorima (Didden i Römbke, 2001).

Dosadašnja istraživanja pokazala su mogućnost upotrebe različitih grupa organizama kao indikatora. Pogodni indikatorski organizmi koji pripadaju mikrofauni tla su praživotinje i oblići. Razlog tome leži u činjenici kako se pripadnici ovih skupina učestalo pojavljuju u različitim ekološkim sustavima, dovoljno su osjetljivi na izloženost zagađivalima i okolišnim stresorima, ali dolaze u dodir samo s tekućom fazom tla (Didden i Römbke, 2001). Osim toga, njihova veličina otežava manipulaciju tijekom terenskih, ali i laboratorijskih istraživanja. Nedostatak mikro i makro člankonožaca kao bioindikatora je njihov izostanak dodira s tekućom fazom tla, zbog čega se dodir sa zagađivalima odvija samo kroz čvrstu i plinovitu fazu. Najpoznatiji modelni organizmi koji se koriste u ekotoksikološkim testovima zasigurno su gujavice (Solé, 2020; van Gestel, 2012). Osim što imaju izrazito važnu ulogu u ekološkim sustavima tla, gujavice se lako uzgajaju u laboratorijskim uvjetima, te su izložene svim fazama u tlu. Često, a ponekad i jedino korištenje vrsta *E. fetida* i *E. andrei* rezultiralo je činjenicom da se mnoga

ekotoksikološka istraživanja provedena s ovim vrstama smatraju manje relevantnima. Naime, ove vrste ne nalazimo u prirodnim ekološkim sustavima tla, već na kompostištima gdje je prisutna iznimno velika količina organske tvari. Razlog čestog korištenja ove dvije vrste leži u činjenice što nativne vrste često imaju izrazito dug životni ciklus, te zahtijevaju specifične uvjete koji otežavaju njihov uzgoj (Römbke i Moser, 1999).

Enhitreje ispunjavaju sve navedene kriterije, te su stoga prikladni modelni organizmi ekotoksikologije tla (Römbke 2003). Početkom 2000-tih krenuo je razvoj standardiziranih laboratorijskih testova koji omogućuju procjenu utjecaja zagađivala i okolišnih stresora na preživljavanje i reprodukciju enhitreja (Römbke i Moser, 2002). Prva laboratorijska istraživanja temeljena na enhitrejama provedena su u vodenom mediju i na agaru (Römbke i Knacker, 1989; Westheide i sur., 1989), te u različitim tipovima tla (Lock i Janssen, 2001; Lock i Janssen, 2002a; Römbke, 1988). Prema protokolima Organizacije za ekonomsku suradnju i razvoj (OECD) preporučena vrsta za provođenje testova reprodukcije je *Enchytraeus albidus* (OECD, 2016), dok se kao alternativne vrste navode *E. luxuriousus* (Lock i Janssen, 2002b), *E. buchholzi* (Jarratt i Thompson, 2009), *E. crypticus* (Castro-Ferreira i sur., 2012), te *E. bulbosus* (Yang i sur., 2021) (Tablica 2).

Tablica 2 - Specifičnosti vrsta roda *Enchytraeus*.

Vrsta	Generacijsko vrijeme*	Temperatura	Fekunditet*	Izvor
E. albidus	33	18°C	120	OECD 2016 ISO, 2014 Amorim i sur., 2005
E. buchholzi	26	20°C	400-600	Voua i sur., 2014 Didden 1991
E. bulbosus	18	18°C	100	Yang i sur., 2021
E. crypticus	18	21°C	200-500	Bicho i sur., 2015 Westheide i Graefe, 1992
E. luxuriosus	19-32	20-21	50	Amorim i sur 2005 Schmelz i Collado, 1999

*Generacijsko vrijeme izraženo je u danima, a fekunditet kao opaženi broj juvenilnih jedinki nakon izlaganja 10 odraslih jedinki u uvjetima standardiziranima za pojedinu vrstu.

Zbog specifičnosti životnog ciklusa pojedinih vrsta enhitreja ekotoksikološki testovi se provode u kraćem vremenskom periodu uz utrošak manje tla i kemikalija u usporedbi s gujavicama. Glavnina suvremenih ekotoksikoloških istraživanja provedena je s dvije najpoznatije vrste enhitreja, *E. albidus* i *E. crypricus*.

1.1.1. *Enchytraeus albidus*

E. albidus je jedna od najpoznatijih i najvećih vrsta roda *Enchytraeus*. Nepigmentirano ili blijedo žuto, segmentirano tijelo obično je dugo između 20 i 30 mm (40 do 65 segmenata), te može težiti do 5 mg (Schmelz i Collado, 2010) (Slika 1).

Slika 1 – *Enchytraeus albidus*. Odrasla jedinka s vidljivim klitelumom. Foto: M. Kovačević

Vrsta *E. albidus* nastanjuje površinske slojeve tla s visokim udjelom organske tvari, te je zastupljena u raznolikim staništima od umjerenih regija do Arktika (Christensen i Dózsa Farkas, 2006). Zbog izrazito raznolikih staništa pojedine populacije ove vrste toleriraju velike fluktuacije u salinitetu i temperaturi između smrzavanja i odmrzavanja. Istraživanja ovog fenomena pokazala su kako *E. albidus* podnosi temperature do -20°C, te se prilagođava salinitetu do 50‰ (Patrício Silva i sur., 2013). Naime u tako ekstremnim uvjetima ove enhitreje sintetiziraju izrazito visoke koncentracije glukoze koja preuzima ulogu krioprotektanta.
Generacijsko vrijeme vrste *E. albidus* je 33 dana pri 18°C i 74 dana pri 12°C (ISO 2014). Prije korištenja u ekotoksikološkim testovima enhitreje vrste *E. albidus* moraju minimalno 5 tjedana provesti u kontroliranim laboratorijskim uvjetima. Taj period se zove aklimatizacijski period. Osim toga, za reprodukcijski test koriste se samo odrasle jedinke s dobro razvijenim klitelumom

i vidljivim jajima. Ove enhitreje svoje kokone oblažu sitnim česticama tla što otežava njihovo uočavanje. Embrionalni razvoj vrste *E. albidus* u prosjeku traje 14 dana.

1.1.2. *Enchytraeus crypticus*

Nepigmentirano tijelo vrste *E. crypticus* sastoji se od najviše 34 segmenta i doseže duljinu između 3 i 12 mm (Schmelz i Collado, 2010) (Slika 2). Za razliku od vrste *E. albidus*, *E. crypticus* se može uzgajati na pločama agara, ima veću stopu reprodukcije, te kraće generacijsko vrijeme (18 dana) što rezultira kraćim vremenom potrebnim za provođenje testova (Castro-Ferreira i sur., 2012). Nadalje, *E. crypticus* ima veću toleranciju na čitav niz okolišnih faktora kao što su pH (4.4-8.2) i tekstura tla (udio gline od 1-29%), te udio organske tvari (1.2 - 42%) (van Gestel i sur., 2011; Kuperman i sur., 2006).

Slika 2 – *E. crypticus*. Kultura organizama uzgojena na tlu. Foto: M. Kovačević

Posebnost ove vrste su kokoni s prozirnom ovojnicom koja omogućava promatranje različitih stadija embrionalnog razvoja. Embrionalni razvoj vrste *E. crypticus* traje 9 dana. Kako je generacijsko vrijeme vrste *E. crypticus* gotovo dvostruko kraće nego *E. albidus*, vrijeme trajanja standardiziranog reprodukcijskog testa smanjeno je s 6 tjedana (standardno vrijeme za vrstu *E. albidus*) na 3 tjedna (van Gestel i sur., 2011).

1.1.3. Primjena enhitreja u ekotoksikološkim istraživanjima

Enhitreje se primjenjuju u ekotoksikološkim istraživanjima više od 20 godina (Amorim i Scott-Forsmand, 2021). Postoje standardizirani protokoli za procjenu utjecaja zagađivala na

preživljavanje i reprodukciju (OECD, 2016; ISO, 2014), bioakumulaciju (OECD, 2010; Amorim i sur 2002), izbjegavanje (ISO, 2008) i čitav niz testova koji omogućuju procjenu dodatnih krajnjih točaka. Neki od njih su testovi embriotoksičnost (Gonçalves i sur., 2015), testovi izlijeganja (Bicho i sur., 2017a; Bicho i sur., 2017b; Santos i sur., 2017; Bicho i sur. 2016), višegeneracijski testovi (Ribeiro i sur 2019; Bicho i sur., 2017b), cjeloživotnih testovi (Gonçalves i sur., 2017), testovi u sustavu više vrsta (engl. Multispecies test systems) (Mendes i sur., 2019; Mendes i sur., 2018; Menezes-Oliveira i sur., 2014; Menezes-Oliveira i sur., 2013), histološki testovi (Bicho i sur., 2021), te testovi koji proučavaju utjecaj na antioksidativni sustav (Maria i sur., 2018; Gomes i sur., 2012; Howcroft i sur., 2011; Howcroft i sur., 2009) i ukupnu energiju organizama (Gomes i sur., 2015a; Gomes i sur., 2015b).

Ekotoksikološka istraživanja temeljena na enhitrejama usmjerena su uglavnom na djelovanje različitih metala i nanočestica. Među njima je zasigurno najbolje proučen utjecaj nanočestica srebra (Bicho i sur., 2021; Mendonça i sur., 2020; Maria i sur., 2018; Topuz i van Gestel, 2017; Bicho i sur., 2016; Ribeiro i sur., 2015; Gomes i sur., 2013). Istraživanja utjecaja nanočestica srebra obuhvatila su mnogobrojne krajnje točke poput preživljavanja, reprodukcije, rasta, uspjeha izlijeganja, bioakumulacije, transfera energije, ponašanja izbjegavanja, lokomotorne aktivnosti, aktivnosti hranjenja, biomarkera oksidativnog stresa, genske ekspresije, genotoksičnosti, imunotoksičnosti, citotoksičnosti te embriotoksičnosti. Iako je utjecaj pesticida na enhitreje također istražen (da Rocha i sur 2020; Chelinho i sur., 2014; Novais i sur., 2014; Novais i sur., 2013; Novais i sur., 2011; Novais i sur., 2010; Kobetičová i sur., 2009; Amorim i sur., 2002; Puurtinen i sur., 1997) velik dio istraživanja usmjeren je na insekticide poput dimetoata, dobro poznate fungicide iz skupine konazola ili modelne fungicide poput karbendazima. Dokazano je kako osim negativnog utjecaja na preživljavanje i reprodukciju pesticidi djeluju na izbjegavanje, uzrokuju disbalans antioksidativnog sustava koji uzrokuje pojavu oksidativnog stresa, te djeluju na količine dostupne energije. Međutim, postojeća istraživanja nisu dostatna za točno razumijevanje mehanizama i dugoročnih učinaka pojedinih pesticida. Osim toga, novije skupine fungicida, koje zauzimaju sve važniju ulogu u poljoprivredi te se zbog izuzetne učinkovitosti primjenjuju u sve većim količinama, nisu dovoljno istražene. To je slučaj i sa strobilurinskim fungicidima, za koje postoje samo saznanja o negativnom utjecaju na preživljavanje i reprodukciju (Gomes i sur., 2021; Leitão i sur., 2014) te utjecaj na mikrobne zajednice probavila enhitreja (Zhang i sur., 2022; Zhang i sur., 2019). Međutim, sve veća pažnja znanstvene zajednice usmjerena je na ovu skupinu fungicida. Naime, zbog prekomjerne primjene i negativnog utjecaja na neciljne organizme izrazito su važna

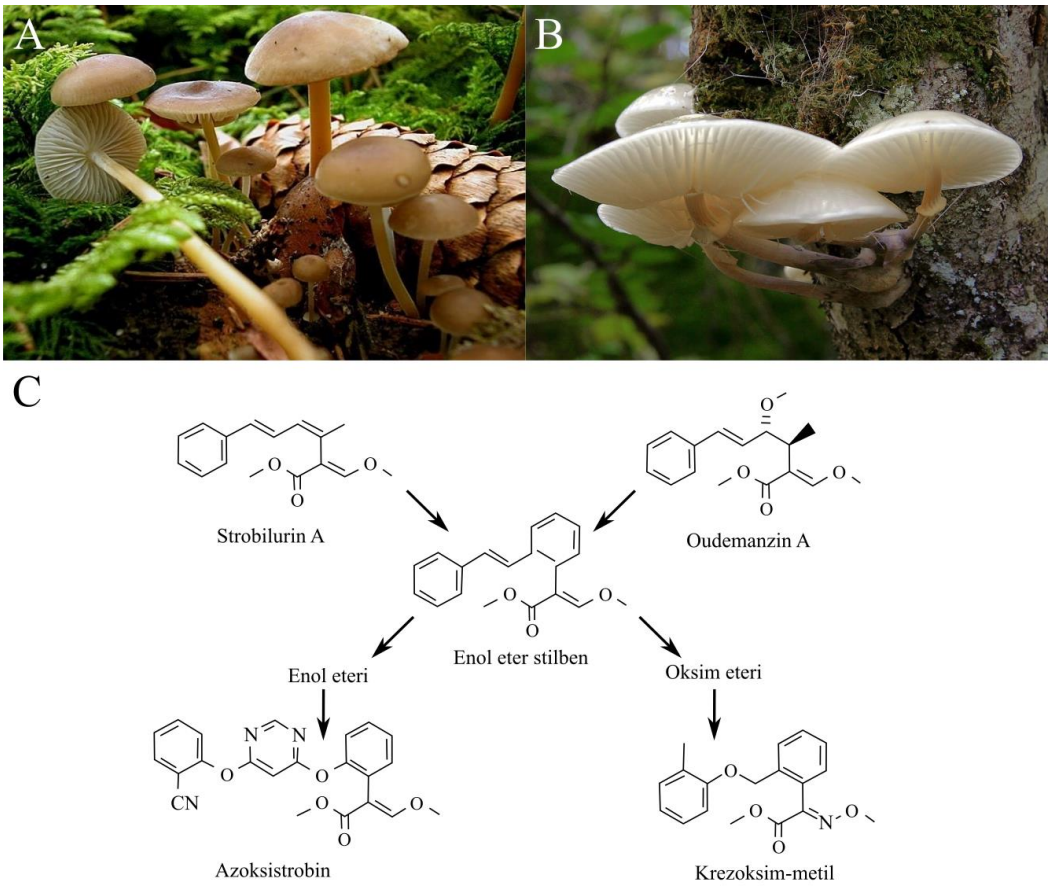
istraživanja koja će omogućiti jasniji uvid u mehanizme djelovanja i dugoročne učinke na populacije enhitreja u tlu.

1.2. Strobilurinski fungicidi

Upotreba pesticida u modernoj poljoprivredi nužna je za izbjegavanje negativnog djelovanja štetnika i osiguravanje dostatnih uroda (Wang i sur., 2021). Među štetnicima poljoprivrednih kultura prema učestalosti infestacija i nastaloj šteti ističu se gljivična oboljenja (Fisher i sur., 2012). Upravo je zbog toga za uspješnu poljoprivrednu proizvodnju ključna primjena fungicida (Strange i sur., 2005).

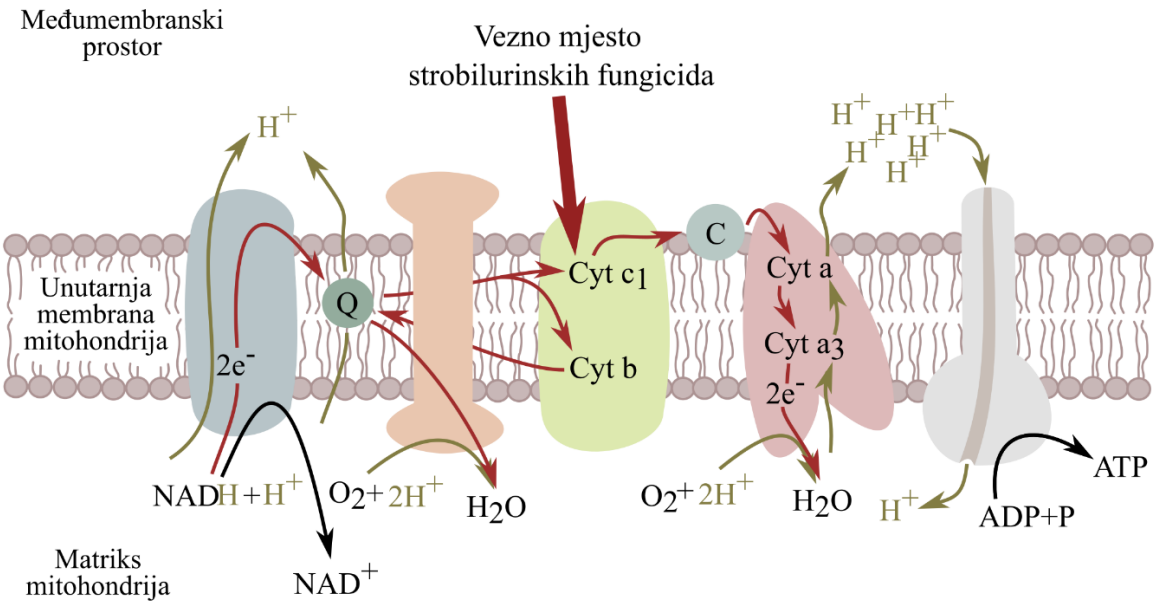
Zbog specifičnosti poljoprivredne proizvodnje i odabira usjeva Europa se smatra glavnim tržištem fungicida (Zubrod i sur., 2019). Naime, od godišnje količine prodanih proizvoda za zaštitu bilja, gotovo 40% čine fungicidi (Eurostat, 2022). Njihova primjena posebice je izražena u vinorodnim područjima, gdje udio fungicida doseže gotovo 90% primijenjenih proizvoda za zaštitu bilja (Roßberg, 2009). Iako su podatci o upotrebi fungicida na području Republike Hrvatske nepotpuni, analiza primjene sredstava za zaštitu bilja na području Osječko-baranjske županije pokazala je da se fungicidima godišnje tretira više od 70% poljoprivrednih površina (Matković, 2016).

Među fungicidima se zbog svoje izrazite efikasnosti i učestalosti primjene ističu strobilurinski fungicidi. Ova skupina fungicida predstavljaju novu prekretnicu nakon razvoja triazolnih fungicida, te su postali jedna od najprodavanijih skupina fungicida na globalnom tržištu (Wang i sur., 2021). Strobilurinski fungicidi temeljeni su na derivatima gljiva stapčarki iz koljena Basidiomycete (Bartlett i sur., 2002) koji su poslužili kao modeli za proizvodnju sintetskih molekula (Slika 3). Najpoznatiji među njima su strobilurin A i oudemazin, produkti gljiva *Strobilurus tenacellus* i *Oudemansiella mucida*. Čitava skupina dobila je ime prema strobilurinu A koji je najjednostavnija prirodna molekula iz koje su razvijene nove strukturne varijacije (Sauter i sur., 1996; Sauter i sur., 1995). Prvotno ime ove skupine bilo je β-metoksiakrilati ili β-MOA prema strukturi toksopore, aktivnog dijela molekule (Ypema i Gold, 1999).



Slika 3 – Nastanak strobilurina. Gljive stapčarke vrste *Strobilurus tenacellus* (A) i *Oudemansiella mucida* (B) iz koljena Basidiomycete čiji su derivati poslužili kao modeli za proizvodnju sintetskih molekula prvih strobilurinskih fungicida. (C) Proces sinteze azoksistrobina i krezoksim-metila iz strobilurina A, odnosno oudemazina (Prilagođeno prema Sauter i sur., 1995.).

Strobilurinski fungicidi inhibiraju respiraciju gljivičnih mitohondrija i blokiraju proizvodnju ATP-a (Zhang i sur., 2020a; Balba, 2007; Kim i sur., 2007; Bartlett i sur., 2002) (Slika 4). Naime, prilikom sinteze energije, energija iz hranjivih tvari najprije se pohranjuje u obliku visokoenergetskih elektrona: reduciranog nikotinamid-adenin dinukleotida (NADH) i reduciranog flavin adenin dinukleotida ($FADH_2$). Elektroni se nakon toga transportiraju kroz takozvani mitohondrijski respiratorni lanac, niz proteinskih kompleksa koji se nalaze uklopljeni u membranu mitohondrija (kompleks I-V), te završavaju ugrađene u krajnjeg elektronakceptora – molekulu kisika. Tijekom ovog procesa energija se pretvara u molekule ATP-a. Međutim, vezanjem strobilurinskih fungicida na takozvano Qo mjesto na citokromu bc1 (kompleks III) blokiran je transfer elektrona između citokroma b i citokroma c1 što u konačnici rezultira inhibicijom produkcije ATP-a (Bartlett i sur., 2002).



Slika 4 – Mehanizam djelovanja strobilurinskih fungicida. Protok elektrona kroz transportni lanac prenosi protone (H^+) preko unutrašnje mitohondrijske membrane. ATP-sintetaza (protonska pumpa) koristi gradijent protona za proizvodnju ATP-a, čime se stanica dobiva energijom za njezin metabolizam. U nekim gljivama i biljkama, inhibitori respiratornog puta i drugi faktori stresa induciraju sintezu alternativne oksidaze. Ovaj alternativni put disanja posredovan oksidazom preusmjerava elektrone na ubikinon (Q) i daje smanjenu osjetljivost na djelovanje strobilurina *in vitro*, ali stvara mnogo manje energije. Q = ubikinon, Cyt b = citokrom b, Cyt c1 = citokrom c1, C = citokrom c, Cyt a = citokrom a, Cyt a3 = citokrom a3. (Prilagođeno prema Ypema i Gold, 1999).

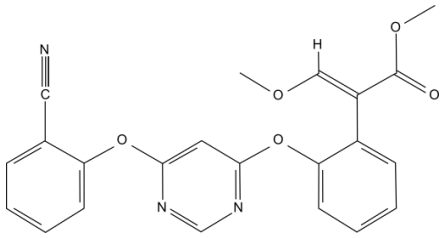
Nakon azoksistrobina razvijene su i registrirane aktivne tvari piraklostrobin, trifloksistrobin, krezoksim-metil, fluoksastrobin, pikoksistrobin i druge. O njihovoj efikasnosti i širokoj primjeni govori i podatak da su 2014. godine komercijalni pripravci temeljeni na azoksistrobinu i piraklostrobinu bili najprodavaniji fungicidi, dok su strobilurini bili jedna od najprodavanijih skupina s udjelom od 22,9% na ukupnom tržištu fungicida (Mao i sur., 2020; Li i sur., 2018; Lu, 2018; McDougall, 2015).

1.2.1. Azoksistrobin

Azoksistrobin je prvi strobilurinski fungicid koji je na tržištu od 1996. godine (Bartlett i sur., 2002), te se od tada uspješno koristi u prevenciji gljivičnih oboljenja. Njegova fizikalno-kemijska svojstva prikazana su u Tablici 3. Primjenjuje se na različitim usjevima, uključujući žitarice, krumpir, voće, orašaste plodove, vinovu lozu, lisnato povrće, ljekovito bilje i začine

(US EPA, 2022). Ponekad se primjenjuje na travnjacima, božićnim drvcima, pamuku, ali i ukrasnom bilju (US EPA, 2020). Azoksistrobin se također dodaje u smjesu prilikom izrade različitih zidnih obloga, kako bi spriječio razvoj plijesni (Cooper i sur., 2020).

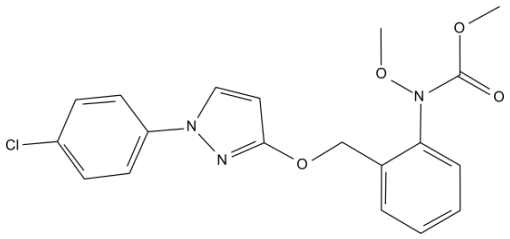
Tablica 3 – Fizikalno-kemijska svojstva azoksistrobina.

Strukturna formula:	
Kemijski naziv prema IUPAC-u:	methyl (E)-2-[2-[6-(2-cyanophenoxy)pyrimidin-4-yl]oxyphenyl]-3-methoxyprop-2-enoate
Kemijska formula:	$C_{22}H_{17}N_3O_5$
CAS registarski broj:	131860-33-8
Molarna masa:	403.4 g/mol
Fizikalno stanje:	bijela kristalna krutina
Gustoća:	1.33 g/cm^3
Topivost u vodi:	6 mg/L pri 20 °C
Talište:	116 °C
Tlak para:	8.3 x 10^{-13} mm Hg pri 25 °C
Particijski koeficijent (log Kow):	2.50 pri 20 °C
Adsorpcijski koeficijent (Koc):	207 - 594 mL/g

1.2.2. Piraklostrobin

Piraklostrobin (Tablica 4), slično kao i azoksistrobin, primjenjuje se na čitavom nizu usjeva među kojima su najzastupljenije žitarice, vinova loza, šećerna repa, soja i krastavci (Joshi i sur., 2014). Zbog visoke toksičnosti za akvatičke organizme primjena piraklostrobina zabranjena je u rižinim poljima (Jiang i sur., 2019a; Li i sur., 2019; Zhang i sur., 2017). Međutim, zbog izrazite efikasnosti i dalje se koristi na prostoru čitave Europe.

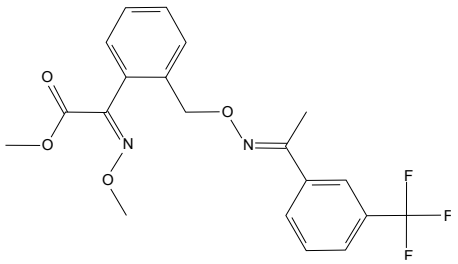
Tablica 4 – Fizikalno-kemijska svojstva piraklostrobina.

Strukturna formula:	
Kemijski naziv prema IUPAC-u:	methyl N-[2-[[1-(4-chlorophenyl)pyrazol-3-yl]oxymethyl]phenyl]-N-methoxycarbamate
Kemijska formula:	$C_{19}H_{18}ClN_3O_4$
CAS registarski broj:	175013-18-0
Molarna masa:	387.8 g/mol
Fizikalno stanje:	bijela ili svijetlo-bež kristalna krutina
Gustoća:	1.285 g/cm^3 pri 20 °C
Topivost u vodi:	1.9 mg/L pri 20 °C
Talište:	63.7 do 65.2 °C
Tlak para:	1.95 x 10^{-10} mm Hg pri 20 °C
Particijski koeficijent (log Kow):	3.99 pri 22 °C
Adsorpcijski koeficijent (Koc):	6 do 16 mL/g

1.2.3. Trifloksistrobin

Komercijalne pripravci temeljeni na trifloksistrobinu (Tablica 5) uglavnom se koriste za prevenciju gljivičnih bolesti na ukrasnom drveću i grmlju, te voćarskim i povrtlarskim kulturama. Iako se smatra da je trifloksistrobin slabo toksičan za sisavce, ptice i pčele, nedavna istraživanja pokazala su kako predstavlja veliki rizik za akvatičke organizme i organizme tla (Junges et al., 2012; Liu i sur., 2020).

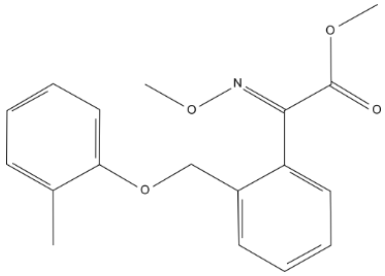
Tablica 5 – Fizikalno-kemijska svojstva trifloksistrobina.

Strukturna formula:	
Kemijski naziv prema IUPAC-u:	methyl (2Z)-2-methoxyimino-2-[2-[[(E)-1-[3-(trifluoromethyl) phenyl]ethylideneamino]oxymethyl]phenyl]acetate
Kemijska formula:	$C_{20}H_{19}F_3N_2O_4$
CAS registarski broj:	141517-21-7
Molarna masa:	408.4 g/mol
Fizikalno stanje:	bijeli prah
Gustoća:	1.36 cm^3 pri 21 °C
Topivost u vodi:	0.610 mg/L pri 25 °C
Talište:	72.9 °C
Tlak para:	2.55 x 10^{-8} mm Hg pri 25 °C
Particijski koeficijent (log Kow):	4.5 pri 25 °C
Adsorpcijski koeficijent (Koc):	351.5 do 811 mL/g

1.2.4. Krezoksim-metil

Krezoksim-metil (Tablica 6) je razvila tvrtka BSF, te je prvo registriran u Kini za prevenciju krastavosti lista i ploda jabuke uzrokovanih vrstom *Venturia inaequalis*, te pepelnice jabuke i krastavaca uzrokovanih vrstom *Podosphaera leucotricha* (Li i sur., 2006). Komercijalne pripravci temeljeni na krezoksim-metilu koriste se za suzbijanje gljivičnih oboljenja u vinogradarstvu, voćarstvu, povrtlarstvu i maslinarstvu.

Tablica 6 – Fizikalno-kemijska svojstva krezoksim-metila.

Strukturna formula:	
Kemijski naziv prema IUPAC-u:	methyl (2E)-2-methoxyimino-2-[2-[(2-methylphenoxy)methyl]phenyl]acetate
Kemijska formula:	$C_{18}H_{19}NO_4$
CAS registarski broj:	143390-89-0
Molarna masa:	313.3 g/mol
Fizikalno stanje:	bijeli kristali
Gustoća:	1.258 g/cm^3
Topivost u vodi:	2 mg/L pri 20 °C
Talište:	99.0 °C
Tlak para:	1.72 x 10^{-8} mm Hg pri 20 °C
Particijski koeficijent (log Kow):	3.40
Adsorpcijski koeficijent (Koc):	1,700 mL/g

1.2.5. Utjecaj strobilurinskih fungicida na neciljne organizme

Strobilurinski fungicidi odlikuju se relativno visokom topivosti u vodi, što može uzrokovati njihovo nakupljanje u vodi, sedimentu i tlu (Zhang i sur., 2020a). Prisutnost ostataka azoksistrobina dokazana je za 22 od 317 uzoraka tla prikupljenih diljem Europe (Silva i sur., 2019). Iako je centralna (medijalna) vrijednost ostataka azoksistrobina bila 0.03 mg/kg tla, maksimalne koncentracije dosezale su 0.25 mg/kg. Nadalje, koncentracije ostataka azoksistrobina u poljoprivrednom tlu u Kini dosežu čak 9.5 mg/kg (Xu i sur., 2021). Informacije o prisutnosti ostalih strobilurinskih fungicida u tlu izrazito su šture, te se većina postojećih istraživanja temelji na procjeni njihovih vrijednosti u vodi.

Intenzivna i prekomjerna primjena strobilurinskih fungicida, te potencijalna toksičnost za ekološke sustave i neciljne organizme privlači sve veću pozornost. Iako se strobilurinski fungicidi smatraju slabo toksičnima za sisavce i ptice, rezultati provedenih istraživanja sugeriraju visoku toksičnost za akvatičke vrste (Cui i sur., 2017; Hartman i sur., 2014; Liu sur., 2013). Wang i sur. (2021) su u svom preglednom radu pokazali kako je piraoksistrobin najtoksičniji za akvatičke organizme, a slijede ga piraklostrobin, trifloksistrobin, pikoksistrobin, krezoksim-metil, fluoksastrobin i azoksistrobin. Najviše pažnje privuklo je djelovanje strobilurinskih fungicida na zebrice (*Danio rerio*). Naime, provedena istraživanja pokazala su kako strobilurinski fungicidi uzrokuju oštećenja mitohondrijskih kompleksa i vezanih enzima (Kumar i sur., 2020; Jiang i sur., 2019a; Jiang i sur., 2019b; Cao i sur., 2018; Luz i sur., 2018) koja su povezana s krajnjim točkama poput rasta i ponašanja. Upravo disfunkcija mitohondrija i pojava oksidativnog stresa pridonose smanjenju dužine tijela ličinki zebrica i promjenama kretanja (Kumar i sur., 2020; Cao i sur., 2018). Nadalje, mitohondrijska disfunkcija povezana je s pojavom kardiotoksičnosti i neurotoksičnosti (Li i sur., 2019), te genotoksičnosti, imunotoksičnosti i endokrine disrupcije. Sve navedene pojave mogu imati utjecaj na cjelokupni organizam uzrokujući odgođeni razvoj, smanjenu reprodukciju i promjene u ponašanju (Kumar i sur., 2020; Cao i sur., 2019; Jiang i sur., 2019a; Jiang i sur., 2019b; Cao i sur., 2018; Zhang i sur., 2017; Cao i sur., 2016; Han i sur., 2016; Zhu i sur., 2015). Najjači utjecaj strobilurinskih fungicida opažen je u stadiju ličinke, juvenilnih jedinki, te za vrijeme embrionalnog razvoja (Jiang i sur., 2019a; Jiang i sur., 2019b; Li i sur., 2018; Jiang i sur., 2018; Wang i sur., 2018). Osim mortaliteta embrija, izlaganja strobilurinskim fungicidima uzrokovalo je disfunkciju mitohondrija i smanjene proizvodnje energije, promjena u genskoj ekspresiji, ali i promjena u lučenju hormona štitnjače. Bez obzira na opažanje štetnog djelovanja, smatra se kako zebrice nisu okolišno relevantna vrsta, te se dobiveni rezultati ne mogu u potpunosti

primijeniti na okolišne vrste. U prilog tome govore istraživanja koja ističu veću osjetljivost vrsta kao što su amur (*Ctenopharyngodon idella*), kalifornijska pastrva (*Oncorhynchus mykiss*) i vrsta *Gobiocypris rarus* (Zhu i sur., 2015). Ovakvi rezultati upućuju na moguću utjecaj strobilurinskih fungicida na različite životne stadije ostalih organizama, među kojima se najviše ističe embrionalni razvoj. Stoga izrazitu važnost imaju buduća istraživanja u ovom području.

1.2.5.1. Utjecaj strobilurinskih fungicida na organizme tla

Utjecaj strobilurinskih fungicida na neciljne organizme tla najbolje je proučen kod gujavice *E. fetida*, dok je utjecaj na enhitreje i skokune do sada slabo istražen (Tablica 7). Iako strobilurinski fungicidi nemaju izražen utjecaj na preživljavanje gujavica, provedena istraživanja su pokazala kako izlaganje vrste *E. fetida* pikoksistrobinu i azoksistrobinu može dovesti do negativnog utjecaja na reprodukciju (Schnug i sur., 2014a; Leitão i sur., 2014; Schnug i sur., 2013). Osim toga primjena pikoksistrobina u okolišu može dovesti do smanjenja raznolikosti lumbrikofaune (Schnug et al., 2015). Nadalje, detaljnije analize djelovanja azoksistrobina, piraklostrobina, fluoksastrobina i trifloksistrobina pokazale su da strobilurinski fungicidi izazivaju pojavu oksidativnog stresa i oštećenja DNA (Hou i sur., 2022; Wu i sur., 2021; Xu i sur., 2021; Liu i sur., 2020; Zhang i sur., 2020b; Ma i sur., 2019; Zhang i sur., 2018b; Han i sur., 2014). Osim utjecaja na gujavice, nekoliko istraživanja se usmjerilo na djelovanje strobilurinskih fungicida na skokune. Iako je dokazana manja osjetljivost u odnosu na gujavice, pokazalo se kako izlaganje pikoksistrobinu utječe na promjene brojnosti testiranih vrsta (Schnug i sur., 2014a) dok izlaganje azoksistrobinu uzrokuje smanjenje reprodukcije vrste *Folsomia candida* (Leitão i sur., 2014). Međutim, najmanji broj istraživanja odnosio se na utjecaj strobilurinskih fungicida na enhitreje. Iako su prema Leitão i sur. (2014) enhitreje vrste *E. crypticus* bile manje osjetljive na djelovanje azoksistrobina u odnosu na gujavice i skokune, uočeno je smanjenje stope reprodukcije. Nadalje, ista pojava uočena je nakon izlaganja komercijalnom pripravku temeljenom na aktivnoj tvari azoksistrobin (Gomes i sur., 2021). Međutim, utjecaj ostalih strobilurinskih fungicida potpuno je nepoznat, kao i njihovo djelovanje na molekularne biomarkere enhitreja. Osim toga, utjecaj strobilurinskih fungicida na embrionalni razvoj organizama tla nije istražen. Ranije navedene posebnosti vrste *E. crypticus* omogućuju provođenje testova embriotoksičnosti i popunjavanje praznina u trenutnom znanju.

Tablica 3 - Pregled istraživanja djelovanja strobilurinskih fungicida na neciljne organizme tla.

Organizam	Fungicid	Vrsta izlaganja	Vrijeme izlaganja	Krajnje točke	Rezultati	Izvor
Eisenia fetida	azoksistrobin pikoksistrobin trifloksistrobin	filter papir test	48 h	LC_{50}	LC_{50}AZO = 2.72 µg/cm^2 LC_{50}PIK = 3.15 µg/cm^2 LC_{50}TRI = >1000 µg/cm^2	Wang i sur., 2012
		izlaganje u tlu	7 dana	LC_{50}	LC_{50}AZO = 362.4 mg/kg LC_{50}PIK = 9.22 mg/kg LC_{50}TRI = 414.1 mg/kg	
			14 dana	LC_{50}	LC_{50}AZO = 327.4 mg/kg LC_{50}PIK = 7.22 mg/kg LC_{50}TRI = 401.3 mg/kg	
Eisenia fetida	pikoksistrobin	izlaganje u tlu	28 dana	preživljavanje i reprodukcija	smanjena stopa reprodukcije	Schnug i sur., 2013
Eisenia fetida *Proisotoma minuta* *Heteromurus nitidus* *Folsomia fimetaria* *Protaphorura fimata* *Folsomia fimetaria* *Hypoaspis aculeifer*	pikoksistrobin	izlaganje u tlu	8 tjedana	brojnost	utjecaj na preživljavanje juvenilnih jedinki *E. fetida*, promjene u brojnosti pojedinih vrsta skokuna	Schnug i sur., 2014a
Folsomia fimetaria	pikoksistrobin	izlaganje u tlu	21 dan	preživljavanje i reprodukcija	slaba toksičnost	Schnug i sur., 2014b
Eisenia fetida	azoksistrobin	izlaganje u tlu	28 dana	antioksidativni odgovor gujavica	oksidativni stres i oštećenje DNA	Han i sur., 2014

Eisenia andrei *Enchytraeus crypticus* *Folsomia candida*	azoksistrobin	izlaganje u tlu	4 tjedna 3 tjedana 28 dana	reprodukcija	EC_{50} = 42 mg/kg EC_{50} = 99.2 mg/kg EC_{50} = 92 mg/kg	Leitão i sur., 2014
Gujavice prisutne u poljoprivrednom tlu	pikoksistrobin	izlaganje u tlu	2 godine	utjecaj na gustoću populacije	jak utjecaj na populaciju gujavica	Schnug et al., 2015
Eisenia fetida	fluoksastrobin	izlaganje u tlu	28 dana	antioksidativni odgovor	oksidativni stres i oštećenje DNA	Zhang i sur., 2018b
Eisenia fetida	piraklostrobin	izlaganje u tlu	28 dana	antioksidativni odgovor	oksidativni stres i oštećenje DNA	Ma i sur., 2019
Eisenia fetida	fluoksastrobin	izlaganje u tlu	28 dana	antioksidativni odgovor	oksidativni stres i oštećenje DNA	Zhang i sur., 2020b
Eisenia fetida	trifloksistrobin	izlaganje u tlu	28 dana	antioksidativni odgovor	oksidativni stres	Liu i sur., 2020
Enchytraeus crypticus	azoksistrobin	izlaganje u tlu	21 dan	preživljavanje i reprodukcija	EC_{50}=37mg/kg	Gomes i sur., 2021
Eisenia fetida	azoksistrobin	izlaganje u tlu	56 dana	antioksidativni odgovor	oksidativni stres i oštećenje DNA	Xu i sur., 2021
Eisenia fetida	trifloksistrobin	izlaganje u tlu	56 dana	antioksidativni odgovor	oksidativni stres i oštećenje DNA	Wu i sur., 2021
Eisenia fetida	piraklostrobin	Izlaganje u tlu	28 dana	antioksidativni odgovor, genotoksičnost	oksidativni stres i oštećenje DNA	Hou i sur., 2022

1.2.6. Razlika djelovanja aktivne tvari i komercijalnih pripravaka

Nedavna istraživanja naglasila su važnost usporedbe djelovanja čiste aktivne tvari i komercijalnih pripravaka, te istraživanja koja bi usporedno proučavala njihov utjecaj na neciljne organizme tla (Gomes i sur., 2021). Naime dokazano je kako je utjecaj pripravaka često jači nego utjecaj čiste aktivne tvari (Gomes i sur., 2021; Marques i sur., 2009; Mesnage i sur., 2014).

Međutim, regulative navode potrebu testiranja čiste aktivne tvari prije njene registracije, te su zbog toga istraživanja temeljena samo na čistoj aktivnoj tvari mnogo češća. Ovakav pristup može dovesti do podcjenjivanja stvarnih učinaka do kojih može doći nakon primjene komercijalnih pripravaka na poljoprivrednim površinama. Veća toksičnost komercijalnih pripravaka povezuje se s dodatcima koji omogućavaju veću bioraspoloživost, ali i stabilnost aktivne tvari čime povećavaju njenu toksičnost za neciljne organizme (Pereira et al., 2009).

Smatra se kako nakon primjene komercijalnih pripravaka dolazi do oslobađanja aktivnog sastojka od dodataka (Flury, 1996). Međutim stopa otpuštanja ovisi o vrsti pripravka i različitim okolišnim faktorima. Najčešće korišteni komercijalni pripravci pesticida su koncentrirane suspenzije (engl. *suspension concentrate* - SC), koncentrati za emulziju (engl. *emulsifiable concentrate* - EC), suspendirane emulzije (engl. *suspoemulsion* - SE), vlaživi prah (engl. *wettable powder* - WP), vododispergirajuće granule (engl. *water-dispersible granule* – WG), topive tekućine (engl. *soluble liquid* - SL) i granule (engl. *granules* - GR) (Cush, 2006). Osim što vrsta pripravka utječe na stopu otpuštanja pesticida, ona utječe i na njegovo ispiranje nakon primjene te akumulaciju u okolišu. Tako su uočene razlike u ispiranju aktivne tvari i različitih pripravaka (Khan i Brown 2016), a najveća razlika opažena je između EC i SC pripravaka. SC pripravci sadrže sitne čestice aktivne tvari otopljene u tekućem mediju, obično vodenoj otopini uz različite dodatke. Među dodatcima nalaze se različite tvari za suspenziju, tvari za vlaženje i zgušnjivači koji povećavaju dostupnost aktivne tvari i mogu utjecati na ponašanje pripravaka u okolišu, te smanjiti ispiranje aktivne tvari i povećati njenu perzistentnost i akumulaciju u okolišu (Khan i Brown, 2016). EC pripravci sadrže emulgatore u organskom otapalu netopljivom u vodi, koje je dizajnirano za stvaranje emulzije ulja u vodi koja nakon razrjeđivanja utječe na ponašanje i aktivnosti pesticida. Oni mogu ograničiti otapanje molekule pesticida u vodi ili mogu usporiti procese koji kontroliraju sorpciju u tlo s uljnim organskim otapalima koja okružuju molekulu pesticida, čime se povećava ispiranje. Kako pomoćna sredstva utječu ne samo na ispiranje fungicida, već i na početnu i ukupnu dostupnost aktivne tvari, naglašena je potreba za testiranjem čiste aktivne tvari u usporedbi s različitim komercijalnim pripravcima. Osim toga, kako bi se što točnije odredilo djelovanje strobilurinskih fungicida na enhitreje nužno je korištenje različitih laboratorijskih testova koji omogućuju procjenu čitavog niza krajnjih točaka.

1.2.7. Testirani fungicidi

Osim čiste aktivne tvari azoksistrobin, tijekom izrade ove disertacije korištena su i četiri komercijalna pripravka temeljena na različitim aktivnim tvarima: Quadris®, Retengo®, Zato 50 WG® i Stroby WG® (Tablica 8). Odabrani komercijalni pripravci temeljeni su na jednoj aktivnoj tvari, te se učestalo koriste za suzbijanje gljivičnih bolesti u ratarstvu, voćarstvu i hortikulturi.

Quadris® je lokalno-sistemični i translaminarni fungicid koji se koristi za preventivno suzbijanje pepelnice (*Uncinula necator*) i plamenjače (*Plasmopara viticola*) vinove loze. Aktivna tvar u ovom pripravku je azoksistrobin s udjelom od 250 g/L. Sredstvo dolazi u obliku

koncentrata za suspenziju (SC). Prilikom primjene ovog sredstva karenca je 35 dana, a primjenjuje se najviše dva puta u sezoni. Međutim kod kultura kao što su tikvenjače, luk, češnjak, mrkva i jagode primjenjuje se do četiri puta u sezoni u vremenskim razmacima od 7 dana (Ministarstvo poljoprivrede, 2022).

Tablica 4 - Testirani fungicidi.

Naziv komercijalnog pripravka	Aktivna tvar	Koncentracija aktivne tvari	Vrsta komercijalnog pripravka	Proizvođač
Quadris®	azoksistrobin	250 g/L	koncentrirana suspenzija	Sygenta
Retengo®	piraklostrobin	200 g/L	koncentrirana suspenzija	BASF
Zato 50 WG®	trifloksistrobin	500 g/kg	močive samodispergirajuće granule	BAYER
Stroby WG®	krezoksim-metil	500 g/kg	močive samodispergirajuće granule	BASF

Lokalno-sistemični i translaminarni fungicid Retengo® koristi se za suzbijanje gljivičnih oboljenja na žitaricama. Dolazi u obliku koncentrata za emulziju (EC), a u svom sastavu ima 200 g aktivne tvari piraklostrobin po litri pripravka. Koristi se za suzbijanje pepelnice, hrđe (žuta i smeđa) i pjegavosti (smeđa pjegavost lista i smeđa pjegavost pljevica) jare i ozime pšenice, ječma uz dvije maksimalne primjene u sezoni. Osim toga jednom u sezoni se može primijeniti na kukuruzu i suncokretu kako bi se suzbile siva pepelnica, pjegavost lista, hrđa, te crna i koncentrična pjegavost (Ministarstvo poljoprivrede, 2022).

Zato 50 WG® i Stroby WG® primjenjuju se u voćarstvu, povrćarstvu i vinogradarstvu za suzbijanje krastavosti, pepelnice, pjegavosti i hrđe. Oba komercijalna pripravka dolaze u obliku vododispergirajućih granula. Komercijalni pripravak Zato 50 WG® sadrži 500 g aktivne tvari trifloksistrobin po kilogramu granula. Maksimalno se primjenjuje tri puta tijekom sezone, a koristi se za prevenciju krastavosti, pepelnice, smeđe pjegavosti i hrđe vinove loze, voćarskih i povrtlarskih kultura, te ukrasnog bilja. Stroby WG® primjenjuje se maksimalno dva puta tijekom jedne sezone, a u kilogramu granula nalazi se 500 g aktivne tvari krezoksim-metil.

Koristi se za suzbijanje krastavosti, smeđe pjegavosti, pepelnice i paunovog oka kod različitih voćarskih i povrtlarskih kultura (Ministarstvo poljoprivrede, 2022).

1.3. Testovi toksičnosti

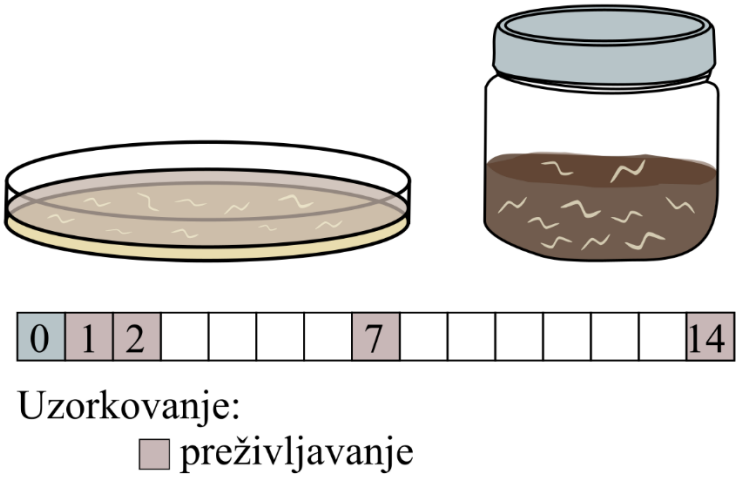
Testovi toksičnosti, između ostalog (u zavisnost od svog dizajna), omogućavaju procjenu djelovanja različitih okolišnih čimbenika ili zagađivala na čitav niz krajnjih točaka. Zbog svog načina života, enhitreje se nalaze u bliskom dodiru s vodom i zrakom u porama tla, ali i samim tlom, što dovodi do izloženosti kroz dermalni, intestinalni i respiratorni sustav (Lock i Janssen, 2003). Standardizirani protokol za reprodukcijski test vrste *E. albidus* propisuje procjenu utjecaja zagađivala na preživljavanje, reprodukciju i bioakumulaciju dok su zbog posebnosti vrste *E. crypticus* uvedene i dodatne krajnje točke temeljene na embrionalnom razvoju (Tablica 9).

Tablica 9 - Toksikološki testovi temeljeni na enhitrejama.

Test	Medij	Trajanje testa	Vrsta	Krajnje točke
Akutni testovi toksičnosti	Agar Tlo	24h, 48h 7,14 dana	*E. albidus*, *E. crypticus*	Preživljavanje
Testovi izbjegavanja	Tlo	24, 48 h	*E. albidus*, *E. crypticus*	Izbjegavanje
Reprodukcijski test	Tlo	6 tjedana 3 tjedna	*E. albidus*, *E. crypticus*	Stopa reprodukcije, molekularni biomarkeri
Test izlijeganja	ISO voda, Tlo	9 dana	*E. crypticus*	Uspjeh izlijeganja

1.3.1. Akutni testovi toksičnosti

Akutni testovi toksičnosti provode se uglavnom s vrstom *E. albidus* zbog njene veličine. Moguće ih je provoditi na pločama agara ili u umjetnom tlu (Slika 5) (OECD 2016; ISO 2014). Akutni testovi toksičnusti traju od 24 h do 14 dana, a najčešće se koriste periodi izlaganja od 48 h, te 7 i 14 dana. U testovima na agaru enhitreje su izložene samo dermalnim putem, dok su prilikom izlaganja u tlu izložene dermalnim, interstinalnim i respiratornim putem. Kao krajnja točka procjenjuje se mortalitet enhitreja, koji se iskazuje u obliku letalnih koncentracija (LCx) i doza-odgovor krivulja.

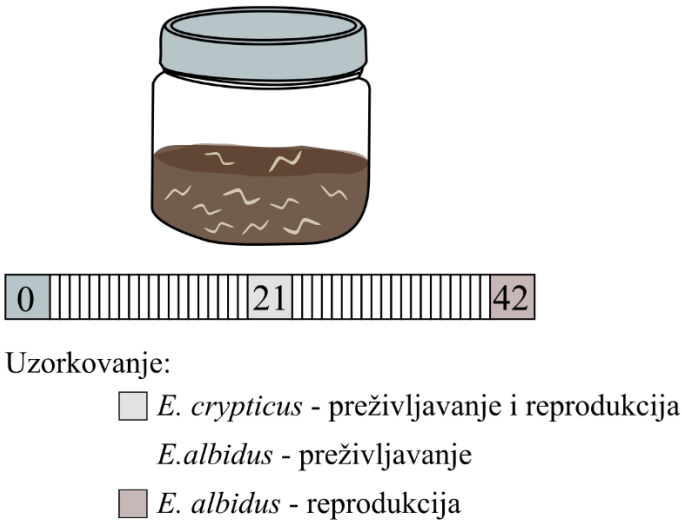


Slika 5 - Shematski prikaz akutnog izlaganja enhitreja. Izlaganje je moguće provesti na agaru (lijevo) ili u tlu (desno). Najčešće se koriste periodi izlaganja od 24 h, 48 h, te 7 i 14 dana nakon čega se utvrđuje djelovanje na preživljavanje.

1.3.2. Reprodukcijski testovi

Reprodukcijski testovi enhitreja (Slika 6) temeljeni su na izlaganju odraslih jedinki s dobro razvijenim klitelumom različitim zagađivalima koji se nalaze u tlu (OECD 2016; ISO 2014). Trajanje reprodukcijskog testa ovisi o vrsti enhitreja. Tako test s vrstom *E. albidus* traje 42 dana, a vrstom *E. crypticus* 21 dan. Naime 10 odraslih jedinki s dobro razvijenim klitelumom izlaže se zagađivalima ili stresorima u 20 g tla. Nakon 21 dana, odrasle jedinke vrste *E. albidus* se uklanjaju, te se mogu koristiti za mjerenje drugih krajnjih točaka poput promjena staničnih i molekularnih biomarkera. Nakon dodatnog 21 dana utvrđuje se broj juvenilnih jedinki. Zbog kraćeg životnog ciklusa vrste *E. crypticus*, nakon 21 dana izlaganja utvrđuje se broj preživjelih odraslih jedinki i broj juvenilnih jedinki. Primarna krajnja točka koja se procjenjuje nakon reprodukcijskog testa je stopa reprodukcije. Inhibicija reprodukcije iskazuje se u obliku efektne koncentracije (ECx) i doza-odgovor krivulje.

Prije brojenja juvenilnih jedinki provodi se njihovo bojenje bojom Bengal rose. Postupak bojenja temelji se na fiksaciji tla i prisutnih jedinki 96%-tnim EtOH. Nakon toga dodaje se 300 µL Bengal rose boje (1% boje otopljen u 96% EtOH) po replici. Nakon 24 h mokrim prosijavanjem uklanjaju se sitne čestice tla i gline čime se sprječava zamućenje prilikom brojenja juvenilnih jedinki. Juvenilne jedinke poprimaju ružičasto obojenje, a zbog njihove veličine brojenje se provodi uz pomoć lupe.

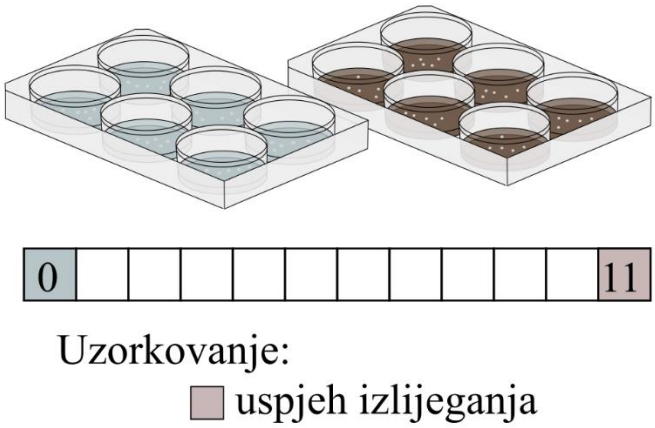


Slika 6 - Shematski prikaz reprodukcijskog testa s vrstama *E. albidus* i *E. crypticus*. Izlaganje se provodi u tlu u periodu od 21 dan za odrasle jedinke obije vrste. Nakon 21 dana utvrđuje se utjecaj na preživljavanje i reprodukciju vrste *E. crypticus*, dok se kod vrste *E. albidus* procjenjuje djelovanje na preživljavanje i uklanjaju odrasle jedinke. Izleženi kokoni ostaju u tlu još 21 dan, a broj juvenilnih jedinki procjenjuje se nakon 42 dana.

1.3.3. Testovi izlijeganja

Razvoj testova embriotoksičnosti i izlijeganja ograničen je svojstvima tla kao medija (Druart et al., 2010). Stoga je provedeno tek nekoliko istraživanja s različitim vrstama puževa (Shoaib i sur., 2010; Iglesias i sur., 2002; Iglesias i sur., 2000). Međutim prozirnost kokona vrste *E. crypticus*, duljina trajanja embrionalnog razvoja i visoka stopa reprodukcije čine ovu vrstu pogodnom za provođenje testova izlijeganja i embriotoksičnosti (Gonçalves i sur., 2015).

Testovi izlijeganja enhitreja (Slika 7) omogućuju procjenu krajnjih točaka poput uspjeha izlijeganja i razvoja embrija (makroskopski ili histološki pristup). Ovakvi testovi započinju izlaganjem sinkroniziranih kokona starosti 1-2 dana. Izlaganje kokona može se provesti u ISO (ISO, 2012) vodi ili tlu. Dok ISO voda omogućuje kontinuirano praćenje embrionalnog razvoja, izlaganje u tlu zahtjeva destruktivno uzorkovanje, te je potreban velik broj replika kako bi bilo moguće kontinuirano praćenje dinamike izlijeganja.



Slika 7 - Shematski prikaz testa izlijeganja vrste *E. crypticus*. Izlaganje se provodi u vodi (lijevo) ili tlu (desno), a broj izleženih jedinki procjenjuje nakon 11 dana.

1.4. Krajnje točke testova toksičnosti

Standardizirani toksikološki testovi za organizme tla uglavnom su usmjereni na preživljavanje i reprodukciju kao ključne krajnje točke (van Gestel, 2012). Međutim, procjena ovih krajnjih točaka može biti dugotrajna, te se često podcjenjuju učinci koji se događaju na molekularnoj ili staničnoj razini, a mogu utjecati na vitalne procese (Howcroft i sur., 2009). U modernoj ekotoksikologiji često se stavlja naglasak na stanične i molekularne biomarkere koji se koriste kao znakovi ranog upozorenja prilikom monitoringa kvalitete okoliša ili procjene utjecaja zagađivala na ekološke sustave (Stegeman i sur., 1992). Upravo se odgovori biomarkera smatraju poveznicom između izvora zagađenja i utjecaja na višim razinama (Suter, 1990). Biomarkeri utjecaja zagađivala na molekularnoj i staničnoj razini pokazali su se pouzdanima za predviđanje učinaka na višim razinama organizacije (Howcroft i sur., 2009). Stoga se njihova upotreba preporuča u svrhu sveobuhvatne procjene djelovanja zagađivala, ali i kako bi se omogućilo povezivanje subletalnih pojava i ekološki relevantnih utjecaja koji se događaju prilikom izlaganja organizama u ekološkim sustavima. U svrhu povezivanja odgovora biomarkera s negativnim utjecajima na višim razinama organizacije odgovor biomarkera bi trebao biti povezan s utjecajem na rast, reprodukciju ili s metaboličkim funkcijama koje izravno utječu na cijeli organizam (Depledge i Fossi, 1994).

1.4.1. Mehanizam multiksenobiotičke otpornosti (MXR)

Mehanizam multiksenobiotičke otpornosti (engl. *multixenobiotic resistance mechanism,* MXR) dio je staničnog detoksikacijskog sustava koji djeluje kao crpka, uklanjajući štetne tvari iz stanice s ciljem smanjenja njihove koncentracije do netoksične razine (Kurelec, 1992). MXR mehanizam temeljen je na transmembranskim proteinima lokaliziranima u citoplazmatskoj membrani. Glavnu ulogu igraju ABC transportni proteini među kojima su zastupljeni P-glikoprotein (P-gp) i multidrug resistance associated protein (MRP) (Leslie i sur., 2005). Ovi proteini prisutni su u svim organizmima uključujući mikroorganizme, biljke, beskralježnjake i kralježnjake (Licht i Schneider, 2011). MXR mehanizam najizraženiji je u različitim epitelnim i endotelnim tkivima koja su direktno ili indirektno povezana s metabolizmom ili izlučivanjem zagađivala (Chu i sur., 2013). Međutim, ksenobiotici, odnosno zagađivala prisutna u okolišu mogu utjecati na aktivnost ovog sustava. Ksenobiotici koji izravno utječu na aktivnost MXR mehanizma imaju dvojako djelovanje. S jedne strane oni mogu djelovati kao induceri i povećati aktivnost MXR sustava, odnosno dovesti do njegove indukcije i povećanog transporta tvari iz stanice. Međutim, s druge strane mogu djelovati poput inhibitora, pri čemu dolazi do smanjenja

ili potpunog prestanka transporta tvari iz stanice i posljedično njihove akumulacije (Epel i sur., 2008; Kurelec 1997). Dosadašnja istraživanja vodenih i kopnenih organizama upućuju na korelaciju između aktivnosti MXR i koncentracije ksenobiotika u okolišu, što ovom mehanizmu daje izraziti potencijal u ekotoksikološkim istraživanjima (Sauerborn Klobučar i sur. 2010; Kurelec 1997).

Iako se promjene aktivnosti MXR sustava učestalo koriste kao biomarker u akvatičkoj ekotoksikologiji (Falfushynska i sur., 2019; Katsumiti i sur., 2018; Cunha i sur., 2017; Franzellitti i sur., 2017; Anselmo i sur., 2012; Faria i sur., 2011) upotreba ovog biomarkera nešto je manje zastupljena u ekotoksikologiji tla (Topić Popović i sur., 2015; Bošnjak i sur., 2014; Velki i Hackenberger, 2012). Metoda razvijena za mjerenje promjena aktivnosti MXR sustava primjenjuje se na gujavicama (Hackenberger i sur., 2012), te se temelji na akumulaciji fluorescentnih supstrata rodamina B i rodamina 123 unutar organizama. Predložena metoda pokazala je kako prisutnost ksenobiotika poput pesticida može izazvati inhibiciju ili indukciju MXR sustava (Velki i sur., 2018; Velki i Hackenberger 2013a; Velki i Hackenberger 2013b; Velki i sur., 2013; Velki i Hackenberger, 2012). Nadalje, nedavno je razvijena metoda za mjerenje ovog biomarkera kod skokuna (Lopes i sur., 2021). Međutim, prisutnost ovog mehanizma kod enhitreja i dalje nije dokazana, te ne postoji prikladna metoda za mjerenje ovog biomarkera. Stoga je potrebno razviti metodu koja bi omogućila mjerenje promjena aktivnosti MXR mehanizma enhitreja, što bi uvelike razjasnila djelovanje pojedinih zagađivala na ove organizme.

1.4.2. Oksidativni stres

Zanimanje za molekularne biomarkere potaknuo je istraživanje antioksidativne obrane (Winston i Di Guli., 1991) koja igra ključnu ulogu u održavanju homeostaze organizama. Tijekom normalne funkcije organizma, u stanicama nastaju reaktivni spojevi kisika (engl. *reactive oxygen species,* ROS) s važnom ulogom u redoks signalizaciji, apoptozi i indukciji obrambenih gena, te mogu imati različite učinke na stanični metabolizam. ROS dijelimo na slobodne radikale i neradikalne produkte. Dva slobodna radikala koji dijele nespareni elektron čine neradikalni oblik ROS-a, dok skupini radikala pripadaju male reaktivne molekule koje sadrže kisik (hidroksilni radikal - OH•, superoksidni radikal - O_2^-, vodikov peroksid - H_2O_2) (Birben i sur., 2012). Prema nastanku slobodni radikali dijele se na endogene i egzogene. Endogeni slobodni radikali nastaju reakcijama uslijed upalnih odgovora, kao posljedica infekcija, starenja ili pretjerane fizičke aktivnosti. Egzogeni slobodni radikali nastaju nakon izlaganja različitim zagađivalima (Valko i sur., 2007).

Slobodni radikali odlikuju se jednim nesparenim, visoko reaktivnim elektronom u vanjskoj orbitali koji napada dvostruke ugljik-ugljik veze polinezasićenih masnih kiselina što dovodi do stvaranja dodatnih međuprodukata - slobodnih radikala. Nastale veze su izuzetno reaktivne i nestabilne pa stupaju u reakciju sa spojevima poput proteina, lipida i ugljikohidrata (Štraus i sur., 2009). U zdravim stanicama nastali ROS uklanja se djelovanjem antioksidansa i specifičnih antioksidacijskih enzima (Halliwell i Gutteridge, 1999). Međutim, izloženost okolišnim stresorima ili zagađivalima može dovesti do stvaranja prekomjernih količina ROS-a što dovodi do disbalansa antioksidativnog sustava i vodi ka potencijalnim dugoročnim oštećenjima stanica i organizma. Stoga je za neometanu funkciju organizma nužno pravovremeno i efikasno uklanjanje ROS-a iz stanica. Ključnu ulogu u tom procesu igraju enzimi antioksidativnog sustava poput superoksid dismutaze (SOD), katalaze (CAT), glutation-s-transferaze (GST) i neenzimske komponente koju čine različiti vitamini. Promjene aktivnosti enzima antioksidacijskog sustava često se koriste kao biomarkeri toksičnih učinaka zagađivala, jer pokazuju izravno mjerljivu manifestaciju nakon izloženosti zagađivalima (Ighodaro i Akinloye, 2018).

Odgovor enzima antioksidativnog sustava dijelimo u dvije faze. Efikasnost prve faze detoksikacije uvelike ovisi o aktivaciji antioksidativnih enzima kao što su SOD i CAT. SOD pripada skupini metaloenzima koji kataliziraju disproporcioniranje superoksidnog aniona (O_2^-) u vodikov peroksid (H_2O_2) (Ighodaro i Akinloye, 2018). Postoji nekoliko formi SOD-a koje kao kofaktore sadrže molekule metala poput bakra, cinka, mangana, željeza i nikla (Abreu i Cabelli, 2010) koje nalazimo u mitohondrijima i citosolu. SOD se smatra prvom linijom obrane u sustavu antioksidativnih enzima (Singh i sur., 2006) te se učestalo koristi kao molekularni biomarker za detekciju oksidativnog stresa. Najčešći odgovor na prisutnost ROS-ova je indukcija enzima, koja je kod enhitreja zabilježena nakon izlaganja različitim vrstama nanočestica (Gomes i sur., 2012; Novais i sur., 2011). H_2O_2 nastao u reakcijama koje katalizira SOD supstrat je za CAT, hematin-sadržavajući enzim koji je prisutan u mitohondrijima, peroksisomima ili citosolu stanica gotovo svih aerobnih organizama. Ovaj enzim kojega čine četiri podjedinice (četiri polipeptidna lanca) s hem prostetičkom grupom u katalitičkom centru, katalizira razgradnju H_2O_2 na molekularni kisik i vodu, bez nastajanja štetnih molekula poput slobodnih radikala (Aebi, 1974). Svojim djelovanjem CAT sprječava transformaciju H_2O_2 u hidroksil radikale (OH^-) koji mogu negativno utjecati na genetski materijal organizama. Zbog izrazite važnosti i efikasnosti ovog enzima, njegova aktivnost osjetljiva je na promjene oksidativnog statusa stanice. Upravo zbog toga promjene aktivnosti CAT služe kao važan biomarker u praćenju djelovanja zagađenja na neciljne organizme.

Međutim ukoliko prva faza ne eliminira sve štetne molekule, aktivira se i druga faza, karakteristična po enzimima poput GST. GST su skupina citosolnih enzima koji imaju važnu ulogu u katalizi konjugacije različitih endogenih i egzogenih elektrofilnih supstrata s endogenim tiolnim antioksidantom, tripeptidom glutationom (GSH) (Palmeira 1999; Clark i sur., 1986). Adicija glutationa na dvostruku vezu temelj je konjugacije, a odvija se razlaganjem epoksidnog mosta, supstitucijom halogena ili drugih odlazećih skupina (Stenersen, 1984). Enzimi druge faze detoksikacije poput GST imaju važnu ulogu u metabolizmu i ekskreciji zagađivala, čime izravno utječu na održavanje homeostaze organizama.

Niske razine lipidne peroksidacije (LPO) stimuliraju stanice na pojačanje antioksidativnih obrambenih mehanizama i aktivaciju signalnih puteva koji pokreću sintezu antioksidanasa ili oksidativnih enzima koji pomažu organizmu u adaptaciji na stres. Međutim, suvišna produkcija ROS-a ili nedovoljno učinkovito uklanjanje mogu odvesti do neuravnoteženosti oksidativnog sustava koje rezultira pojavom lipidne peroksidacije (LPO) i mogućih oštećenja tkiva. LPO je složena lančana reakcija potaknuta ROS-om, a tijekom nje dolazi do oksidacije višestruko nezasićenih masnih kiselina (Štefan i sur., 2007). LPO se dijeli na tri stupnja: inicijaciju, propagaciju i terminaciju, a najčešće ju uzrokuje hidroksilni radikal. Glavni produkt lipidne peroksidacije je malondialdehid (MDA) koji se učestalo koristi za mjerenje stupnja LPO u stanicama (Duryee i sur., 2010).

Dokazano je kako strobilurinski fungicidi uzrokuju oksidativni stres i lipidnu peroksidaciju kod gujavica (Wu i sur., 2021; Liu i sur., 2020; Ma i sur., 2019), međutim informacije o djelovanju strobilurinskih fungicida na oksidativni status enhitreja ne postoje.

1.4.3. Energetski status

Dostupna energija i njena ravnoteža presudne su za odvijanje ključnih procesa u organizmima poput bazalnog metabolizma, rasta i reprodukcije (Novais i sur., 2013). Međutim, izloženost različitim zagađivalima može uzrokovati metaboličke promjene i smanjenje energetskih rezervi, osobito prilikom dugotrajnog izlaganja, što u konačnici može rezultirati utjecajem na rast i reprodukciju jedinki, ali i na cjelokupnu dinamiku i strukturu populacije (de Coen i Janssen, 2003). Tako se i utjecaj zagađivala može utvrđivati neizravno, procjenom utjecaja na reprodukciju ili izravno, određivanjem količine ukupne dostupne energije. Istraživanja dostupnih energetskih rezervi vrste *E. albidus* ukazala su na potencijalnu primjenu količine dostupne energije kao biomarkera prilikom izlaganja okolišnim stresorima (Amorim i sur., 2012), metalima (Novais i sur., 2013) i pesticidima (Novais i Amorim, 2013).

Kako bi se odredila količina dostupne energije nekog organizma učestalo se koristi metodologija koju su propisali de Coen i Janssen (1997, 2003). Za nju je potrebno odrediti ukupni sadržaj ugljikohidrata, lipida i proteina koji se uz pomoć energetskih ekvivalenata pretvaraju u energiju. Istraživanja energetskog statusa enhitreja vrste *E. albidus* pokazala su kako se energetske rezerve sastoje uglavnom od proteina (50-55%) i lipida (35-40%), dok ugljikohidrati zauzimaju najmanji udio (približno 10%) (Novais i sur., 2013; Amorim i sur., 2012).

Iako su zastupljeni s najmanjim udjelom, ugljikohidrati imaju izuzetno važnu ulogu u metabolizmu. Prema građi, ugljikohidrati su aldehidi ili ketoni s više hidroksilnih skupina, a služe kao pričuvna energija, gorivo i metabolički produkt. Naime, upravo se ugljikohidrati učestalo koriste kao prvi izvor energije prilikom izloženosti organizama stresnim uvjetima (Moolman i sur., 2007), a brza mobilizacija ugljikohidrata uočena je kod enhitreja izloženih metalima (Novais i sur., 2013) i pesticidima (Novais i Amorim, 2013). Uz ugljikohidrate, lipidi se odlikuju visokom efikasnošću skladištenja energije. Kako su neproteinske komponente poželjniji izvori energije, lipidi se mobiliziraju prije ili zajedno s ugljikohidratima prilikom izloženosti zagađivalima (Smolders i sur., 2003). Smanjenje koncentracije lipida može biti posljedica smanjene aktivnosti hranjenja i povećane mobilizacije, ali i posljedica oštećenja koja nastaju uslijed LPO. Nasuprot tome, povećana akumulacija lipida nakon izlaganja različitim pesticidima (Novais i Amorim, 2013) povezuje se s upalnim stresom (Gomes i sur., 2015b). Iako se ne koriste neposredno poput ugljikohidrati i lipida, proteini mogu biti važan izvor energije (Smolders i sur., 2003). Međutim, u nekim slučajevima povećanje koncentracije proteina povezuje se s indukcijom sinteze proteina koji se koriste za različite detoksifikacijske ili ostale mehanizme (Sokolova i sur., 2012; Tripathi i sur., 2010; Smolders i sur. 2003). Među proteinima koji se sintetiziraju u stresnim uvjetima ističu se metalotioneini (MT) i proteini toplinskog stresa (engl. *heat shock proteins* - HSP) koji su izravno uključeni u mehanizme detoksikacije.

Izlaganje enhitreja metalima pokazalo je očitu poveznicu između povećanja metaboličke aktivnosti, procesa detoksikacije i ponovnog uspostavljanja stanične ravnoteže (Gomes i sur., 2015; Novais i sur., 2013), međutim promjene dostupne energije prilikom izloženosti strobilurinskim fungicidima nisu proučavane.

1.5. Statističke metode

Statistička analiza podataka provedena je računalnom okruženju R (R Development Core Team, 2022) pomoću R korisničkog sučelja RStudio (RStudioTeam, 2022). Distribucija dobivenih rezultata testirana je Shapiro-Wilkovim testom, dok je za testiranje homogenosti varijance korišten Bartlettov test. Za daljnju analizu podataka s normalnom distribucijom korišteni su parametarski testovi. Pri usporedbi dvije skupine podataka korišten je t-test, dok je za analizu više skupina podataka korištena analiza varijance (ANOVA) u kombinaciji s odgovarajućim post hoc testom (Dunnet post hoc test ($p \leq 0.05$)). Za skupine podataka koji su odstupali od normalne distribucije korišten je ne parametarski test (Kruskal Wallis) i odgovarajući post hoc test (Gao tests).

Za izračun letalnih (LCx), efektnih (ECx) i inhibitornih (ICx) koncentracija, te opisivanje doza-odgovor krivulja korišten je paket drc (Ritz i sur., 2015). Prilikom izračuna LCx podatci o preživljavanju modelirani su uz pomoć tri parametarske logističke krivulje. Za izračun ECx korišteni su podatci o reprodukciji, uspjehu i vremenu izlijeganja koji su modelirani prema tri ili četiri parametarskim logističkim krivuljama. Za opisivanje odgovora promjenama aktivnosti MXR mehanizma i izračuna ICx korištena je tri parametarska logistička funkcija.

1.6. Ciljevi istraživanja

S obzirom na nedostatke postojećeg znanja, ciljevi ove disertacije bili su nadopuniti postojeća istraživanja novim znanjima o utjecaju strobilurinskih fungicida na enhitreje vrste *E. crypticus* i *E. albidus* ispitivanjem sljedećih hipoteza:

Enhitreje posjeduju MXR transportere i na njihovu aktivnost se može utjecati modelnim inhibitorima i inducerima;
Promjene aktivnosti MXR sustava enhitreja mogu se koristiti kao vrijedan komplementarni biomarker u ekotoksikološkim istraživanjima;
Ksenobiotici prisutni u okolišu mogu djelovati na aktivnost MXR sustava enhitreja uzrokujući njegovu inhibiciju ili indukciju;
Komercijalni pripravci strobilurinskih fungicida mogu negativno utjecati na preživljavanje i reprodukciju enhitreja vrste *E. crypticus*;
Komercijalni pripravci strobilurinskih fungicida temeljeni na različitim aktivnim tvarima pokazat će različito djelovanje na neciljnu vrstu *E. crypticus*;
Istraživanje toksičnosti čiste aktivne tvari nije dostatno za procjenu okolišnog rizika jer se dodatcima u komercijalnim pripravcima bitno mijenjaju toksikološka, tj. ekotoksikološka svojstva aktivne tvari;
Procjena djelovanja čiste aktivne tvari i različitih komercijalnih pripravaka strobilurinskih fungicida na aktivnosti MXR mehanizma, preživljavanje, reprodukciju, uspjeh izlijeganja, te oksidativni i energetski status dat će cjelovitu sliku djelovanja strobilurinskih fungicida na enhitreje;
Kako bi se predvidjelo djelovanje komercijalnih pripravaka u okolišu uz standardne krajnje točke, preživljavanje i reprodukciju, nužno je uvesti i istražiti nove krajnje točke koje će pomoći pri točnijem određivanju mehanizama toksičnosti.

2. ZNANSTVENI RADOVI

2.1. Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology

Chemosphere 279 (2021) 130549

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology

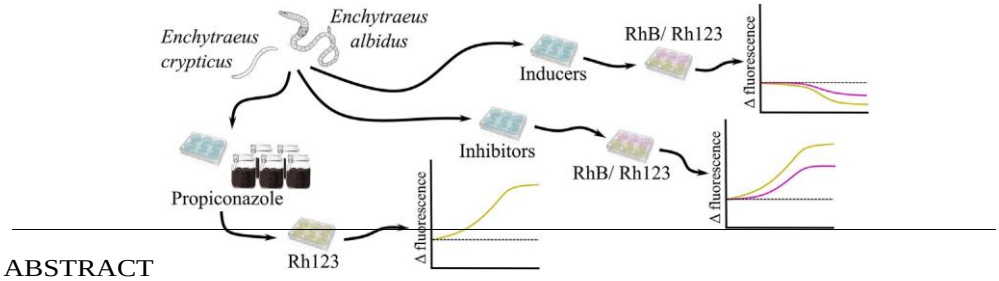
Marija Kovačević, Davorka K. Hackenberger, Željka Lončarić, Branimir K. Hackenberger*

University of Osijek, Department of Biology, Cara Hadrijana 8A, HR-31000, Osijek, Croatia

HIGHLIGHTS

- The expression of ABC efflux trans- porters in enchytraeids was demonstrated.
- Cyclosporine A, ivermectin and verapamil inhibited enchytraeid MXR activity.
- Dexamethasone and rifampicin acted as inducers of MXR system in enchytraeids.
- Propiconazole acts as a chemo- sensitizer, inhibiting the MXR activity.
- Dye efflux assay is applicable in standard toxicity test conducted in soil.

GRAPHICAL ABSTRACT



ARTICLEINFO

Article history:
Received 1 October 2020
Received in revised form
5 April 2021
Accepted 8 April 2021
Available online 12 April 2021

Handling Editor: James Laorchak

Keywords:
Enchytraeus
Efflux pump
Chemosensitize
rsMXR protein

ABSTRACT

The multixenobiotic resistance (MXR) mechanism is the first defense line against xenobiotics. Enchytraeids, a model organism in soil ecotoxicology, are often exposed to various xenobiotics, some of which may influence MXR activity. Since MXR activity has not been studied in these organisms, the aim of this paper was to establish a methodology for the implementation of the dye assay in enchytraeids. *Enchytraeus albidus* and *Enchytraeus crypticus* were exposed to model chemosensitizers: cyclosporine A (CA), dexamethasone (DEX), ivermectin (IVM), rifampicin (RIF), verapamil (VER), and fungicide propiconazole (PCZ). Thereafter, a dye assay with specific fluorescent dyes rhodamine B and rhodamine 123 was performed. Changes in MXR activity caused by variations in dye accumulation were measured fluorometrically. CA, IVM, and VER were found to inhibit the MXR system and increase the fluorescence 2.2- fold, while DEX and RIF induced the MXR system and decreased the fluorescence. CA was the strongest inhibitor in both *E. albidus* (IC_{50} 5.48 ± 1.25 μM) and *E. crypticus* (IC_{50} 5.20 ± 3.10 μM). In the validation experiment, PCZ was found to inhibit the MXR system. The IC_{50} varied between species and exposure substrates: water (*E. albidus* - IC_{50} 0.74 ± 0.24 mg/L; *E. crypticus* - 1.31 ± 0.24 mg/L) or soil (*E. albidus* - 1.79 ± 0.42 mg/kg; *E. crypticus* - 1.79 ± 0.17 mg/kg). In conclusion, the tested compounds changed the MXR activity, which confirms the applicability of this method as a valuable complementary biomarker in soil ecotoxicolog.

Abbreviations: MXR, Multixenobiotic resistance (protein); P-gp, permeability glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; ABC, adenosine triphosphate (ATP)-binding cassette; RhB, rhodamine B; Rh123, rhodamine 123; CA, cyclosporine A; VER, verapamil; IVM, ivermectin; DEX, dexamethasone; RIF, rifampicin; PCZ, propiconazole; AS, artificial soil; IC, inhibitory concentration; EC, an effective concentration.

* Corresponding author.

E-mail address: hackenberger@biologija.unios.hr (B.K. Hackenberger).

https://doi.org/10.1016/j.chemosphere.2021.130549 0045-6535/

Introduction

Every year, humans discard into the environment considerable quantities of chemical compounds. To be able to survive in such conditions, organisms develop a set of highly specific cellular de- fense mechanisms. One of these is the multixenobiotic resistance (MXR) mechanism, which is considered the first defense line against xenobiotics (Kurelec, 1992). MXR is based on the activity of adenosine triphosphate (ATP)-binding cassette (ABC) superfamily members: P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) (Leslieet al., 2005). The ABC proteins are present in all organisms, including microbes, plants, invertebrates, and vertebrates (Licht and Schneider, 2011). Although P-gp and MRPs overlap in the structure and substrate specificity, they differ in the transport mechanism and substrate types (Leslie et al., 2005). P-gp trans- porters are involved in the efflux of non-modified xenobiotics (moderately amphipathic, hydrophobic, neutral, or positively charged planar organic molecules of low molecular weight with basic nitrogen atoms), while MRPs are involved in the transport of the products of different metabolism phases (Litman et al., 2001). P-gp and MRP transporters, the primarily studied MXR proteins involved in the efflux of xenobiotics, are commonly known as the efflux pumps.

The MXR mechanism is highly expressed in different epithelial and endothelial tissues directly or indirectly involved in the metabolism or excretion of contaminants (e.g., mussel and fish gills,liver, kidney, intestines, blood-brain barrier, and placenta) (Chu et al., 2013). This mechanism protects against environmental contamination by pumping out endogenous chemicals from the cells, thus preventing their accumulation and toxic effects (Kurelec, 1992). Chemicals and xenobiotics that block MXR transporters' function and alter cell sensitivity are known as chemosensitizers (Kurelec, 1997). Regarding the mechanism of action, chemo- sensitizers are classified into competitive and non-competitive inhibitors. While competitive inhibitors overwhelm the efflux pump's substrate binding capacity, non-competitive inhibitors block the ATPase activity of the efflux pump (Faria et al., 2011). Both of these inhibitor types target the MXR system. In contrast to in-hibitors, MXR system inducers increase the efflux pump activity, induce P-gp and MRP, and reduce xenobiotics accumulation. Envi- ronmental chemosensitizers lead to an intracellular accumulation of xenobiotics, consequently increasing their toxic effects. A wide range of environmental pollutants has been shown to affect the MXR system activity (Smital and Kurelec, 1998; Ečimović et al., 2018; Lackmann et al., 2018; Vehovszky et al., 2018; Moreira et al., 2019). Due to the xenobiotic presence in the environment and their influence on the MXR system activity, changes in MXR activity are used as a biomarker of environmental pollution (Kurelec et al., 2000).

MXR activity and its modulation by various chemosensitizers is commonly assessed with a cell-based transport assay with fluo- rescent substrates, known as the dye assay. This fast and easy to use method enables preliminary testing of the compounds and their interaction with MXR proteins (Cunha et al., 2017). The dye assay is based on the accumulation of fluorescent dyes, mostly rhodamine B (RhB) and rhodamine 123 (Rh123). While RhB, commonly used in marine and freshwater invertebrates, is a P-gp substrate model, Rh123 is a substrate of both P-gp and MRP (Daoud et al., 2000).

Briefly, this *in vivo* assay is based on the accumulation of fluo- rescent dye in the organism after exposure to model chemo- sensitizers. Model inhibitors include immunosuppressive agent cyclosporine A (CA) and calcium channel blocker verapamil (VER). These agents are known for their ability to inhibit both P-gp and the MRP, and to subsequently increase the accumulation of fluorescent substrates in the cell (Epel et al., 2008). Additionally, an antipara- sitic drug, macrocyclic lactone, ivermectin (IVM) is reported as a suitable substrate and potent P-gp inhibitor (Lespine et al., 2006). Among P-gp efflux inducers, the most frequently used are synthetic glucocorticoids, such as dexamethasone (DEX), or macrolide anti- biotics, such as rifampicin (RIF) (Jackson and Kennedy, 2017). The experiments with model inhibitors or inducers are the first step in the study of MXR inducibility before conducting the experiments with environmental xenobiotics.

Dye assays and changes in MXR activity have been used for screening the substrates and inhibitors of ABC transporters (Luckenbach et al., 2014). The MXR is mostly studied in aquatic invertebrates and vertebrates in various life stages, for example in echinoid larvae (Anselmo et al., 2012), zebrafish embryos (Cunha et al., 2017) and hepatocyte cell line (Moreira et al., 2019; Lopes et al., 2019), mussel larvae (Faria et al., 2011; Franzellitti et al., 2017), adult mussel (Falfushynska et al., 2019), and mussel cells (Katsumiti et al., 2018). Besides aquatic invertebrates, it is also studied in soil invertebrates. An important model for the study of efflux transport in terrestrial annelids are earthworms (Hackenberger et al., 2012; Bošnjak et al., 2014). In earthworm models, changes in efflux pump activity are used as a biomarker of exposure to environmental pollutants, mostly pesticides (Velki and Hackenberger, 2012; Velki et al., 2013) or wastewater and sludge (Topić Popović et al., 2015). Differences in the efflux pump activity were found between species (Smital et al., 2000; Velki and Hackenberger, 2012) and between populations from clean and polluted sites that correlate with the sensitivity to chemicals (Kurelec et al., 1996; Smital et al., 2004).

The most commonly used fluorescent dyes in the phylum Annelida are RhB and Rh123 (Hackenberger et al., 2012; Bošnjak et al., 2014; Vehniäinen and Kukkonen, 2015) in combination with known MXR inhibitors and inducers (i.e., model chemosensitizers).

Besides earthworms, another group of organisms that plays an essential role in the decomposition of organic matter and soil bioturbation are enchytraeids (Briones, 2014). These organisms are ecologically relevant soil-dwelling annelids widespread in many types of soil. Enchytraeids are particularly important in the areas where earthworms are absent, such as in the Arctic and subarctic tundra, moorlands, montane grasslands, agricultural land in the taiga zone, and coniferous forest soils (Swift et al., 1998). *Enchy- traeus albidus* and *Enchytraeus crypticus* are model species widely used in soil ecotoxicology (Didden and Römbke, 2001; Castro-Ferreira et al., 2012). Their use in laboratory tests is recom-mended by the Organisation for Economic Co-operation and Development ((OECD 220 2016)) and International Organization for Standardization ((ISO 16387 2004)) guidelines, and they are frequently used to assess the effects of environmental pollutants, mostly pesticides (Amorim et al., 2005; Leitão et al., 2014; Bart et al., 2017) and nanoparticles (Amorim and Scott-Fordsmand, 2012; Bicho et al., 2016, 2017) or their combination (Hackenberger et al., 2019). Although enchytraeids are commonly used model species and they are assumed to have an MXR system that helps in the defense against xenobiotics, so far it has not been studied whether this system actually exists and how it is affected by modulators and xenobiotics. Understanding the MXR mechanism's role in altering chemical toxicokinetics and characterizing its regulation can aid in studying the chemical defense mechanisms. Hence, this study aimed to establish a methodology for imple- menting the dye assay in enchytraeids, confirm the presence of efflux transporters and their activity, and determine whether enchytraeids are suitable organisms for this type of test. The

experiments involved known MXR activity modulators (inhibitors (CA, VER, IVM) and inducers (DEX, RIF)). Additionally, to verify the results, an experiment with fungicide propiconazole (PCZ) - an environmentally relevant modulator of efflux pump activity, was conducted.

Materials and methods

Test organisms

The study used adult potworms with a well-developed clitellum from a synchronized culture. The potworms *E. albidus* (Henle, 1837) and *E. crypticus* (Westheide and Graefe, 1992) were obtained from the culture maintained at the Department of Biology in Osijek (Croatia) for several years according to the OECD 220 (2016), ISO 16387 (2004), and Bicho et al. (2015) guidelines. Shortly, the cul- tures were kept under controlled conditions in a climate room at a constant temperature (18 ± 1 °C), relative humidity of 60%, and a photoperiod of 16:8 h (light: dark). *E. albidus* cultures were main- tained in moist soil media, while *E. crypticus* cultures were kept in agar plates and were prepared with the salt solution of 2 M $CaCl_2$, 1 M $MgSO_4$, 0.05 mM $NaHCO_3$, and 0.01 mM KCl. Both species were fed with ground rolled oatmeal *ad libitum*. Synchronized cultures of *E. crypticus* were prepared according to Bicho et al. (2015). Briefly, adults with a well-developed clitellum were transferred into fresh agar plates to lay cocoons. After two days, the synchronized co- coons were transferred with a brush to fresh agar plates. *E. albidus* cultures were synchronized by transferring the cocoons into fresh soil. The process consisted of gently picking cocoons from the culture using a brush. At the beginning of the test, the age of adult *E. crypticus* was 25 days and three months for *E. albidus*.

Test materials

All reagents were of analytical grade. Cyclosporine A (CAS Number 59865-13-3, molecular weight 1202.635 g/mol), dexa- methasone (CAS Number 50-02-2, molecular weight 392.461 g/ mol), rifampicin (CAS Number CAS 13292-46-1, molecular weight 822.94 g/mol), and Rhodamine 123 (CAS Number 62669-70-9, molecular weight 380.82 g/mol) were purchased from ACROS Or- ganics (Geel, Belgium). Ivermectin (CAS Number 70288-86-7, mo- lecular weight 875.1 g/mol) and Rhodamine B (CAS Number 81-88- 9, molecular weight 479.02 g/mol) were obtained from Sigma (St Louis, MO, USA) and verapamil (VER, CAS Number 152-11-4, mo- lecular weight 491.07 g/mol) purchased from J&K Scientific GmbH (Pforzheim, Germany). Dimethyl sulfoxide (DMSO, CAS Number 67- 68-5, molecular weight 78.13 g/mol) was purchased from Gram- mol (Zagreb, Croatia). A commercial preparation of pesticide Bumper 25 EC (Chromos Agro) with active ingredient propicona- zole (PCZ) (250 g/L) was used.

Test media

Water

Standard ISO water, containing 2 M $CaCl_2$, 1 M $MgSO_4$, 0.05 mM $NaHCO_3$, and 0.01 mM KCl, was used (ISO, 2012). The test conditions were based on the aquatic toxicity test for enchytraeids described by Römbke and Knacker (1989) and reproduced by Gomes et al. (2015). For additional details on test procedures please see the supplementary material.

Soil

The artificial soil (AS) was prepared according to the OECD (2016) protocol with sphagnum peat (10%), kaolin clay (20%), and air-dried quartz sand (70%). The soil pH was adjusted to 6 ± 0.5 by the addition of $CaCO_3$.

Dye accumulation experiment

A dye accumulation experiment was conducted to select the appropriate fluorescent dye concentration and exposure time needed to avoid passive diffusion. The test was performed with different RhB and Rh123 concentrations (0, 1.25, 2.5, 5, 10, and 20 μM) dissolved in distilled water. Enchytraeids were exposed to 5 ml of fluorescent dye solutions with ISO water in six-well plates for 1, 2, 4, and 6 h in the dark at 20 °C. The treatments were replicated five times, and each replicate consisted of seven *E. albidus* or 20 *E. crypticus* individuals. At the end of the exposure, enchytraeids were prepared for fluorescence measurement ac- cording to the procedure described below.

MXR model inhibitors and inducers experiment

Enchytraeids were exposed to a range of model chemo-sensitizers concentrations (inhibitors: CA 0, 2, 5, 10, and 15 μM; VER 0, 5, 10, 20, and 50 μM; IVM 0, 0.1, 1, 10, and 20 μM and inducers: DEX 0, 0.1, 1, 10, and 20 μM; RIF 0, 0.1, 1, 10, and 20 μM). The con- centrations of inhibitors and inducers were chosen based on pre- liminary experiments. The exposure was conducted in 5 ml of chemosensitizer solution in six-well plates for 6 h in the dark at 20 °C. For the negative control, only ISO water was used. As DMSO proportion in the exposure solution was less than 5×10^{-4} (v: v) and no effect of DMSO in the preliminary study was observed, a solvent control was not used. Furthermore, DMSO toxicity at con- centrations above 0.5% was not reported (Galvao et al., 2013; Sumida et al., 2011). Details about stock solution and working so- lution preparations are included in Supplementary information. After the exposure to chemosensitizers, a dye assay was conducted. Based on the dye accumulation experiment (2.4.), 10 μM RhB and 5 μM Rh123 concentrations and 1 h exposure was chosen as optimal for dye assay.

Validation experiment

To test the assay validity, enchytraeids were exposed to fungi- cide PCZ, a known MXR inhibitor. Two experiments were con- ducted: one in ISO water and one in AS. Nonlethal pesticide concentrations were chosen based on previous research (Hackenberger et al., 2019). For water exposure, the pesticide sus- pensions were prepared using ISO water to obtain the following concentrations: 0, 0.3125, 0.625, 1.25, 2.450, and 4.9 mg/L. Water exposure was performed in six-well plates containing 5 ml of pesticide solution in ISO water. For AS exposure, the selected PCZ concentrations were prepared with distilled water and added into each test vessel in the amount needed for 60% soil WHC (water holding capacity). The soil was homogeneously mixed and allowed to equilibrate for 24 h before the start of the tests. The tested concentrations were 0, 0.3125, 0.625, 1.25, 2.450, and 4.9 mg/kg. The water exposure test was run at 20 °C in the dark for 6 h , and the AS exposure test was run at 20 °C and 16:8 h photoperiod for seven days. After exposure, enchytraeids were washed in distilled water and transferred to six-well plates containing 5 ml of 5 mM Rh123 in ISO water and exposed for 1 h in the dark, at 20 °C. Each replicate consisted of seven *E. albidus* or 20 *E. crypticus* individuals. All treatments were done in a pentaplicate. At the end of the exposure, enchytraeids were homogenized and prepared for fluorescence measurement as described below.

Sample preparation and measurement of rhodamine amount

After exposure to RhB or Rh123, potworms were quickly washed with distilled water and weighted. Whole potworms were ho- mogenized in pools (seven in a pool for *E. albidus*, and 20 for *E. crypticus*) immediately after the exposure in 250 mL of cold po- tassium phosphate buffer (0.1 M, pH 7.4) with the IKA RW20 digital homogenizer. The homogenates were centrifuged for 30 min at 9000 g and 4 °C to yield the post-mitochondrial fraction (S9). Immediately after the centrifugation, RhB and Rh123 amount in S9 tissue fraction was measured with a spectrofluorometer (Varian Cary Eclipse). The samples were kept in the dark to avoid RhB and Rh123 photodegradation. The fluorescence was determined at the wavelengths of 544 nm (excitation) and 590 nm (emission) for RhB,and at 490 nm (excitation) and 544 nm (emission) for Rh123. The measured fluorescence values were normalized by organisms' weight. Dye accumulation in the treatments was quantified as a fold increase over control.

Data analysis

Statistical analyses were performed with the statistical software R version 3.4.0 (R Development Core R Core Team, 2017) and RStudio (R Studio Team, 2016). The data were tested for normality with the Shapiro-Wilk test and the homogeneity of variance with the Bartlett test. As the data were distributed normally, the sig- nificance of differences in Rh123 and RhB accumulation was assessed by ANOVA followed by the Dunnett *post hoc* test. For curvefitting and calculation of 50% inhibitory concentration (IC50) values, R package drc (Ritz et al., 2015) was used. Fluorescent substrate accumulation during different exposure times was fitted to the three-parameter log-logistic model.

Results

Dye accumulation experiment

RhB and Rh123 accumulation dynamics depended on fluores- cent dye concentration and exposure length (Fig. 1). In this exper- iment, no mortality was observed. The kinetics of fluorescent dye accumulation differed between the species. Significant dye accu- mulation, detected as fluorescence increase, was observed in *E. albidus* when it was exposed to 20 mM Rh123 (Fig. 1A) and when it was exposed to 1.25, 2.5, 5, and 20 mM RhB, both for 6 h (Fig. 1C).In *E. crypticus*, a significant fluorescence increase was observed only after it was exposed to the highest Rh123 concentration for 6 h (Fig. 1B) and when it was exposed to the highest RhB concentration for two, four, and 6 h (Fig. 1D). The results obtained after 6 h exposure to 20 mM Rh123 for both species and after two, four, and 6 h exposure to 20 mM RhB for *E. crypticus* indicate passive dye diffusion. Rh123 showed a stronger response than RhB. Namely, Δfluorescence was significantly higher (5 times higher) in both species exposed to 20 mM Rh123 for 6 h than to the same RhB concentration.

Efflux pump activity modulation

E. albidus

The exposure of *E. albidus* to CA significantly ($p < 0.05$) increased Rh123 fluorescence in a dose- dependent manner (up to 1.6-fold), but it increased RhB fluorescence only at 10 mM (1.3-fold) (Fig. 2A). IVM increased the fluorescence of both substrates (Fig. 2C) up to 1.3-fold. Furthermore, VER increased Rh123 accumulation ($p < 0.05$) in a dose-dependent manner, with an increase ranging from 1.1 to 1.5-fold compared with the control (Fig. 2E). It also increased RhB fluorescence 1.65-fold at the lowest concentration applied (5 mM VER), but decreased it at higher concentrations (Fig. 2E). DEX significantly decreased both substrates' fluorescence at all concentrations (Fig. 3A) e Rh123 fluorescence decreased 0.3-fold and RhB fluorescence decreased 0.4-fold. RIF significantly ($p < 0.05$) decreased the fluorescence of both substrates in a dose- dependent manner (Fig. 3C).

Increased fluorescence enabled the calculation of IC_{50} for CA and VER in a dose-dependent manner. IC_{50} for VER in combination with Rh123 was 13.77 ± 5.92 μM and for VER in combination with RhB it was 10.10 ± 3.54 μM IC_{50} for CA in combination with Rh123 was 7.05 ± 2.13 μM) and for CA in combination with RhB it was 5.48 ± 1.25 μM.

E. crypticus

The accumulation of both fluorescent substrates in *E. crypticus* exposed to model inhibitors resulted in fluorescence increase. CA treatment significantly ($p < 0.05$) increased the fluorescence in a dose-dependent manner (Fig. 2B) - Rh123 fluorescence increased from 1.1 to 2.1-fold and RhB fluorescence increased from 1.3 to 1.8-fold compared with the control. IVM I ncreased Rh123 fluorescence up to 1.45-fold, and it increased RhB fluorescence up to 0.5-fold (Fig. 2D). Unlike in *E. albidus*, VER caused a dose-dependent in- crease ($p < 0.05$) of both substrates (Fig. 2F). At higher concentra- tions, such as at 50 μM, VER increased Rh123 fluorescence up to 2.2-fold, and RhB fluorescence up to 1.7-fold. Both inducers (DEX and RIF) significantly decreased Rh123 fluorescence and slightly decreased RhB fluorescence (Fig. 3B, D).

The most potent inhibitor of the MXR system was CA in com- bination with RhB (IC_{50} 5.20 ± 3.10 μM) and Rh123 (IC_{50} 6.59 ± 1.71 μM). IC_{50} for IVM in combination with Rh123 was 8.35 ± 5.34 μM and for IVM in combination with RhB it was 7.90 ± 3.90 μM. IC for VER in combination with Rh123 was 19.55 ± 6.75 μM and for VER in combination with RhB it was 7.90 ± 3.90 μM.

Validation experiment

No mortality was observed after 6 h exposure to PCZ in either ISO water or AS tests. In both media, PCZ inhibited efflux pump activity. Namely, it increased fluorescence in a dose-dependent manner in both species and in both exposure substrates (Fig. 4).In *E. albidus*, it significantly increased Rh123 fluorescence at con- centrations higher than 1.25 mg PCZ/kg (above 2-fold at 2.45 and above 2.2-fold at 4.9 mg PCZ/kg) in ISO water (Fig. 4A). In soil, the same PCZ concentrations caused a slightly lower Rh123 fluores- cence increase (1.6-fold at 2.4 and 4.9 mg PCZ/kg). In *E. crypticus*, Rh123 fluorescence increased above 2-fold at 2.45 and 4.9 mg PCZ/kg in ISO water, while in soil, it increased for more than 3-fold(4.9 mg PCZ/kg) (Fig. 4B).

IC_{50} for PCZ obtained by ISO water exposure was 0.74 ± 0.24 mg/L for *E. albidus* and 1.31 ± 0.24 mg/L for *E. crypticus*. These values were slightly higher after soil exposure, with almost the same values for both species: 1.79 ± 0.42 mg/kg for *E. albidus* and 1.79 ± 0.17 mg/kg for *E. crypticus*.

Discussion

The exposure to known inhibitors (CA, VER, IVM) and inducers (DEX, RIF) changed Rh123 and RhB accumulation, which confirmed the presence of efflux transporters and their activity in *E. albidus* and *E. crypticus*.

In the dye accumulation experiment, Rh123 uptake at the same concentration of fluorescent dye was time-dependent in both enchytraeid species, while RhB uptake significantly changed only in

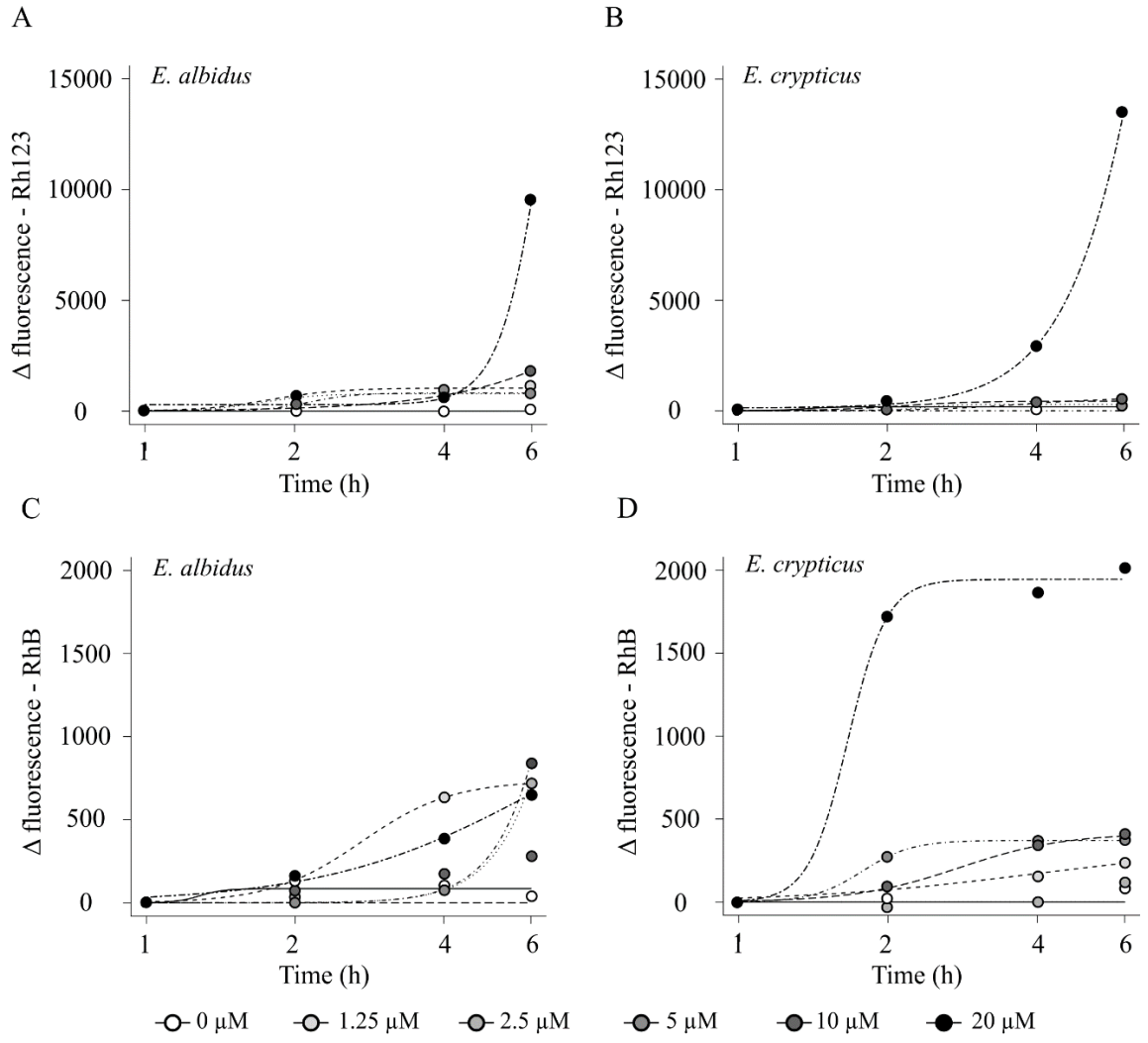


Fig. 1. Fitted substrate accumulation (three-parameter log-logistic model) as a function of exposure time (1, 2, 4, and 6 h) and substrate concentrations: Rh123 (0, 1.25, 2.5, 5,10, and 20 µM) (A, C) and RhB (0, 1.25, 2.5, 5, 10, and 20 µM) (B, D) in *E. albidus* (A, C) and *E. crypticus* (B, D). D fluorescence is expressed as a change in fluorescent units compared with the control obtained from (n = 5).

E. crypticus. This difference may be attributed to different proper- ties, binding sites, and fluorescent ability of fluorescent substrates. Furthermore, RhB is reported to have lower fluorescence intensity in combination with phosphate buffer (Sauer et al., 1995), while it does not have an impact on Rh123 fluorescence intensity.

The fluorescent dye concentration should be high enough to allow a sufficient dye uptake but not too high since this could induce passive diffusion across biological membranes. Although in human hepatocellular carcinoma cell line Rh123 uptake was re- ported to be an active process at concentrations below 2 µM (Forster et al., 2012), in zebrafish hepatic cells *in vivo* passive diffusion was not reported at 20 µM Rh123 concentration (Jackson and Kennedy, 2017). Our results suggest that in *E. albidus* passive diffusion occurred after 6-h exposure to 20 µM Rh123, while RhB fluorescence increase was not significant, and passive diffusion occurred at concentrations higher than tested. In *E. crypticus*, pas- sive diffusion also occurred after 6-h exposure to 20 µM Rh123, and after 20 µM RhB exposure longer than 1 h. Hence,, 1 h was chosen as an optimal exposure time, while the concentrations of 5 µM for Rh123 and 10 µ M for RhB were selected based on fluorescence Intensity and fluorescence change. At these concentrations, the active dye uptake is sufficien t to conduct an assay but low enough to avoid passive diffusion. Additionally, at these concentrations, the substrate fluorescence is high enough to be detected.

Almost all used model inhibitors increased Rh123 and RhB accumulation and, consequently, their fluorescence, in a dose- dependent manner. For both species and substrates, the most potent inhibitor was CA. It is a known non-competitive inhibitor that blocks the P-gp ATP-ase activity necessary for ATP-mediated efflux (Twentyman, 1992). Model competitive inhibitor VER in *E. crypticus* inhibited the efflux pump in combination with both fluorescent substrates, and in *E. albidus* only in combination with Rh123. Interestingly, even though VER increased RhB fluorescence in earthworm *Eisenia andrei* (Hackenberger et al., 2012) and pot- worm *E. crypticus*, it caused a decrease in *E. albidus* and *Eisenia fetida* (Bošnjak et al., 2014). Such a response presumably results from the interaction between the fluorescent substrate and the inhibitor or binding site due to VER's mode of action. Namely, VER is a competitive inhibitor that acts as a substrate with a high affinity to the P-gp binding site, preventing the binding and active

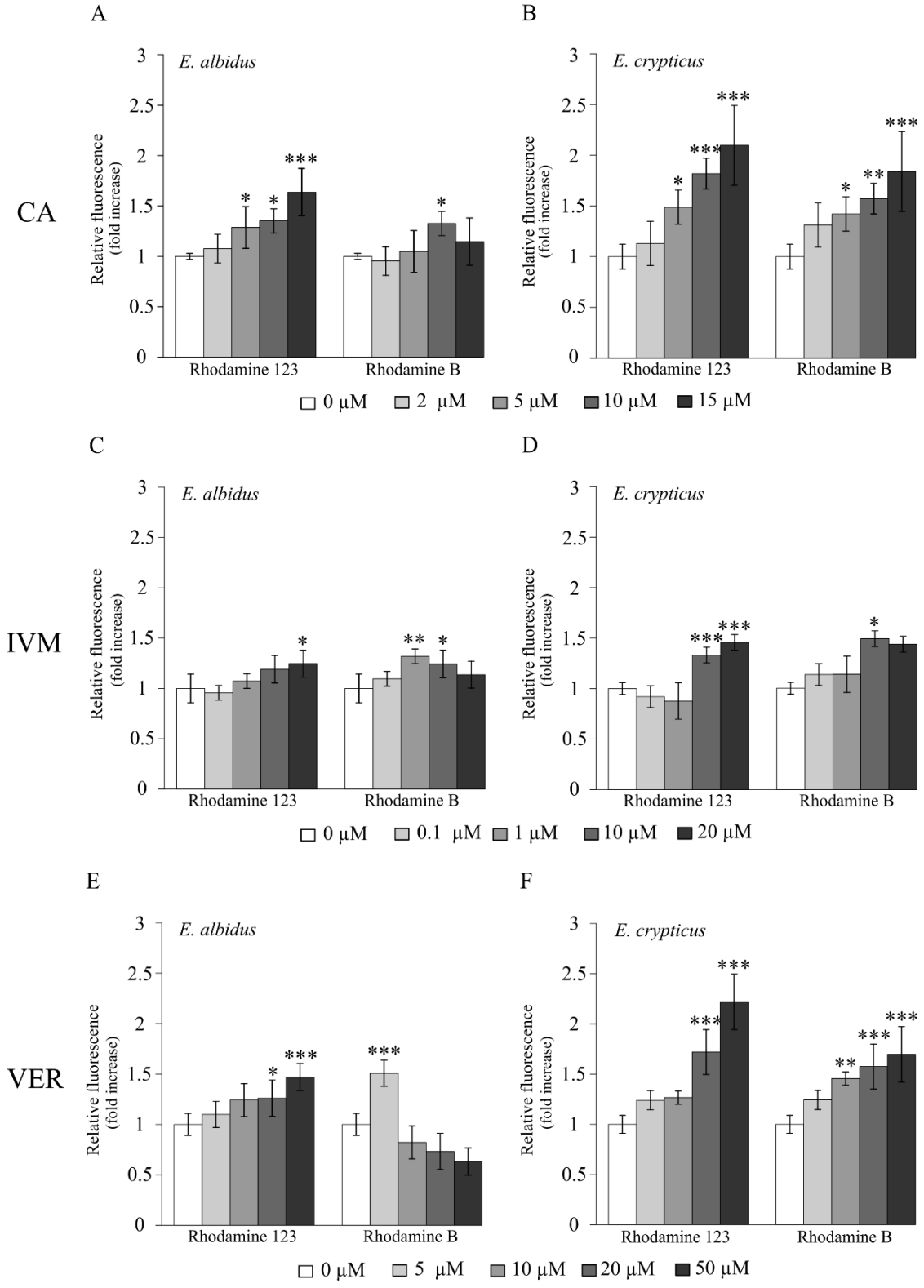


Fig. 2. Rh123 (5 μM) and RhB (10 μM) relative fluorescence in *E. albidus* (A, C, E) and *E. crypticus* (B, D, F) exposed to different concentrations of inhibitors. Cyclosporine A (0, 2, 5,10, and 15 μM) (A, B); ivermectin (0, 0.1, 1, 10, and 20- μM) (C, D); verapamil (0, 5, 10, 20, and 50 μM) (E, F). The relative fluorescence was expressed as a fold increase compared with the control. The results are expressed as the mean ± SD (n = 5). Significant differences obtained with the *post hoc* test compared with the control are labeled as ***p < 0.001, **p < 0.01, and *p < 0.05.

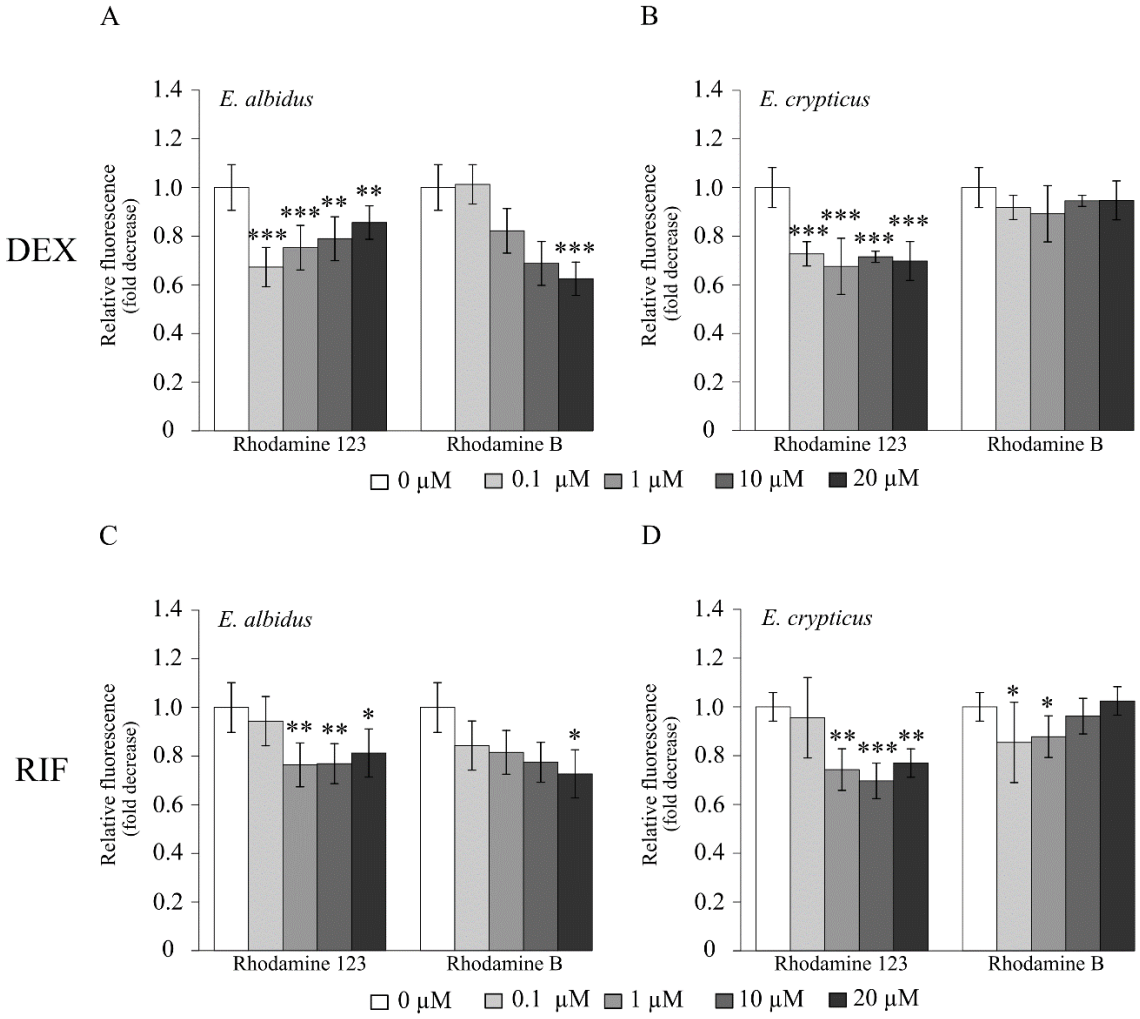


Fig. 3. Rh123 (5 µM) and RhB (10 µM) relative fluorescence in *E. albidus* (A, C) and *E. crypticus* (B, D) exposed to different concentrations of inducers. Dexamethasone (0, 0.1, 1,10 and 20 µM) (A, B); rifampicin (0, 0.1, 1,10 and 20 µM) (C, D). The relative fluorescence was expressed as a fold decrease compared with the control. The results are expressed as the mean ± SD (n = 5). Significant differences obtained with the *post hoc* test compared with the control are labeled as ***p < 0.001, **p < 0.01, and *p < 0.05.

Transport of other substrates or xenobiotics. The increased fluo- rescence at higher CA or VER concentrations, particularly notice- able in *E. crypticus* with Rh123 as a substrate, can be associated with the transport not only with P-gp but also with proteins from the MRP subfamily (Haimeur et al., 2005). These results suggest species-specific ratios of P-gp and MRP proteins in enchytraeid species. Furthermore, although RhB can serve as a MRP substrate, it is a model P-gp substrate, being commonly used as a fluorescent dye in marine and freshwater invertebrates (Smital et al., 2000). In contrast, Rh123, recognized as a substrate for both P-gp and MRP, is suggested as a dye of choice for earthworm models (Bošnjak et al.,2014). Our results point to Rh123 being a better fluorescent sub- strate for enchytraeid experiments as well. This is likely related to species-specific ratios of P-gp and MRP proteins, which could be similar to those in earthworms since both of these species belong to the annelid phylum. Additionally, Žaja et al. (2007) demonstrated that CA, VER, reversin 205, MK571, probenecid, and indomethacin were more potent in combination with Rh123. In our study, the third tested inhibitor, IVM, inhibited the efflux and increased Rh123and RhB accumulation in both species. The same pattern was observed by Lespine et al. (2006), who showed that IVM inhibited P-gp transport function and MRPs in various modified cell lines. In their study, IVM inhibited P-gp proteins at lower concentrations, while MRP inhibition required higher concentrations (Lespine et al.,2006). Still, it has to be taken into consideration that MRP does not compensate for P-gp absence, which leads to neurotoxicity in ani- mals exposed to IVM. Furthermore, IVM is highly toxic to soil or- ganisms (Jensen et al., 2003), and in our preliminary experiment study concentrations higher than 50 µM were fatal for both species. CA was also lethal for both enchytraeid species at concentrations higher than 50 µM, while RIF and DEX showed lethal effects at 100 µM. VER was least toxic, with no lethal effects even at the highest concentration (100 µM).

P-gp inducers DEX and RIF (Séréé et al., 1998) decreased RhB and Rh123 accumulation, which indicates that they induced the efflux pump mechanism, i.e., enhanced the removal of fluorescent sub- strates from the cells, thereby decreasing fluorescence. The same induction effect was observed, for example, in earthworm *Eisenia andrei* exposed to DEX (Hackenberger et al., 2012) and porcine brain capillary endothelial cells treated with RIF (Ott et al., 2009). Furthermore, in our study both inducers caused a difference in induction between the species and fluorescent dyes. Thus, in

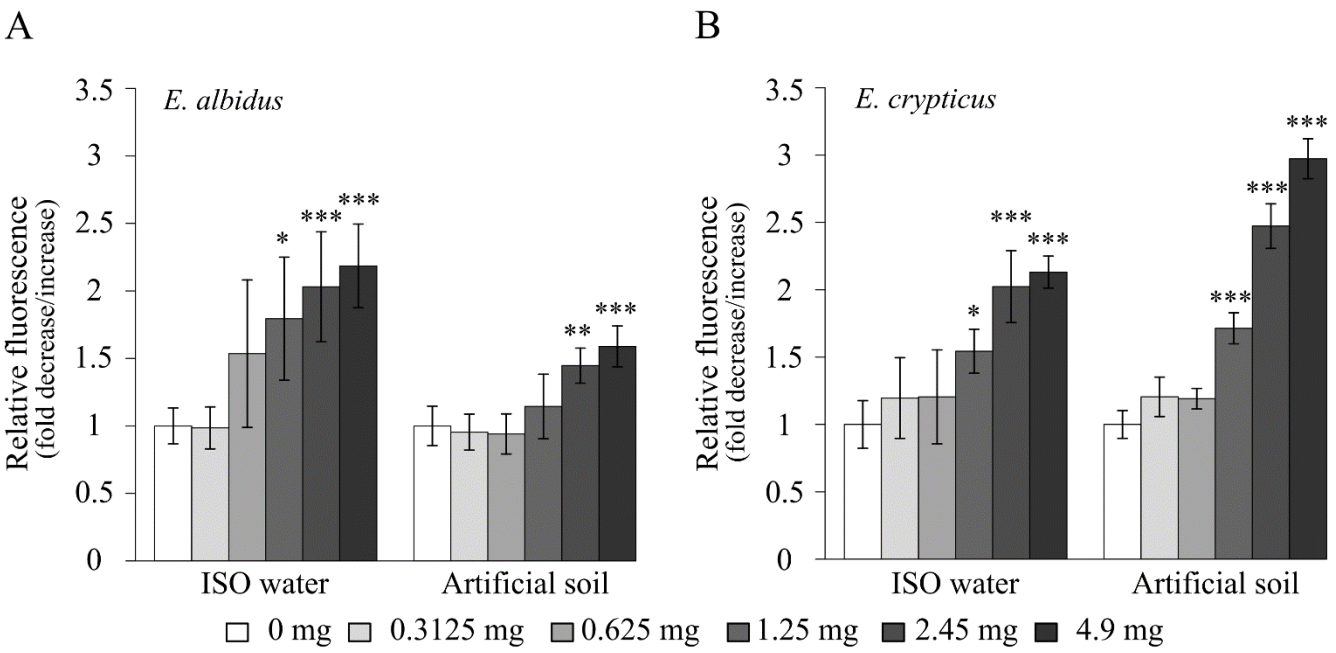


Fig. 4. Rh123 (5μM) relative fluorescence in *E. albidus*(A) and *E. crypticus*(B) exposed to different concentrations of PCZ in ISO water (0, 0.3125, 0.625,1.25, 2.45, and 4.9 mg a.i. L-1)and AS (0, 0.3125, 0.625, 1.25, 2.45, and 4.9 mga.i.kg^{-1} $_{d.w.\ soil}$). The relative fluorescence was expressed as a fold increase/decrease compared with the control. The results are expressed as the mean±SD (n = 5). Significant differences obtained with thepost hoctest compared with the control are labeled as***p<0.001,**p<0.01, and*p<0.05.

E. crypticus there was little or no induction in combination with RhB, while a significant induction was observed in combination with Rh123. In *E. albidus*, lower DEX concentrations caused higher induction, which decreased with increasing DEX concentration. On the contrary, RIF induction was dose dependent. Such differences can probably be attributed to different protein structures of the twospecies, but also to different types of interaction between fluores- cent substrates and inhibitors.

To avoid interference with or binding to soil substances, tests were performed in ISO water, making chemosensitizers maximallyavailable to enchytraeids (Römbke and Knacker, 1989). Aquatic tests are excellent for short-term and range-finding tests, facili- tating continuous monitoring of the behavior during exposure. In our study, ISO water tests enabled us to quickly and easily assess acute exposure and possible adverse effects of different substances.However, as enchytraeids are a soil organism, experiments con- ducted in that medium allow a more realistic exposure and assessment. The validation experiment with PCZ showed the ISO water test to be comparable with the AS test, indicating that both media can be used to investigate the effect of a substance on efflux pump activity in enchytraeids. Furthermore, the dye assay proved to be an easy to use and effective method to study efflux pump activity after enchytraeid exposure to various chemosensitizers.

An efficient MXR system increases xenobiotic resistance. Namely, high efflux transport activity allows only a few of the potentially harmful substances to reach a toxic level (Bard, 2000; Epel et al., 2008). In a marine environment, most pollutants in- crease the efflux transporters activity, but some of them act as in- hibitors (Jeong et al., 2017). These chemosensitizers are not necessarily substrates of the efflux system, but they may interact either on the substrate-binding site or on the regulatory part of the transporter molecules and affect the xenobiotic accumulation (Kurth et al., 2017). Some pesticides show MXR inhibitory potential at environmentally relevant concentrations. For example, organo- phosphate and neonicotinoid insecticides, as well as conazole fungicides, inhibited the P-gp efflux activity in various organisms (Velki and Hackenberger, 2012, 2013a, 2013b; Mazur et al., 2015; Guseman et al., 2016; Vehovszky et al., 2018; Velki et al., 2018). The inhibition of efflux pump activity after exposure to PCZ observed in our study corresponds to previous findings on conazole fungicides. An analysis of PCZ transport in membrane vesicles demonstrated that PCZ acted as a P-gp inhibitor rather than a substrate (Mazur et al., 2015). Furthermore, P-gp modulation or inhibition can significantly affect the absorption and excretion of xenobiotics, anddrugs' pharmacokinetics and safety. As expected, PCZ has a more pronounced impact during exposure in water. The fluorescence increase observed after enchytraeids were exposed to the highest PCZ concentration in water is comparable to the increase caused bymodel inhibitors (CA and VER). Similar results were observed in a study using NIH-3T3/MDR1 cells, which showed that PCZ and VER inhibited P-gp at a comparable level (Pivčević and Žaja 2006). Previous studies of the PCZ effect on *E. albidus* (Hackenberger et al.,2019) showed that EC50 for reproduction was 3.7 mg/kg, as well as that GST activity and lipid peroxidation significantly increased at concentrations ranging from 2.45 mg/kg to 4.9 mg/kg. As MXR activity inhibition took place already at 1.25 mg/kg after one week of exposure, the changes in efflux pump activity could be used as an early and complementary biomarker of soil pollution in enchytraeids.

Efflux transporters inhibition can enhance the toxicity of some xenobiotics, as the potential for xenobiotic accumulation inside the cell depends on the efflux pump activity. Therefore, it is useful to determine the effects of xenobiotics and chemosensitizers on the efflux pump activity in different organisms and understand themechanisms underlying these processes. Additionally, the syner- gistic action of some xenobiotics can lead to more potent adverse effects at lower concentrations. All this makes it important to investigate the impact of environmental pollutants on efflux pump activity in enchytraeids and to assess the effect of changes of efflux pump activity on the sensitivity towards other chemicals or envi- ronmental stressors.

Conclusion

Both tested enchytraeid species were shown to be suitable models for the established dye assay. Furthermore, functional

experiments demonstrated the expression of ABC efflux transporters in enchytraeids. The efflux pump activity in enchytraeids can be modulated by common substrates and chemosensitizers used in earthworm models. However, the difference in the fluorescence substrate accumulation suggests the presence of particular MXR proteins (P-gp and MRP). Further research should use various types of MXR inhibitors and specific substrates to determine the dominant type of MXR transporters in enchytraeids and their gene expression. The validation test results showed that this assay was applicable in a standard toxicity test conducted in the soil. Besides, the exposure to PCZ showed that environmental xenobiotics could affect the MXR system in enchytraeids, which warrants further research on the effects of different pollutants and abiotic factors on efflux pump activity. In conclusion, the changes of MXR activity could be a valuable complementary biomarker in ecotoxicological research and risk assessment not only in aquatic but also in soil organisms.

Declaration of competing interest

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

Acknowledgment

This research was conducted in the framework of the DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) funded by the Croatian Science Foundation (HrZZ) under the contract number: IP-09-2014-4459 and "Young Researchers' Career Development Project e Training of Doctoral Students" funded by the Croatian Science Foundation (HrZZ) (DOK-2018-01-4257).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130549.

Credit author statement

Marija Kovačević; - writing - original draft, Investigation, Davorka K. Hackenberger - investigation, writing, Visualization, Željka Lončarić - investigation, Formal analysis, Branimir K. Hackenberger - conceptualization, Supervision, funding acqusition, Writing e review & editing.

References

Amorim, M.J.B., Scott-Fordsmand, J.J., 2012. Toxicity of copper nanoparticles and $CuCl_2$ salt to *Enchytraeus albidus* worms: survival, reproduction and avoidance responses. Environ. Pollut. 164, 164e168. https://doi.org/10.1016/ j.envpol.2012.01.015.

Amorim, M.J.B., Römbke, J., Soares, A.M.V.M., 2005. Avoidance behavior of *Enchytraeus albidus*: effects of Benomyl, Carbendazim, phenmedipham and different soil types. Chemosphere 59, 501e510. https://doi.org/10.1016/ j.chemosphere.2005.01.057.

Anselmo, H.M.R., Van Den Berg, J.H.J., Rietjens, I.M.C.M., Murk, A.T.J., 2012. Inhibition of cellular efflux pumps involved in multi xenobiotic resistance (MXR) in echinoid larvae as a possible mode of action for increased ecotoxicological risk of mixtures. Ecotoxicology 21, 2276e2287. https://doi.org/10.1007/s10646-012-0984-2.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357e389. https://doi.org/10.1016/S0166-445X(00)00088-6.

Bart, S., Laurent, C., Péry, A.R.R., Mougin, C., Pelosi, C., 2017. Differences in sensitivity between earthworms and enchytraeids exposed to two commercial fungicides. Ecotoxicol. Environ. Saf. 140, 177e184. https://doi.org/10.1016/ j.ecoenv.2017.02.052.

Bicho, R.C., Santos, F.C.F., Gonçalves, M.F.M., Soares, A.M.V.M., Amorim, M.J.B., 2015. Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test-an optional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology 24, 1053e1063. https://doi.org/10.1007/s10646-015-1445-5.

Bicho, R.C., Ribeiro, T., Rodrigues, N.P., Scott-Fordsmand, J.J., Amorim, M.J.B., 2016. Effects of Ag nanomaterials (NM300K) and Ag salt (AgNO3) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*. J. HazardMater. 318, 608e614. https://doi.org/10.1016/j.jhazmat.2016.07.040.

Bicho, R.C., Santos, F.C.F., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. Effects of copper oxide nanomaterials (CuONMs) are life stage dependent e full life cycle in *Enchytraeus crypticus*. Environ. Pollut. 224, 117e124. https://doi.org/10.1016/ j.envpol.2017.01.067.

Bošnjak, I., Bielen, A., Babić, S., Šver, L., Popović, N.T., Strunjak-Perović, I., Čož-Rakovac, R., Klobučar, R.S., 2014. First evidence of the P-glycoprotein gene expression and multixenobiotic resistance modulation in earthworm. Arh. Hig. Rada. Toksikol. 65, 67e75. https://doi.org/10.2478/10004-1254-65-2014-2421.

Briones, M.J.I., 2014. Soil fauna and soil functions: a jigsaw puzzle. Front. Environ. Sci. 2 https://doi.org/10.3389/fenvs.2014.00007.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., Amorim, M.J.B., 2012. *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87, 1222e1227. https://doi.org/10.1016/ j.chemosphere.2012.01.021.

Chu, X., Korzekwa, K., Elsby, R., Fenner, K., Galetin, A., Lai, Y., 2013. Intracellular drug concentrations and transporters: measurement modelling and implications for the liver. Nature 94, 126e141.

Cunha, V., Burkhardt-Medicke, K., Wellner, P., Santos, M.M., Moradas-Ferreira, P., Luckenbach, T., Ferreira, M., 2017. Effects of pharmaceuticals and personal care products (PPCPs) on multixenobiotic resistance (MXR) related efflux transporter activity in zebrafish (*Danio rerio*) embryos. Ecotoxicol. Environ. Saf. 136, 14e23. https://doi.org/10.1016/j.ecoenv.2016.10.022.

Daoud, R., Kast, C., Gros, P., Georges, E., 2000. Rhodamine 123 binds to multiple sites in the multidrug resistance protein (MRP1). Biochemistry 39, 15344e15352.

Didden, W., Römbke, J., 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicol. Environ. Saf. 50, 25e43. https://doi.org/10.1006/eesa.2001.2075.

Ečimović, S., Velki, M., Vuković, R., Šolfa Čamagajevac, I., Petek, A., Bošnjaković, R., Grgić, M., Engelmann, P., Bodó, K., Filipović-Marijić, V., Ivanković, D., Erk, M., Mijošek, T., Lončarić, Z., 2018. Acute toxicity of selenate and selenite and their impacts on oxidative status, efflux pump activity, cellular and genetic parameters in earthworm *Eisenia andrei*. Chemosphere. https://doi.org/10.1016/ j.chemosphere.2018.08.095.

Epel, D., Luckenbach, T., Stevenson, C.N., Macmanus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: newly appreciated roles in protection against pollutants. Environ. Sci. Technol. 42, 3914e3920. https://doi.org/ 10.1021/es087187v.

Falfushynska, H., Sokolov, E.P., Haider, F., Oppermann, C., Kragl, U., Ruth, W., Stock, M., Glufke, S., Winkel, E.J., Sokolova, I.M., 2019. Effects of a common pharmaceutical, atorvastatin, on energy metabolism and detoxification mechanisms of a marine bivalve *Mytilus edulis*. Aquat. Toxicol. 208, 47e61. https:// doi.org/10.1016/j.aquatox.2018.12.022.

Faria, M., Navarro, A., Luckenbach, T., Pinna, B., Barata, C., 2011. Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants. Aquat. Toxicol. 101, 78e87. https://doi.org/10.1016/j.aquatox.2010.09.004.

Forster, S., Thumser, A.E., Hood, S.R., Plant, N., 2012. Characterization of Rhodamine-123 as a tracer dye for use in *in vitro* drug transport assays. PloS One 7 (3), e33253. https://doi.org/10.1371/journal.pone.0033253.

Franzellitti, S., Striano, T., Pretolani, F., Fabbri, E., 2017. Investigating appearance and regulation of the MXR phenotype in early embryo stages of the Mediterranean mussel (*Mytilus galloprovincialis*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 199, 1e10. https://doi.org/10.1016/j.cbpc.2016.11.004.

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M.R., Cordeiro, M.F., 2013. Unexpected low-dose toxicity of the universal solvent DMSO. The FASEBJournal article fj 13e235440.

Gomes, S.I.L., Caputo, G., Pinna, N., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Effect of 10 different TiO_2 and ZrO_2 (nano)materials on the soil invertebrate *Enchytraeus crypticus*. Environ. Toxicol. Chem. 34, 2409e2416. https://doi.org/10.1002/etc.3080.

Guseman, A.J., Kaliah, M., Kunkle, G., Dively, G.P., Pettis, J.S., Evans, J.D., Van Engelsdorp, D., Hawthorne, D.J., 2016. Multi-drug resistance transporters and a mechanism-based strategy for assessing risks of pesticide combinations to honey bees. PloS One 11, e0148242. https://doi.org/10.1371/ journal.pone.0148242.

Hackenberger, B.K., Velki, M., Stepić, S., Hackenberger, D.K., 2012. First evidence for the presence of efflux pump in the earthworm *Eisenia andrei*. Ecotoxicol. Environ. Saf. 75, 40e45. https://doi.org/10.1016/j.ecoenv.2011.06.024.

Hackenberger, D.K., Stjepanović, N., Lončarić, Ž, Hackenberger, B.K., 2019. Effects of single and combined exposure to nano and bulk zinc-oxide and propiconazoleon *Enchytraeus albidus*. Chemosphere 224, 572e579. https://doi.org/10.1016/ j.chemosphere.2019.02.189.

Haimeur, A., Conseil, G., Deeley, R.G., Cole, S.P.C., 2005. The MRP-related and BCRP/ABCG2 multidrug resistance proteins: Biology, substrate specificity and regulation. Curr. Drug Metabol. 5, 21e53. https://doi.org/10.2174/

1389200043489199.
Henle, F.G.J., 1837. Ueber Enchytraeus, eine neue Anneliden-Gattung. Arch. Anat. Physiol. Med. 74e90.
International Organization for Standardization (ISO), 2004. Soil QualitydEffects of Pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of Effects on Reproduction and Survival. ISO 16387:2004.
International Organization for Standardization (ISO), 2012. Water quality Deter- mination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) - acute toxicity test. ISO 6341:2012.
Jackson, J.S., Kennedy, C.J., 2017. Regulation of hepatic abcb4 and cyp3a65 gene expression and multidrug/multixenobiotic resistance (MDR/MXR) functional activity in the model teleost, *Danio rerio* (zebrafish). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 200, 34e41. https://doi.org/10.1016/j.cbpc.2017.06.004.
Jensen, J., Krogh, P.H., Sverdrup, L.E., 2003. Effects of the antibacterial agents tia- mulin, olanquindox and metronidazole and the anthelmintic ivermectin on the soil invertebrate species *Folsomia fimetaria* (Collembola) and *Enchytraeus crypticus* (Enchytraeidae). Chemosphere 50, 437e443. https://doi.org/10.1016/ S0045-6535(02)00336-3.
Jeong, C.B., Kim, H.S., Kang, H.M., Lee, J.S., 2017. ATP-binding cassette (ABC) proteins in aquatic invertebrates: evolutionary significance and application in marine ecotoxicology. Aquat. Toxicol. 185, 29e39. https://doi.org/10.1016/ j.aquatox.2017.01.013.
Katsumiti, A., Thorley, A.J., Arostegui, I., Reip, P., Valsami-Jones, E., Tetley, T.D., Cajaraville, M.P., 2018. Cytotoxicity and cellular mechanisms of toxicity of CuO NPs in mussel cells in vitro and comparative sensitivity with human cells. Toxicol. Vitro 48, 146e158. https://doi.org/10.1016/j.tiv.2018.01.013.
Kurelec, B., 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22, 23e43. https://doi.org/10.3109/10408449209145320.
Kurelec, B., 1997. A new type of hazardous chemical: the chemosensitizers of multixenobiotic resistance. Environ. Health Perspect. 105, 855e860.
Kurelec, B., Krča, S., Lucić, D., 1996. Expression of multixenobiotic resistance mechanism in a marine mussel *Mytilus galloprovincialis* as a biomarker of exposure to polluted environments. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 113, 283e289. https://doi.org/10.1016/0742-8413(96) 84525-7.
Kurelec, B., Smital, T., Pivcevic, B., Eufemia, N., Epel, D., 2000. Multixenobiotic resistance, P-glycoprotein, and chemosensitizers. Ecotoxicology 9, 307e327.
Kurth, D., Lips, S., Massei, R., Krauss, M., Luckenbach, T., Schulze, T., Brack, W., 2017. The impact of chemosensitization on bioaccumulation and sediment toxicity. Chemosphere 186, 652e659. https://doi.org/10.1016/ j.chemosphere.2017.08.019.
Lackmann, C., Velki, M., Seiler, T.B., Hollert, H., 2018. Herbicides diuron and flua- zifop--butyl affect avoidance response and multixenobiotic resistance p ac- tivity in earthworm.
Leitão, S., Cerejeira, M.J., Van den Brink, P.J., Sousa, J.P., 2014. Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial in- vertebrates using a natural Mediterranean soil. Appl. Soil Ecol. 76, 124e131. https://doi.org/10.1016/j.apsoil.2013.12.013.
Leslie, E.M., Deeley, R.G., Cole, S.P.C., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204 (3), 216e237. https://doi.org/10.1016/j.taap.2004.10.012.
Lespine, A., Dupuy, J., Orlowski, S., Nagy, T., Glavinas, H., Krajcsi, P., Alvinerie, M.,2006. Interaction of ivermectin with multidrug resistance proteins (MRP1, 2 and 3). Chem. Biol. Interact. 159, 169e179. https://doi.org/10.1016/ j.cbi.2005.11.002.
Licht, A., Schneider, E., 2011. ATP binding cassette systems: structures, mechanisms, and functions. Cent. Eur. J. Biol. 6, 785e801. https://doi.org/10.2478/s11535-011-0054-4.
Litman, T., Druley, T.E., Stein, W.D., Bates, S.E., 2001. From MDR to MXR: new un- derstanding of multidrug resistance systems, their properties and clinical sig- nificance. Cell. Mol. Life Sci. 58, 931e959. https://doi.org/10.1007/PL00000912.
Lopes, F.M., Sandrini, J.Z., Souza, M.M., 2019. Modulation of the multixenobiotic resistance mechanism in *Danio rerio* hepatocyte culture (ZF-L) after exposure to glyphosate and Roundup®. Chemosphere 228, 159e165. https://doi.org/10.1016/ j.chemosphere.2019.04.140.
Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug trans- porters in fish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 165, 28e52. https://doi.org/10.1016/j.cbpc.2014.05.002.
Mazur, C.S., Marchitti, S.A., Zastre, J., 2015. P-glycoprotein inhibition by the agri-cultural pesticide propiconazole and its hydroxylated metabolites: implications for pesticide-drug interactions. Toxicol. Lett. 232, 37e45. https://doi.org/ 10.1016/j.toxlet.2014.09.020.
Moreira, L.F., Zomer, S.J., Marques, S.M., 2019. Modulation of the multixenobioticresistance mechanism in *Danio rerio* hepatocyte culture (ZF-L) after exposure to glyphosate and Roundup®. Chemosphere. https://doi.org/10.1016/ j.chemosphere.2019.04.140.
Organization for Economic Cooperation and Development (OECD), 2016. Test No. 220: OECD Guideline for Testing of Chemicals Enchytraeid Reproduction Test. OECD Publishing, Paris Paris France.
Ott, M., Fricker, G., Bauer, B., 2009. Pregnane X receptor (PXR) regulates P-glyco- protein at the blood-brain barrier: functional similarities between pig and human PXR. J. Pharmacol. Exp. Therapeut. 329, 141e149. https://doi.org/ 10.1124/jpet.108.149690.
Pivcević, B., Žaja, R., 2006. Pesticides and their binary combinations as P- glyco- protein inhibitors in NIH 3T3/MDR1 cells. Environ. Toxicol. Pharmacol. 22, 268e276.
R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project. org/.
R Studio Team, 2016. RStudio. Integrated Development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com.
Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PloS One 10, e0146021.
Römbke, J., Knacker, T., 1989. Aquatic toxicity test for enchytraeids. Hydrobiologia 180, 235e242.
Sauer, M., Han, K.T., Müller, R., Nord, S., Schulz, A., Seeger, S., Wolfrum, J., Arden-Jacob, J., Deltau, G., Marx, N.J., Zander, C., Drexhage, K.H., 1995. New fluorescent dyes in the red region for biodiagnostics. J. Fluoresc. 5 (3), 247e261. https://doi.org/10.1007/BF00723896.
Sérée, E., Villard, P.H., Hevér, A., Guigal, N., Puyoou, F., Charvet, B., Point-Scomma, H., Lechevalier, E., Lacarelle, B., Barra, Y., 1998. Modulation of MDR1 and CYP3A expression by dexamethasone: evidence for an inverse regulation in adrenals. Biochem. Biophys. Res. Commun. 252, 392e395. https://doi.org/10.1006/ bbrc.1998.9662.
Smital, T., Kurelec, B., 1998. The chemosensitizers of multixenobiotic resistance mechanism in aquatic invertebrates: a new class of pollutants. Mutat. Res. Mol. Mech. Mutagen. 399, 43e53. https://doi.org/10.1016/S0027-5107(97)00265-0.
Smital, T., Sauerborn, R., Pivcević, B., Krča, S., Kurelec, B., 2000. Interspecies differ- ences in P-glycoprotein mediated activity of multixenobiotic resistance mech- anism in several marine and freshwater invertebrates. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 126, 175e186. https://doi.org/10.1016/S0742-8413(00)00110-9.
Smital, T., Luckenbach, T., Sauerborn, R., Hamdoun, A.M., Vega, R.L., Epel, D., 2004. Emerging contaminants - pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic or- ganisms. Mutat. Res. Fund Mol. Mech. Mutagen 552, 101e117. https://doi.org/ 10.1016/j.mrfmmm.2004.06.006.
Sumida, K., Igarashi, Y., Toritsuka, N., Matsashita, T., Abe-Tomizawa, K., Aoki, M., Urushidani, T., Yamada, H., Ohno, Y., 2011. Effects of DMSO on gene expressionin human and rat hepato-cytes. Hum. Exp. Toxicol. 30, 1701. https://doi.org/10.1177/0960327111399325.
Swift, M.J., Andren, O., Brussaard, L., Briones, M., Couteaux, M.M., Ekschmitt, K., Kjoller, A., Loiseau, P., Smith, P., 1998. Global change, soil biodiversity, and ni- trogen cycling in terrestrial ecosystems: three case studies. Global Change Biol.4, 729e743.
Topić Popović, N., Strunjak-Perović, I., Klobučar, R.S., Barišić, J., Babić, S., Jadan, M., Kepec, S., Kazazić, S.P., Matijatko, V., Ljubić, B., Car, I., Repec, S., Stipanicev, D., Klobučar, G.I.V., Čož-Rakovac, R., 2015. Impact of treated wastewater on organismic biosensors at various levels of biological organization. Sci. Total Environ. 538, 23e37. https://doi.org/10.1016/j.scitotenv.2015.08.044.
Twentyman, P.R., 1992. Cyclosporins as drug resistance modifiers. Biochem. Phar- macol. 43 (1), 109e117. https://doi.org/10.1016/0006-2952(92)90668-9.
Vehniäinen, E.R., Kukkonen, J.V.K., 2015. Multixenobiotic resistance efflux activity in *Daphnia magna* and *Lumbriculus variegatus*. Chemosphere 124, 143e149. https://doi.org/10.1016/j.chemosphere.2014.12.014.
Vehovszky, Á., Farkas, A., Csikós, V., Székács, A., Mörtl, M., Győri, J., 2018. Neon- icotinoid insecticides are potential substrates of the multixenobiotic resistance (MXR) mechanism in the non-target invertebrate. *Dreissena* sp. Aquat. Toxicol. 205, 148e155. https://doi.org/10.1016/j.aquatox.2018.10.013.
Velki, M., Hackenberger, B.K., 2012. Species-specific differences in biomarker re- sponses in two ecologically different earthworms exposed to the insecticide dimethoate. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 156, 104e112. https://doi.org/10.1016/j.cbpc.2012.05.001.
Velki, M., Hackenberger, B.K., 2013a. Biomarker responses in earthworm *Eisenia andrei* exposed to pirimiphos-methyl and deltamethrin using different toxicity tests. Chemosphere 90, 1216e1226. https://doi.org/10.1016/ j.chemosphere.2012.09.051.
Velki, M., Hackenberger, B.K., 2013b. Inhibition and recovery of molecular bio- markers of earthworm *Eisenia andrei* after exposure to organophosphate dimethoate. Soil Biol. Biochem. 57, 100e108. https://doi.org/10.1016/ j.soilbio.2012.09.018.
Velki, M., Stepić, S., Hackenberger, B.K., 2013. Effects of formalin on some biomarker activities of earthworms pre-exposed to temephos. Chemosphere 90, 2690e2696. https://doi.org/10.1016/j.chemosphere.2012.11.048.
Velki, M., Weltmeyer, A., Seiler, T.B., Hollert, H., 2018. Acute toxicities and effects on multixenobiotic resistance activity of eight pesticides to the earthworm *Eisenia andrei*. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-018-3959-x.
Westheide, W, Graefe, U, 1992. Two new terrestrial *Enchytraeus* species (Oli- gochaeta, Annelida). J. Nat. Hist. 26 (3), 479e488. https://doi.org/10.1080/ 00222939200770311.
Žaja, R., Klobučar, R.S., Smital, T., 2007. Detection and functional characterization of Pgp1 (ABCB1) and MRP3 (ABCC3) efflux transporters in the PLHC-1 fish hepa- toma cell line. Aquat. Toxicol. 81, 365e376. https://doi.org/10.1016/ j.aquatox.2006.12.015.

2.2. Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifloxystrobin) on survival, reproduction and hatching success of *Enchytraeus crypticus*

Science of the Total Environment 790 (2021) 148143

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifloxystrobin) on survival, reproduction and hatching success of *Enchytraeus crypticus*

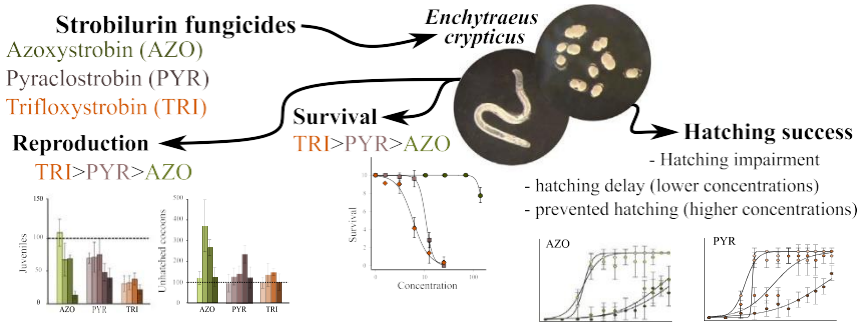
Marija Kovačević, Davorka K. Hackenberger, Branimir K. Hackenberger *

University of Osijek, Department of Biology, Cara Hadrijana 8A, HR-31000 Osijek, Croati

HIGHLIGHTS

- Effects of three strobilurin fungicides on enchytraeids were compared.
- Enchytraeid survival, reproduction, and hatching dynamics were evaluated.
- Trifloxystrobin was the most detrimen- tal on *E. crypticus* survival and reproduc-tion.
- Azoxystrobin and pyraclostrobin caused a hatching impairment and delay.
- Hatching test could improve risk assess-ment in the soil.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 January 2021
Received in revised form 19 April 2021
Accepted 25 May 2021
Available online 29 May 2021

Editor: Jose Julio Ortega-Calvo

Keywords:

Hatching impairment
Strobilurin fungicides
Enchytraeus Hatching
test Hatching dynamic

ABSTRACT

Large quantities of strobilurin fungicides (SFs) are used worldwide, resulting in adverse effects on non-target organisms. SFs affect the reproduction and embryonic development of aquatic organisms, while the impact on soil organisms has been insufficiently researched. Therefore, we investigated the effects of three SFs (azoxystrobin (AZO), pyraclostrobin (PYR), and trifloxystrobin (TRI)) on the survival, reproduction, and hatching success of the non-target soil oligochaete *Enchytraeus crypticus*. The standard enchytraeid reproduction test (ERT) showed that, regarding survival, TRI (LC_{50} = 2.34 mg/kg) was the most toxic, followed by PYR (LC_{50} = 4.26 mg/kg) and AZO (LC_{50} ≥150 mg/kg). Reproduction was affected in the same order (TRI EC_{50} = 0.045 mg/kg, PYR EC_{50} = 1.85 mg/kg, and AZO EC_{50} = 93.10 mg/kg). Exposure to AZO and PYR showed a negative impact on hatching success with a significant increase in the number of unhatched cocoons. Prolonged hatching test was consequently carried out. As a result, a hatching delay was observed at lower AZO and PYR concentrations, while at higher concentrations hatching was completely stopped as the cocoons were no longer viable. Hence, hatching test enabled a discrimination between hatching delay and hatching impairment. Besides demonstrating the adverse effects of AZO, PYR, and TRI on the survival, reproduction, and hatching success of *E. crypticus*, the obtained results indicate the convenience of using several endpoints in reproduction tests. The usage of prolonged hatching tests and monitoring of hatching dynamics could fill the gap between standard reproduction tests and multigeneration tests and allow a better understanding of the adverse effects on reproduction.

Introduction

Strobilurin fungicides (SFs) became an important and commonlyused class of agricultural fungicides. They bind to the mitochondrial

* Corresponding author.
E-mail address: hackenberger@biologija.unios.hr (B.K. Hackenberger).

https://doi.org/10.1016/j.scitotenv.2021.148143 0048-9697/

respiratory complex III and block electron transfer from quinol to cytochrome *c*1, thus acting on mitochondrial respiration and blocking ATP production (Bartlett et al., 2002). Azoxystrobin (AZO), pyraclostrobin (PYR), trifloxystrobin (TRI), fluoxastrobin (FLUO), picoxystrobin (PICO), and kresoxim-methyl (KRE) are considered the most widely used SFs types (Zhang et al., 2020c). Due to their efficiency, SFs are used in large quantities worldwide. This led to the accumu- lation of SFs in surface waters at high concentrations and could se- verily affect many soil ecosystems and harm non-target soil organisms (Ma et al., 2019). Among non-target soil organisms, enchytraeids play an important role in soil ecosystems. As environ- mentally relevant soil-dwelling annelids, they are involved in soil organic matter decomposition and bioturbation. In addition, they are widely distributed in many soil types and have a particularly important role in Arctic and subarctic regions where earthworms are absent.

Despite recent increased attention to the potential toxicity of SFs to various organisms, data on the toxicity of these fungicides to soil ecosystems and organisms are inconclusive, and existing information is sparse. Zhang et al. (2020c) provided an overview of the research conducted on the effects of SFs on different groups of non-target organisms. Most research on the effect of SFs on soil organisms has been conducted on the earthworm *Eisenia fetida* (Han et al., 2014; Schnug et al., 2014; Schnuget al., 2015; Ma et al., 2019; Zhang et al., 2020b), where they affected survival, reproduction, caused oxidative stress and DNA damage. SFs also reduced the reproductive rate of springtails (Leitão et al., 2014; Schnug et al., 2014). In enchytraeids, only the adverse effects of AZO on *Enchytraeus crypticus* reproduction have been demonstrated(Leitão et al., 2014; Gomes et al., 2021), while the effects of other SFs are unknown.

Embryotoxicity tests have been developed mainly for aquatic organisms, while for soil organisms such tests are rare. Standardized ecotoxicological tests for soil invertebrates primarily focus on assessing a survival and number of juveniles. However, assessing the effects on early life stages is both time-efficient and highly sensitive, especially for soil organisms (Bart et al., 2019). The need to develop a full life cycle test and tests that assess effects on early life stages has been highlighted in the regulatory context, particularly for specific com- pounds such as endocrine disruptors (Crane et al., 2010). Embryotoxicity tests in soil organisms have only recently been developed (Gonçalves et al., 2015). Such tests are performed on the potworm *E. crypticus*, which is one of the most commonly used model organisms in soil eco- toxicology due to its characteristics (Castro-Ferreira et al., 2012). Namely, a higher reproduction rate and shorter generation enable faster culture synchronization compared to *E. albidus*. Moreover, the ability tocultivate this species on agar plates allows easy collection and observa- tion of cocoons. The development of new tests and the awareness that embryonic development is one of the most sensitive points in the life cycle have led to an increase in the number of studies addressing this issue. Although the number of studies on the influence of metals and nanoparticles on embryonic development and different life stages of soil invertebrates increased (Bicho et al., 2015; Bicho et al., 2016; Bichoet al., 2017; Santos et al., 2017; Gomes et al., 2018), the influence of pes-ticides, including SFs, has not been studied yet. As SFs are known to affectembryo development in aquatic organisms, it would be interesting to as- sess if they have the same effect on enchytraeids. For example, studies on zebrafish have shown that embryos are the most sensitive life stagewhen exposed to AZO, KRE, PICO, PYR, and TRI (Jiang et al., 2019; Kimet al., 2020). Moreover, the exposure of zebrafish larval stage to FLUO led to delayed hatching, induced developmental toxicity, and oxidativestress (Zhang et al., 2020a).

Therefore, this study aimed to assess the effects of three SFs (azoxystrobin (AZO), pyraclostrobin (PYR), and trifloxystrobin (TRI)) on the potworm *E. crypticus* and evaluate the potential adverse effectsof SFs in terrestrial ecosystems studying multiple endpoints: survival, reproduction and hatching success.

Material and methods

Test organisms

The test species *Enchytraeus crypticus* (Westheide and Graefe, 1992) (Oligochaeta: Enchytraeidae) was used. Potworms were obtained froma culture maintained for several years at the Department of Biology in Osijek (Croatia). Cultures were maintained under controlled conditionsin a climate room at a constant temperature of 18 ± 1 °C, a relative humidity of 60%, and photoperiod of 16:8 h (light: dark) in agar plates prepared with a salt solution of $CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$. Potworms were fed *ad libitum* with ground rolled oatmeal.

Synchronized cultures of enchytraeids were prepared according toBicho et al. (2015). Briefly, adults with well-developed clitellum are transferred to fresh agar plates to lay cocoons. For the reproduction test, 25-day-old synchronized adults with well-developed clitellum were used. One day old cocoons were used for the hatching test.

Test soil

LUFA 2.2. standard natural soil (Speyer, Germany) was used. The main characteristics were pH (0.01 M $CaCl_2$) of 5.5, 1.73% organic mat-ter, 45.8% WHC (water holding capacity), 8.3% clay, 14.9% silt and 76.8% sand in terms of particle size distribution.

Test pesticides and spiking

Commercial formulations of the fungicides QUADRIS® (Syngenta) with the active ingredient azoxystrobin (AZO) (250 g/L), RETENGO® (Bayer AG) with the active ingredient pyraclostrobin (PYR) (200 g/L) and ZATO WG® (BASF SE) with the active ingredient trifloxystrobin (TRI) (500 g/kg) were used. The concentrations tested for the enchytraeid reproduction tests (ERT) were 0, 10, 25, 75, and 150 mg $_{a.i.}$/kg of d.w. soil for AZO and 0, 0.1, 0.3125, 0.625, 1.25, 2.5, 5 and 10 mg $_{a.i.}$/kg of d.w. soil for PYR and TRI. The selected concentrations were based on the resultsof a preliminary study. A careful examination of the cocoons after ERT showed a significant increase of unhatched cocoons in AZO and PYR treatments, while no difference in cocoon numbers in TRI treatments was observed. Furthermore, the cocoons in AZO and PYR treatments were almost fully developed, while in TRI treatments the cocoons were completely damaged and underdeveloped. Therefore, only AZO and PYR were selected for subsequent hatching test. The selected concentrations for the hatching test based on results of ERT were 0, 25, 75, and 150 mg $_{a.i.}$/kg of d.w. soil for AZO and 0, 0.625, 1.25 and 2.5 mg $_{a.i.}$/kg of d.w. soil for PYR.

As the commercial formulations are water soluble, stock solutions for each test substance were prepared and diluted. The required concentrations of commercial formulations dissolved in water were added separately to each replicate in the amount required for 60% of the WHC. The soil was mixed homogeneously and equilibrated for 24 h before starting the tests. To confirm nominal pesticide concen- trations, an LC-MS/MS (HRN EN 15662:2018) analysis were per- formed using Agilent 6470 LC/MS system. The results showed that the deviation between nominal and actual concentrations of chemicalswas less than 20% (Tables S1, S2). Therefore, the results are given in nominal concentrations.

Test procedures

The enchytraeid reproduction test (ERT) was performed accordingto standard guidelines (ISO, 2004; OECD, 2016). Briefly, 10 adult *E. crypticus* with well-developed clitellum were introduced into each test vessel containing 20 g of moist spiked soil, after which 2 mg of oat-meal was added for food. Food and water were replenished weekly. Thetest ran at 20 °C and 16:8 h photoperiod for 21 days. All treatments werecarried out in five replicates. To extract organisms from the soil,

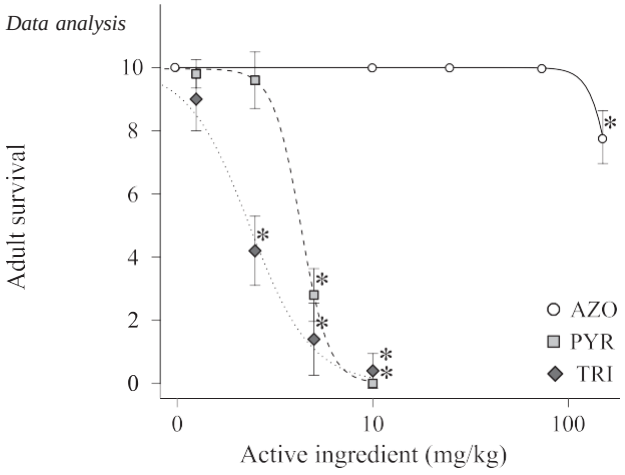


Fig. 1. Survival of adult *Enchytraeus crypticus* in the Enchytraeid Reproduction test (ERT) after exposure to azoxystrobin (AZO), pyraclostrobin (PYR) and trifloxystrobin (TRI). Results are expressed as average ± SD. The lines represent the model fitted to data. *: $p < 0.05$ (Dunnett's).

replicates were fixed with 96% ethanol and Bengal rose (1% solution in ethanol). After 2 h, soil samples were sieved through a mesh(63 μm) to remove small clay particles and prevent water blur- ring. The number of adults, juveniles, and unhatched cocoons was counted using a stereomicroscope. As *E. crypticus* does not coat its cocoons with soil particles, they are coloured as juvenile and adult individuals, which makes them easy to spot and count under a stereomicroscope.

The hatching test was performed on 6-well plates containing 5 g LUFA 2.2. and 5 synchronized (1-day old) *E. crypticus* cocoons per well. The procedure consisted of carefully picking the cocoons off the agar plates with a brush. It is important to spread the cocoons evenly and cover them with soil to avoid desiccation and ensure ex- posure and hatching success. For the prolonged hatching test, 30 replicates per test concentration were used, three for each of the 10 sampling days. Replicates were sampled from the 10th to 19th day of exposure. The duration of the test was chosen based on the hatch- ing dynamics in preliminary experiments. The endpoint for the test was the number of hatched juveniles and the number of unhatched cocoons. To extract the organisms from the soil, the same procedure as for ERT was used.

Statistical analyses were performed using R statistical software ver-sion 3.4.0 (R Development Core Team, 2020) and RStudio (R Studio Team, 2020). Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test). Normally distributed data were analysed with ANOVA, follows by Dunnett *post hoc* test ($p \leq 0.05$). Data that deviated from normality where analysed using KruskalWallis test followed by Gao *post hoc* tests (Gao et al., 2008).

For curve fitting and calculation of effect concentrations (ECx) and lethal concentrations (LCx), package *drc* (Ritz et al., 2015) was used. EC estimates were performed for the various endpoints (survival, repro- duction, hatching success, and hatching time). Dose response curves were fitted to the logistic models. For the three-parameter log-logistic models, *LL.3* function was used, while *LL.4* function was used for four-parameter log-logistic models. The function *confint* was used to deter- mine the confidence interval while a function *EDcomp* was used to test for significant differences between EC values. Differences betweenlinear regressions were determined by ANOVA and Tukey *post hoc* analysis.

Results

Enchytraeid reproduction test (ERT)

The performed tests fulfilled the validity criteria according to the OECD guidelines (OECD, 2016). Namely, in controls the adult mortalitywas less than 20%, the number of juveniles was higher than 50, with acoefficient of variation lower than 50%. ERT results showed stronger ef-fects of TRI and PYR compared to AZO in terms of survival (Fig. 1) andreproduction (Fig. 2). Adult mortality had a significant dose- dependent effect. TRI (LC_{50} = 2.34 (2.13–2.55) mg/kg) was the most toxic followed by PYR (LC_{50} = 4.26 (4.06–4.47) mg/kg), while AZO ($LC_{50} \geq 150$ mg/kg) showed the lowest impact (Table 1).

Accordingly, the number of hatched juveniles was significantly re- duced with TRI treatment (at all concentrations; $p < 0.05$) (Fig. 2C). PYR and AZO caused a dose-dependent reduction in the number of hatched juveniles, with a significant decrease at the highest concentra- tions applied (Fig. 2). The EC values are summarized in Table 1.

Moreover, a significant increase in the number of unhatched co- coons was observed in the AZO and PYR treatments (Fig. 2). The number of unhatched cocoons increased almost threefold at a concentration of25 mg $_{AZO}$ /kg and 1.25 mg $_{PYR}$ /kg compared to the control. Additionally,a dose-dependent decrease in hatching rate was observed in the AZO

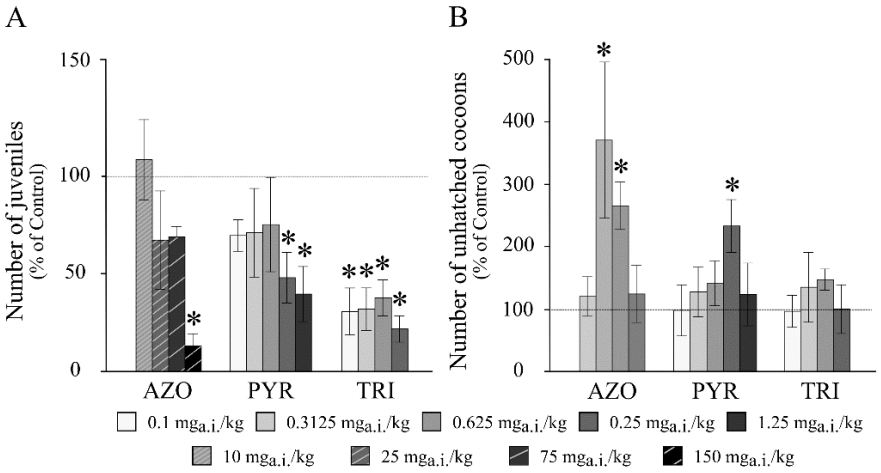


Fig. 2. Reproduction of *Enchytraeus crypticus* after exposure to strobilurin fungicides azoxystrobin (AZO), pyraclostrobin (PYR) and trifloxystrobin (TRI) in LUFA 2.2. soil presented as the number of juveniles (A) and the number of unhatched cocoons (B). Results are expressed as average ± SD.

Table 1
Summary of the effect concentrations (ECx) expressed as mg $_{a.i.}$/kg d.w. soil estimated for *Enchytraeus crypticus* after exposure to azoxystrobin (AZO), pyraclostrobin (PYR) and trifloxystrobin (TRI) in LUFA 2.2 soil in Enchytraeid Reproduction test (ERT). Results show EC and the 95% confidence intervals (in brackets).

		LC_{10}	LC_{50}	LC_{90}
Survival	AZO	132 (100–157)	>150	>150
	PYR	2.95 (2–3.20)	4.26 (4.06–4.47)	6.17 (5.2–6.57)
	TRI	1.06 (0.95–1.50)	2.34 (2.13–2.55)	5.18 (4.3–6.1)
		EC_{10}	EC_{50}	EC_{90}
Reproduction	AZO	57 (40–75)	93 (79–103)	150 (110) 160)
	PYR	0.50 (0.20–0.80)	1.85 (1.5–2.3)	3.80 (2.1–4.3)
	TRI	0.000613	0.045 (0.031–0.079)	3.37 (1.2–3.9)

Table 2
Summary of the effect time (ETx) expressed in days, for cocoons of *Enchytraeus crypticus* when exposed to azoxystrobin (AZO) and pyraclostrobin (PYR) in LUFA 2.2 soil. Results show EC and the 95% confidence intervals (in brackets).

	Active ingredient	ET_{10} (days)	ET_{50} (days)	ET_{90} (days)
Control	0 mg/kg	9.78 (9-9.9)	10.38 (10.23-10.55)	11.01 (10-11.4)
AZO	25 mg/kg	9.17 (9.1-10.1)	10.21 (9.94-10.49)	11.77 (10-11.9)
	75 mg/kg	12.84 (9.9-13)	16.80 (10.6-11.96)	24.44 (12.4-26.5)
	150 mg/kg	13.27 (9.8-14.2)	19.08 (13.53-18.11)	27.45 (14.1-31)
PYR	0.625 mg/kg	9.38 (9-9.8)	10.13 (9.87-10.39)	11.27 (11-13.2)
	1.25 mg/kg	9.95 (9.3-10.5)	13.63 (11.89-13.31)	15.85 (13.4-18.2)
	2.5 mg/kg	12.19 (11.6-14.2)	17.86 (11.2-21.4)	26.17 (14-29)

and PYR treatments. Namely, while more than 85% cocoons hatched in the control, these percentages decreased significantly with increase of SFs concentrations. In contrast, there were no significant changes in the number of unhatched cocoons or hatching rate after TRI applicationat any concentration applied.

Hatching dynamics

After prolonged exposure experiment, changes in hatching dynam-ics were observed between the tested concentrations (Fig. 3). Compared to the control at day 11, the tested SFs significantly inhibitedhatching rate at 75 mg $_{AZO}$ /kg, 150 mg $_{AZO}$ /kg, 1.25 mg $_{PYR}$ /kg and 2.5 mg $_{PYR}$ /kg, respectively. However, the hatching rate changed with the exposure time and, at the end of the experiment, a significant difference was observed only between the control group and two highest SFs concentrations applied (150 mg $_{AZO}$ /kg and 2.5 mg $_{PYR}$ /kg). While the hatching rate reached maximum (between 90 and 100%) and remained the same until the end of the experiment in the control, it showed a time-related increase in treatments with maximum after the 11th day. In contrast to the control group where all juveniles hatched, number of hatched juveniles from day 11 to day 19 of exposure in treatments differed and were concentration dependent. At the end, the number of hatched juveniles reached almost 100% at 25 mg $_{AZO}$/kg, 60% at 75 mg $_{AZO}$/kg, and almost 50% at 150 mg $_{AZO}$/kg, respectively. The same pattern occurred with the PYR treatment. The percentage of hatched juveniles (comparing 11th and 19th day) increased from 85% to more than 90% at 0.625 mg $_{PYR}$ /kg, from 50% to almost 100% at 1.25 mg $_{PYR}$/kg and from 13% to 65% at 2.5 mg $_{PYR}$/kg.

Prolonged hatching tests allowed the study of hatching dynamics and the calculation of the effective time (ETx) for hatching success at different exposure days. The differences in time required for hatching are summarized in Table 2. Apart from the differences in the hatching time required, the prolonged exposure also affected the calculated effective concentrations (ECx) (Table S3). The EC_{50} differed significantly between day 11 and 19, and increased from 34 (25–51) to 104 (62–147) mg/kg for AZO and from 1.08 (0.88–1.29) to >2.5 mg/kg for PYR (Table S3).

Discussion

This study demonstrated an adverse effect of three SFs on survival, reproduction and hatching success in *E. crypticus*. Furthermore, the prolonged hatching test showed that AZO and PYR caused hatching impairment and lead to hatching delay (at lower concentrations) or prevent it completely (at higher concentrations). The difference in sensitivity between life stages of soil organisms exposed to different classes of chemicals has been confirmed in previous research (Castro-Ferreira et al., 2012; Gomes et al., 2018). In this research the adults were less sensitive to the tested fungicides than juveniles as well.

It is challenging to prevent AZO from reaching the soil ecosystems knowing that it is the best-selling fungicide worldwide (Zhang et al., 2019). AZO was thought to degrade relatively rapidly in the environment and therefore a low risk for soil organisms was predicted (Bartlett et al., 2002). However, AZO is more persistent in aerobic than in anaerobic soils and the degradation time depends on the chemical and microbial properties of the soil (Rodrigues et al., 2013). Furthermore, a median dissipation time between 56 and 279 days is enough to affect several generations of enchytraeids. Due to poor mobility, it mostly remains in the soil and becomes persistent in the soil environment (Wu et al., 2016). Our results showed that AZO was the least toxic comparedto all three tested SFs, with an LC_{50} > 150 mg/kg. These values were

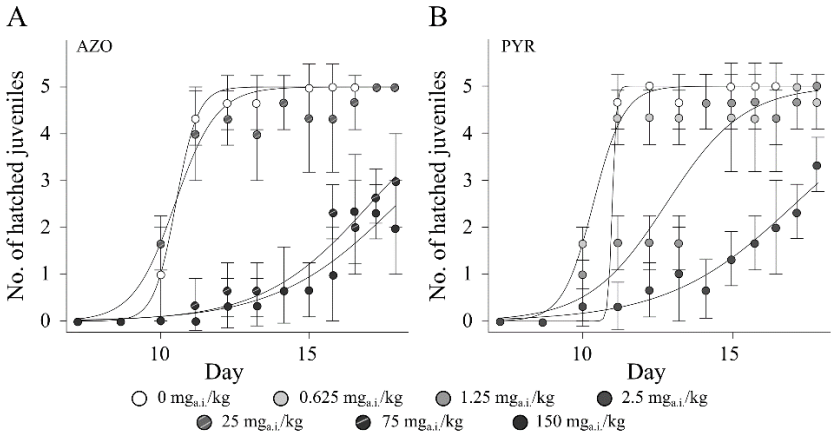


Fig. 3. Results of the Hatching test (HT) of synchronized *Enchytraeus crypticus* cocoons exposed to azoxystrobin (AZO) (A) and pyraclostrobin (PYR) (B). Results are expressed as average ± SD. The lines represent the model fitted to data.

higher than the ones reported by Gomes et al. (2021) (LC_{50} 39 (34–45) mg/kg). However, those were obtained with pure active substance, while we used the commercial formulation. The same difference was with the EC_{50} for reproduction, with lower values in experiment with pure substance (EC_{50} = 37 mg/kg, Gomes et al., 2021), than with commercial formulation as is ours (EC_{50} 93 (79–103) mg/kg) and from Leitão et al. (2014) (EC_{50} = 99.2 (73.3–125.7) mg/kg), which were similar. These results suggest different effect size and toxicity of commercial formulations and pure compounds. Furthermore, treatment with AZO significantly increased the number of unhatched cocoons at 25 mg/kg and 75 mg/kg indicating hatching impairment rather than preventing cocoons production.

In this study, PYR showed stronger adverse effects than AZO against *E. crypticus* in terms of both survival and reproduction. As AZO, PYR also caused hatching impairment, resulting in a hatching delay. To our knowledge, PYR has not been tested on enchytraeids before. So far the only research was on earthworms reporting that PYR disrupts the oxidative balance in *E. fetida* (Ma et al., 2019). However, potentially negative impacts on reproduction, growth, and development are unknown.

Although the data on the effect of TRI on soil environment and soil organisms are insufficient, Wang et al. (2012) reported a negative effect of TRI on *E. fetida* in terms of survival with an LC_{50} of 401.3 (309.4–539.1) mg/kg obtained with filter paper contact test. Soil exposure to TRI of earthworm *E. fetida* showed that TRI and its main metabolite TRI acid exhibited biochemical toxicity and genotoxicity (Liu et al., 2020). Compared to the results obtained in this study, at the same concentration range, TRI exhibited higher toxicity toward enchytraeids than earthworms.

In contrast to soil organisms, the effects of SFs in aquatic organisms, particularly in zebrafish *Danio rerio*, are widely studied (Zhang et al., 2020c). The unique feature of the zebrafish is the possibility of using it for embryotoxicity tests. The zebrafish embryo standard toxicity test (OECD, 2013) is a good example where embryotoxicity has been extensively studied. Many SFs have been shown to affect embryonic development. Thus it is known that AZO, FLUO, PICO, PYR, TRI and KRE led to high embryonic development toxicity (hatching inhibition, mortality, and teratogenic rates, reduce respiration and decreased mitochondrial function) (Jiang et al., 2019; Kim et al., 2020; Kumar et al., 2020; Mao et al., 2020; Zhang et al., 2020a). One of the adverse effects is the slowing of embryonic development, which leads to the prolonged time required for complete embryonic development and hatching, which is observed in PYR, KRE and FLUO exposed zebrafish (Maoet al., 2020; Zhang et al., 2020a). We observed the same effect after a prolonged hatching test with synchronized *E. crypticus* cocoons exposedto AZO and PYR. Namely, after 11 days of exposure, more than 90% of ju-veniles hatched in control, which proved to be a sufficient time for *E. crypticus* to complete embryonic development and hatch (Bicho et al., 2015). However, while in the control the hatching rate reached maximum (between 90 and 100%) and remained the same until the end of the experiment, it showed a time-related increase in treatmentswith maximum after the 11th day. This suggests that SFs at lower concentrations affect embryonic development and hatching success more than the cocoon production itself. Furthermore, after 19 days of exposure, the remaining unhatched cocoons were dry or damaged and would not hatch even after prolonged exposure. Observation of hatch- ing dynamics after exposure to AZO and PYR showed that some concentrations led to delayed hatching, while higher concentrations led to a hatching impairment. Comparison of time needed for hatching in control and pesticides treatments is of high ecological relevance, as a delay, not impairment, will influence population dynamics (Rodriguesetal., 2020). The hatching delay has been observed after exposure of *E. crypticus* to some other chemicals as well. Namely, full life cycle test exposure to Ni nanoparticles caused a hatching delay and only after25 days the number of organisms was similar to the control (Santos et al., 2017). Prolonged exposure (11–17 days) of 1–2 days old *E. crypticus* cocoons to different Ag (nano) materials also allowed discrimination between hatching delay and impairment (Rodrigues et al., 2020). A study with the earthworm *Aporrectodea caliginosa* reported that the most sensitive endpoint was the hatching success of the cocoons produced by exposed adults (Bart et al., 2019). Furthermore,the cocoons produced by exposed adults took additional five days to hatch and had a lower hatching success than cocoons produced by non- exposed adults. This finding could also explain the difference between a large number of unhatched cocoons after exposure to AZO (25 mg/kg) in ERT and 80% of hatched juveniles at the same concentration in prolonged hatching test. Such results suggest that AZO does not only alter cocoonproduction at higher concentrations but also their viability at lower con- centrations. A similar effect was observed with the PYR treatments. It isnoticeable that AZO and PYR at lower concentrations caused a hatchingdelay that was not previously described in enchytraeids. Furthermore, hatching dynamics showed a different response in the control group and at lower concentrations than it was the case at higher exposure con- centrations. Namely, at control treatments and at lower concentrationsthe hatching dynamics shows a logarithmic growth, while in the case of higher concentrations this growth is exponential (Fig. 3). Furthermore, the implementation of the prolonged hatching test enables monitoringof the hatching dynamics and emphasizes importance of the experimentduration on the obtained results. Besides, the hatching test can serve as ascreening tool that will show whether a substance causes the hatching delay or completely prevents it. Hence, it would be effective to conduct research that will monitor the impact of SFs throughout the life cycle of an organisms over several generations.

Current toxicological studies on SFs focus on acute or chronic toxicity, but the toxicological mechanisms are still unclear (Zhang et al., 2020c). AZO and PYR induced oxidative stress, genotoxicity, and neurotoxicity in zebrafish (Li et al., 2019) suggesting the need to investigate its effect on oxidative stress, neurotoxicity and genotoxicity biomarkers in enchytraeidsas well. In addition, it is necessary to investigate the influence of otherSFs such as PICO, FLUO, and KRE that are listed as high toxic for zebrafishand can cause oxidative stress and affect larval development (Jiang et al.,2019; Mao et al., 2020), but has not been tested with enchytraeids.

Conclusion

This study demonstrated the adverse reproduction effects of threeSFs on the soil non-target organism *E. crypticus*. Overall, TRY exerted the strongest toxicity on both survival and reproduction compared to PYR and AZO. Application of standard ERT estimated the effects of pes-ticides on survival and number of juveniles, yet the prolonged hatching test allowed recognizing the difference between hatching delay and complete hatching impairment (non-viability of cocoons) at lower exposure concentrations. Obtained results highlighted the importance of further research of SFs impact on enchytraeids. Furthermore, an application of test with different endpoints regarding reproduction provides a better insight into the action of an individual substance. Such tests could be implemented in future studies together with different bio- markers of oxidative stress, neurotoxicity or genotoxicity to further un-derstand the mechanism of SFs toxicity.

CRediT authorship contribution statement

Marija Kovačević Writing – original draft, Investigation. **Davorka K. Hackenberger**: Investigation, Writing – original draft, Writing – review & editing, Visualization. **Branimir K. Hackenberger:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Acknowledgment

This research was conducted in the framework of the DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) funded by the Croatian Science Foundation (HrZZ) under the contract number: IP-09-2014-4459 and "Young Researchers' Career Development Project – Training of Doctoral Students" funded by the Croatian Science Foundation (HrZZ) (DOK-2018-01-4257).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148143.

References

Bart, S., Barraud, A., Amossé, J., Péry, A.R.R., Mougin, C., Pelosi, C., 2019. Effects of two common fungicides on the reproduction of *Aporrectodea caliginosa* in natural soil. Ecotoxicol. Environ. Saf. 181, 518–524. https://doi.org/10.1016/j.ecoenv.2019.06.049.

Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., Parr-Dobrzanski, B., 2002. The strobilurin fungicides. Pest Manag. Sci. 58, 649–662. https://doi.org/10.1002/ ps.520.

Bicho, R.C., Santos, F.C.F., Gonçalves, M.F.M., Soares, A.M.V.M., Amorim, M.J.B., 2015. Enchytraeid reproduction TestPLUS: hatching, growth and full life cycle test—an op- tional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology 24, 1053–1063. https://doi.org/10.1007/s10646-015-1445-5.

Bicho, R.C., Ribeiro, T., Rodrigues, N.P., Scott-Fordsmand, J.J., Amorim, M.J.B., 2016. Effects of Ag nanomaterials (NM300K) and Ag salt (AgNO3) can be discriminated in a full lifecycle long term test with *Enchytraeus crypticus*. J. Hazard. Mater. 318, 608–614. https://doi.org/10.1016/j.jhazmat.2016.07.040.

Bicho, R.C., Santos, F.C.F., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in *Enchytraeus crypticus*. Environ. Pollut. 224, 117–124. https://doi.org/10.1016/j. envpol.2017.01.067.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., Amorim, M.J.B., 2012. *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87, 1222–1227. https://doi.org/10.1016/j.chemosphere.2012.01.021.

Crane, M., Gross, M., Matthiessen, P., Ankley, G.T., Axford, S., Bjerregaard, P., Brown, R., Chapman, P., Dorgeloh, M., Galay-Burgos, M., Green, J., Hazlerigg, C., Janssen, J., Lorenzen, K., Parrott, J., Rufli, H., Schäfers, C., Seki, M., Stolzenberg, H.C., Van Der Hoeven, N., Vethaak, D., Winfield, I.J., Zok, S., Wheeler, J., 2010. Multi-criteria decisionanalysis of test endpoints for detecting the effects of endocrine active substances in fish full life cycle tests. Integr. Environ. Assess. Manag 6, 378–389. https://doi.org/10.1002/ieam.43.

Gao, X., Alvo, M., Chen, J., Li, G., 2008. Nonparametric multiple comparison procedures for unbalanced one-way factorial designs. J. Stat. Plan. Inference 138 (8), 2574–2591. https://doi.org/10.1016/j.jspi.2007.10.01.

Gomes, S.I.L., Gonçalves, M.F.M., Bicho, R.C., Roca, C.P., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2018. High-throughput gene expression in soil invertebrate em- bryos – mechanisms of Cd toxicity in *Enchytraeus crypticus*. Chemosphere 212, 87–94. https://doi.org/10.1016/j.chemosphere.2018.08.068.

Gomes, S.I.L., Ammendola, A., Casini, S., Amorim, M.J.B., 2021. Toxicity of fungicides to terrestrial non-target fauna – formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – a case study with *Enchytraeus crypticus* (Oligochaeta). Sci. Total Environ. 754, 142098. https://doi.org/10.1016/j.scitotenv.2020.142098.

Gonçalves, M.F.M., Bicho, R.C., Rêma, A., Soares, A.M.V.M., Faustino, A.M.R., Amorim, M.J.B., 2015. Development of an embryotoxicity test for *Enchytraeus crypticus* - the effect of Cd. Chemosphere 139, 386–392. https://doi.org/10.1016/j.chemosphere.2015.07.021.

Han, Y., Zhu, L., Wang, Jinhua, Wang, Jun, Xie, H., Zhang, S., 2014. Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin. Ecotoxicol.Environ. Saf. 107, 214–219. https://doi.org/10.1016/j.ecoenv.2014.06.006.

International Organization for Standardization (ISO), 2004. Soil quality—effects of pollut-ants on Enchytraeidae (*Enchytraeus* sp.) - determination of effects on reproduction and survival ISO 16387:2004.

Jiang, J., Wu, S., Lv, L., Liu, X., Chen, L., Zhao, X., Wang, Q., 2019. Mitochondrial dysfunction, apoptosis and transcriptomic alterations induced by four strobilurins in zebrafish (*Danio rerio*) present early life stages. Environ. Pollut. 253, 722–730. https://doi.org/10.1016/j.envpol.2019.07.081.

Kim, C., Choe, H., Park, J., Kim, G., Kim, K., Jeon, H.J., Moon, J.K., Kim, M.J., Lee, S.E., 2020. Molecular mechanisms of developmental toxicities of azoxystrobin and pyraclostrobin toward zebrafish (*Danio rerio*) embryos: visualization of abnormal development using two transgenic lines. Environ. Pollut. https://doi.org/10.1016/j.envpol.2020.116087.

Kumar, N., Willis, A., Satbhai, K., Ramalingam, L., Schmitt, C., Moustaid-Moussa, N., Crago,J., 2020. Developmental toxicity in embryo-larval zebrafish (*Danio rerio*) exposed to strobilurin fungicides (azoxystrobin and pyraclostrobin). Chemosphere 241, 124980. https://doi.org/10.1016/j.chemosphere.2019.124980.

Leitão, S., Cerejeira, M.J., Brink, P.J. Van Den, Paulo, J., 2014. Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. Appl. Soil Ecol. 76, 124–131. https://doi.org/ 10.1016/j.apsoil.2013.12.013.

Li, H., Zhao, F., Cao, F., Teng, M., Yang, Y., Qiu, L., 2019. Mitochondrial dysfunction-based cardiotoxicity and neurotoxicity induced by pyraclostrobin in zebrafish larvae. Environ. Pollut. 251, 203–211. https://doi.org/10.1016/j.envpol.2019.04.122.

Liu, T., Liu, Y., Fang, K., Zhang, X., Wang, X., 2020. Transcriptome, bioaccumulation and toxicity analyses of earthworms (*Eisenia fetida*) affected by trifloxystrobin and trifloxystrobin acid. Environ. Pollut. 265, 115100. https://doi.org/10.1016/j.envpol.2020.115100.

Ma, J., Cheng, C., Du, Z., Li, B., Wang, Jinhua, Wang, Jun, Wang, Z., Zhu, L., 2019. Toxicological effects of pyraclostrobin on the antioxidant defense system and DNA damage in earthworms (*Eisenia fetida*). Ecol. Indic. 101, 111–116. https://doi.org/10.1016/j.ecolind.2019.01.015.

Mao, L., Jia, W., Zhang, L., Zhang, Y., Zhu, L., Sial, M.U., Jiang, H., 2020. Embryonic development and oxidative stress effects in the larvae and adult fish livers of zebrafish (*Danio rerio*) exposed to the strobilurin fungicides, kresoxim-methyl and pyraclostrobin. Sci.Total Environ. 729, 139031. https://doi.org/10.1016/j.scitotenv.2020.139031.

Organization for Economic Cooperation and Development (OECD), 2013. Test no. 210: fish, early-life stage toxicity test. OECD Guidelines for Testing of Chemicals. OECD Publishing, Paris France.

Organization for Economic Cooperation and Development (OECD), 2016. Test no. 220: enchytraeid reproduction test. OECD Guidelines for Testing of Chemicals. OECD Publishing, Paris France.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.

R Studio Team, 2020. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA http://www.rstudio.com.

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLOs ONE598 (10), e0146021.

Rodrigues, E.T., Lopes, I., Pardal, M.A., 2013. Occurrence, fate and effects of azoxystrobin in aquatic ecosystems: a review. Environ. Int. 53, 18–28. https://doi.org/10.1016/j.envint.2012.12.005.

Rodrigues, N.P., Scott-Fordsmand, J.J., Amorim, M.J.B., 2020. Novel understanding of toxicity in a life cycle perspective – the mechanisms that lead to population effect – the case of Ag (nano)materials. Environ. Pollut. 262. https://doi.org/10.1016/j.envpol.2020.114277.

Santos, F.C.F., Gomes, S.I.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. Hazard assessment of nickel nanoparticles in soil—the use of a full life cycle test with *Enchytraeus crypticus*. Environ. Toxicol. Chem. 36, 2934–2941. https://doi.org/10.1002/etc.3853.

Schnug, L., Jensen, J., Scott-Fordsmand, J.J., Leinaas, H.P., 2014. Toxicity of three biocides to springtails and earthworms in a soil multi-species (SMS) test system. Soil Biol. Biochem. 74, 115–126. https://doi.org/10.1016/j.soilbio.2014.03.007.

Schnug, L., Ergon, T., Jakob, L., Scott-Fordsmand, J.J., Joner, E.J., Leinaas, H.P., 2015. Responses of earthworms to repeated exposure to three biocides applied singly and as a mixture in an agricultural field. Sci. Total Environ. 505, 223–235. https://doi.org/10.1016/j.scitotenv.2014.09.089.

Wang, Y., Wu, S., Chen, L., Wu, C., Yu, R., Wang, Q., Zhao, X., 2012. Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. Chemosphere 88, 484–491. https://doi.org/10.1016/j.chemosphere.2012.02.086.

Westheide, W., Graefe, U., 1992. Two new terrestrial *Enchytraeus* species (Oligochaeta, Annelida). J. Nat. Hist 26 (3), 479–488. https://doi.org/10.1080/00222939200770311.

Wu, P., Wu, W.Z., Han, Z.H., Yang, H., 2016. Desorption and mobilization of three strobilurin fungicides in three types of soil. Environ. Monit. Assess. 188, 363.

Zhang, Q., Zhu, D., Ding, J., Zheng, F., Zhou, S., Lu, T., Zhu, Y.G., Qian, H., 2019. The fungicide azoxystrobin perturbs the gut microbiota community and enriches antibiotic resis- tance genes in *Enchytraeus crypticus*. Environ. Int. 131, 104965. https://doi.org/ 10.1016/j.envint.2019.104965.

Zhang, C., Zhang, J., Zhu, L., Du, Z., Wang, Jun, Wang, Jinhua, Li, B., Yang, Y., 2020a. Fluoxastrobin-induced effects on acute toxicity, development toxicity, oxidative stress, and DNA damage in *Danio rerio* embryos. Sci. Total Environ. 715, 137069. https://doi.org/10.1016/j.scitotenv.2020.137069.

Zhang, C., Zhou, T., Du, Z., Juhasz, A., Zhu, L., Wang, Jun, Wang, Jinhua, Li, B., 2020b. Applying fungicide on earthworms: biochemical effects of *Eisenia fetida* exposed to fluoxastrobin in three natural soils. Environ. Pollut. 258, 113666. https://doi.org/ 10.1016/j.envpol.2019.113666.

Zhang, C., Zhou, T., Xu, Y., Du, Z., Li, B., Wang, Jinhua, Wang, Jun, Zhu, L., 2020c. Ecotoxicology of strobilurin fungicides. Sci. Total Environ. 742, 140611. https://doi.org/10.1016/j.scitotenv.2020.140611.

2.3. Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product

Pesticide Biochemistry and Physiology 187 (2022) 105198

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product

Marija Kovačević, Nikolina Stjepanović, Davorka K. Hackenberger, Željka Lončarić, Branimir K. Hackenberger *

University of Osijek, Department of Biology, Cara Hadrijana 8A, HR-31000 Osijek, Croatia

ARTICLE INFO

Keywords: Survival
Reproduction
Oxidative stress
Energy available
Strobilurin

ABSTRACT

Due to the often-excessive usage of fungicides, increasing attention is being paid to their impact on soil and non-target organisms. Risk assessments are usually based on the pure active ingredient and not on the formulated products applied in the environment. The aim of this study was therefore to investigate how azoxystrobin, the best-selling strobilurin fungicide, affects non-target soil organisms *Enchytraeus albidus*. To investigate the effects of the different types of azoxystrobin, *E. albidus* was exposed to the pure active ingredient, AZO_AI, and the formulated product, AZO_FP. Survival, reproduction, and molecular biomarkers of *E. albidus* were determined for different exposure durations (seven and 21 days). AZO_FP (LC_{50} = 15.3 $mg_{a.i.}$/kg) showed a slightly strongereffect on survival than AZO_AI (LC_{50} = 16.8 $mg_{a.i.}$/kg), yet the impact on reproduction was much stronger. Namely, while the tested concentrations of AZO_AI (EC_{50}≥ 8 $mg_{a.i.}$/kg) had almost no effect on reproduction, AZO_FP (EC_{50} = 2.9 $mg_{a.i.}$/kg) significantly inhibited reproduction in a dose-dependent manner. Changes in enzyme activities (superoxide dismutase, catalase, glutathione-s-transferase) and malondialdehyde levels in both treatments indicated oxidative stress. Although AZO_FP had a stronger negative effect, the impact depended on the exposure time and the tested concentration. The higher toxicity of AZO_FP was a consequence of increased bioavailability and activity of the active ingredient due to the presence of adjuvants. Overall stronger adverse effects of AZO_FP suggest that the toxicity of azoxystrobin in the agricultural environment on the enchytraeid population may be underestimated. Furthermore, the results of this study highlighted the importance of comparing the toxicity of the active ingredient and the formulated product.

1. Introduction

To ensure high crop yields and protection against plant diseases and pests, plant protection products (PPPs) are commonly used in modern agriculture. Among the PPPs sold in the European Union (EU) in 2019, fungicides represented 40% of sales (Eurostat). Strobilurin fungicides(Sfs) are one of the essential fungicides used in large quantities world- wide due to their efficacy. SFs act by blocking ATP production and thus impairing mitochondrial respiration (Bartlett et al., 2002). Azoxystrobin (AZO) is one of the most important and highly efficient broad-spectrum SFs. Due to its wide use, AZO enters the aquatic and soil ecosystem through agricultural and urban runoff. The presence of azoxystrobin residues was detected in 22 out of 317 soil samples collected acrossEurope (Silva et al., 2019). The reported median concentration of azoxystrobin was 0.03 mg/kg, while the maximum concentration reached 0.25 mg/kg. Moreover, concentrations of AZO up to 9.5 mg/kg were detected in Chinese soils (Xu et al., 2021). Namely, due to the increase in average humidity and temperatures, the possibility of fungal disease development in crops has increased, which has consequently led to an increased application of all PPPs, and especially fungicides.

Among SFs, AZO has attracted scientific attention because of its overapplication and ecotoxicity. AZO has been shown to affect various non-target organisms, including mammals, amphibians, aquatic, and soil organisms (Zhang et al., 2020). While the impact on aquatic or- ganisms have been extensively studied (for a review see Rodrigues et al., 2013), most of the research among soil organisms has been conductedon earthworms (Wang et al., 2012; Han et al., 2014; Zhang et al., 2018;Zhang et al., 2020; Xu et al., 2021; Wu et al., 2021). AZO tested as an active ingredient was highly toxic (Wang et al., 2012) and causedoxidative stress to earthworm *Eisenia fetida*, leading to lipid peroxidation and DNA damage (Han et al., 2014). However, testing only the active ingredient may lead to an underestimation of the ecotoxicological

* Corresponding author.
E-mail address: hackenberger@biologija.unios.hr (B.K. Hackenberger).

https://doi.org/10.1016/j.pestbp.2022.105198
Received 5 June 2022; Received in revised form 28 July 2022; Accepted 4 August 2022
Available online 8 August 2022

effects of AZO. Namely, due to recommendations in regulatory guidelines, the use of pure active ingredients instead of formulated products is common. Nevertheless, it has been shown that the effect of a formulated product is often stronger than that of a pure active ingredient (Marqueset al., 2009; Mesnage et al., 2014; Gomes et al., 2021). Recent research has also highlighted the importance of studies that include both a pure active ingredient and a formulated product (Gomes et al., 2021).

Enchytraeids are recognised as indicators of soil quality (Didden and Römbke, 2001; Castro-Ferreira et al., 2012; Pelosi and Römbke, 2016). They play an important role in soil ecology, especially in soils under tillage pressure, where the number of earthworms is reduced. Namely, enchytraeids affect the decomposition of organic matter, bioturbation, and the circulation of nutrients in the soil, thus improving soil structure, porosity, and quality (Briones, 2014; Maraldo et al., 2011). Due to their features, *Enchytraeus albidus* and *E. crypticus* are commonly used in ecotoxicology tests. *E. albidus*, the larger of the two species, can be found in the surface soil layers, especially in soils with a high content of organic matter. It can be found in diverse habitats, from temperate re- gions to the arctic (Christensen and Dózsa-Farkas, 2006). Due to its wide distribution, this species is considered a more relevant indicator or- ganism compared to the earthworms *Eisenia fetida* and *E. andrei* (Römbke and Moser, 1999). Moreover, *E. albidus* has a generation time of 33 days at 18 °C (ISO, 2004), which allows the performance of the reproduction test in six weeks (OECD, 2016). Furthermore, in additionto the effect on survival and reproduction, it is possible to determine the effect on a wide range of endpoints and molecular biomarkers. However, existing information on the impact of strobilurin fungicides on this species is insufficient. Recent results addressed only the impact of AZO on *E. crypticus* (Leitão et al., 2014; Gomes et al., 2021). In addition to effects on survival and reproduction, a hatching delay has been demonstrated (Kovačević et al., 2021a). However, the impact on mo- lecular biomarkers of enchytraeids has not been studied. Biomarkers can be used as an early warning system to detect stress following exposure tospecific toxicants or stressors (Lam and Gray, 2003). The assessment of biomarkers at different levels of an organism's organization providesbetter insight into response to contaminants.

The multixenobiotic resistance mechanism (MXR) found in aquatic and soil organisms, including enchytraeids (Kovačević et al., 2021b), serves as a defence system and acts by pumping harmful substances out of the cell (Kurelec, 1992). Its activity can be assessed by measuringchanges in the accumulation of fluorescent substrates. MXR effects fungicide toxicity by active pumping fungicides out of the cell, thus reducing their accumulation. However, if the MXR system is inhibited,the fungicides accumulate in the cell and toxic effects occur. Simulta-neously, when an organism comes into contact with harmful substances, free radicals are produced and antioxidant defence is activated. The role and effectiveness of the first phase of detoxification depend on the activation of antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT). If the first phase does not eliminate all harmful molecules, second phase enzymes, such as glutathione-s-transferase (GST), are activated. Sometimes, however, the action of toxic sub- stances is too strong and lipid peroxidation (LPO) occurs, indicating long-term damage. To obtain a more comprehensive picture of the effect of the toxicant, it is important to also assess the impact on the energy status of the organism. Namely, available energy and energy balance are crucial for key processes in an organism such as basal metabolic rate, growth, and reproduction (Novais et al., 2013). Therefore, measuring protein, lipid, and carbohydrate content are essential to evaluate available energy reserves.

In this research, the impact of AZO on *Enchytraeus albidus* was evaluated to obtain new information on the differences between the toxicity of the pure active ingredient and the formulated product. Therefore, multiple endpoints were assessed. In addition to survival and reproduction, effects on MXR activity, oxidative status, and available energy reserves were measured.

2. Material and methods

Test organism and soil

As a model organism, the soil annelid *Enchytraeus albidus* (Oli- gochaeta: Enchytraeidae) from the culture maintained in the laboratory at the Department of Biology in Osijek (Croatia) were used (OECD 220, 2016; ISO 16387, 2012). The culture was established in 2014 from or- ganisms provided by the Complutense University of Madrid. The or-ganisms were kept in moist soil in a climate room and fed ad libitum with oatmeal. The temperature was set at 20 1 °C, relative humidity at 60% with a photoperiod of 16 h of light and 8 h of darkness.

All experiments were carried out in artificial soil (AS) (OECD 220, 2016) that consists of air-dried quartz sand (70%), kaolin clay (20%)and sphagnum peat (10%). As prescribed in the guidelines, the soil pH was modified by adding $CaCO_3$ up to 6 ± 0.5.

Test materials and spiking

The active ingredient azoxystrobin (AZO_AI) (Pestanal®, analytical standard, 98.0%) and the fungicide formulated product Quadris® (AZO_FP) (Syngenta) were used. AZO_FP based on the active substance azoxystrobin (25%) contains adjuvants 1,2-benzisothazol-3(2H)-one (0.025–0.05%), naphthalene and alkyl naphthalene sulfonic acid form-aldehyde condensate and sodium salts (1 ≤ 10%). The recommended application dose for AZO_CP in the field is 1 L/ha or 0.17 mg $_{a.i.}$/kg $_{soil}$. Priory the final experiment, a range-finding test was conducted. Tested concentrations were 0, 1, 2, 3, 4, 5, 6, 8, 10, 14, 18, 22, 25, 52,75, 100 and 150 mg $_{a.i.}$/kg $_{soil}$ for AZO_AI and AZO_FP. Concentrationsfor the final test were selected based on the recommended applicationdoses for AZO_FP and according to the results of the range-finding test.The concentrations used in the test were 0, 0.085, 0.17, 1.45, 2.7, 4 and8 mg $_{a.i.}$/kg $_{soil}$ for AZO_AI and AZO_FP. The AZO_AI was prepared bydissolving in organic solvent (acetone) and diluted to the tested con-centrations. After dissolving, the required concentrations of AZO_AIwere added in 2 mL of acetone to each replicate, homogeneously mixed,and left for 24 h to evaporate. Solvent controls were run in parallel withthe test. The soil moisture was adjusted with water until 60% of thewater holding capacity (WHC). As AZO_FP is water-soluble, a stock so-lution was prepared and diluted. The required concentrations of AZO_FPwere dissolved in water and separately added to each replicate in theamount required for 60% of the water holding capacity (WHC). The soilwas mixed homogeneously and allowed to equilibrate for 24 h before thestart of the tests. Clean soil with the required amount of water was used as a control.

Test procedures and sample preparation

The range-finding test was conducted in three replicates per test concentration. 10 adult organisms with well-developed clitellum were exposed to different concentrations of AZO_FP and AZO_AI for seven days. To determine LC values survival was determined after 24 h, 48 h, 72 h, 96 h and seven days.

The reproduction test was carried out according to the standardised enchytraeid reproduction test (ERT) guidelines (ISO, 2012; OECD, 2016). Ten adults with well-developed clitellum were introduced into each test vessel containing 20 g of moist soil and food supply (auto-claved rolled oats). The test was carried out at 20 ± 1 °C and a photo-period of 16: 8 h for six weeks (42 days). Water and food were replenished weekly, according to weight loss. Five replicates per test concentration were used. After 21 days, the surviving adults were extracted and counted, pooled per replicate, weighted, and stored at−80 °C. 21 days after removing adults (42 days after beginning the experiment), the juvenile organisms were fixed with ethanol and stained with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through the mesh (63 μm) to separate enchytraeids from soil and

facilitate counting with a stereomicroscope.

Simultaneously with the ERT, a seven-day exposure with identical settings was conducted. To provide a better understanding of the effects of fungicides and to allow a comprehensive consideration of changes in multiple endpoints at different periods.

Parallel with the above tests, ten replicates with ten organisms pertest concentration were used to measure MXR activity. MXR activity was measured on day seven (5 replicates per test concentration) and day 21(5 replicates per test concentration) as the accumulation of Rh123 according to Kovačević et al. (2021b).

Oxidative status and energy reserves were measured in the whole organisms that were pooled per replicate and homogenized (IKA RW20 digital homogenizer) in a cold potassium phosphate buffer (0.1 M, pH7.4) (1: 15, w: v ratio). Post-mitochondrial fraction (S9) was obtained after centrifugation of homogenates for 30 min at 9000*g* and 4 °C. Ho- mogenates and S9 fractions were stored at 80 °C until further analysis. Homogenates were used for measuring MDA, lipid and carbohydrate content while in S9 protein content and activities of enzymes SOD, CAT and GST werw mwasured.

Oxidative status

SOD was measured according to McCord and Fridovich (1969), CAT activity was determined with the method of Claiborne (1985) and GST activity was evaluated with the method described by Habig et al. (1974). LPO was determined as the malondialdehyde (MDA) content according to Gagne (2014). All enzyme activities and MDA contents were calcu- lated per protein content and expressed relative to their respective control.

Energy reserves

To determine the available energy reserves, the total protein, lipid, and carbohydrate content were quantified. The method described by Bradford (1976) was used to measure protein content in the S9 fraction, while the lipid and carbohydrate contents were determined in homog- enate using the methods described by Frings et al. (1972) and Jermyn (1975). For transforming energy sources into energetic equivalents, the enthalpy combustion method described by De Coen and Janssen (1997, 2003) was used. Results were expressed relative to their respective control.

Data analysis

Data analyses were performed with statistical software R version 4.3.0 (R Development Core Team, 2021) and RStudio (RStudio Team,2021). Data normality was tested using the Shapiro-Wilk test and ho-mogeneity of variance was assessed by the Bartlett test. As data werenormally distributed, ANOVA, followed by a Dunnett post hoc test ($p \leq 0.05$) was used. Effect concentration (ECx) and lethal concentration (LCx) were calculated by fitting the curve in the package *drc* to the three-parameter log-logistic models (function LL.3) (Ritz et al., 2015).

Results

The test validity criteria described in OECD 220 (2016) were met. Namely, adult mortality in the reproduction test was below 10% and the number of juveniles was higher than 50. The range-finding test showed a slightly stronger negative effect of AZO_FP on survival (Table 1). The results of the reproduction experiment (21 days of exposure) showed a significant inhibition (ANOVA, $p < 0.05$) of reproduction with the highest treatment of AZO_AI (8 $mg_{a.i.}/kg_{soil}$) and most AZO_FP treat- ments (1.45, 2.7, 4, and 8 $mg_{a.i.}/kg_{soil}$) (Fig. 1). Additionally, the calculated EC_{50} values show that AZO_CP has a stronger adverse effect on reproduction compared to AZO_AI (Table 1). Moreover, an increased number of unhatched cocoons was observed in some AZO_FP treatments

Lethal (LCx) and effect (ECx) concentrations expressed as mg $_{a.i.}$/kg $_{soil}$ calculated for *Enchytraeus albidus* after exposure to azoxystrobin as the pure active ingredient (AZO_AI) and formulated product (AZO_FP). Results are expressed as LC/EC and the 95% confidence intervals.

	Exposure time	LC_{10}	LC_{50}	LC_{90}
AZO_AI	**24 h**	16.31 (15.20–17.42)	20.54 (19.76-21.32)	29.84 (26.03–32.93)
	48 h	19.89 (13.73–16.05)	18.76 (18.19–19.33)	23.64 (21.84–25.44)
	72 h	12.66 (11.69–13.63)	17.65 (17.30–17.99)	21.53 (20.50–22.63)
	7 days	11.95 (11.21–12.70)	16.76 (16.51–17.01)	19.14 (18.27–20.00)
AZO_CP	**24 h**	12.19 (11.41–12.98)	18.44 (16.62–20.26)	33.72 (29.97–37.48)
	48 h	11.67 (11.17–12.17)	18.12 (17.47–17.76)	31.58 (29.36–33.80)
	72 h	11.61 (10.98–12.50)	16.98 (16.47–17.50)	30.84 (28.12–33.56)
	7 days	10.65 (10.14–11.17)	15.29 (14.88–15.71)	26.98 (24.99–28.97)
		EC_{10}	**EC_{50}**	**EC_{90}**
AZO_AI	**21 day**	>8	>8	>8
AZO_CP	**21 day**	1.23 (0.40–2.06)	2.94 (2.41–3.73)	6.21 (3.84–7.78)

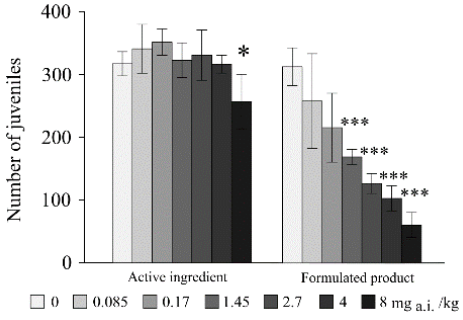


Fig. 1. The number of juveniles after exposure of *Enchytraeus albidus* to azoxystrobin as a pure active ingredient (AZO_AI) and formulated product (AZO_FP) presented as the number of juveniles. Results are expressed as the average number of juveniles ± SD.

(> 15 at 1.45 and > 8 at 2.7 $mg_{a.i.}/kg_{soil}$) and highest AZO_AI treatment(8 $mg_{a.i.}/kg_{soil}$).

In addition to population biomarkers, both AZO_AI and AZO_FP demonstrated negative effects on cellular and molecular biomarkers. MXR activity was only affected after seven days of exposure to AZO_AI and AZO_FP. AZO_AI significantly induced (ANOVA, $p < 0.05$) MXR activity at lower concentrations (0.17, 1.45 $mg_{a.i.}/kg_{soil}$), while AZO_FP inhibited MXR activity and caused the accumulation of R123 within cells at 4 and 8 $mg_{a.i.}/kg_{soil}$ (Fig. 2).

Moreover, exposure to both AZO_AI and AZO_FP induced changes in measured enzyme activities (Fig. 3). Namely, both substances tested led to a significant induction (ANOVA, $p < 0.05$) of SOD and CAT activities after seven days of exposure. After 21 days of exposure, SOD and CAT activities returned to control levels. While AZO_AI induced GST activity after seven days, AZO_FP caused induction after 21 days. The MDA content increased significantly (ANOVA, $p < 0.05$) after 21 days of exposure to AZO_AI (2.7 $mg_{a.i.}/kg_{soil}$) and AZO_FP (4 and 8 $mg_{a.i.}/kg_{soil}$).

The total available energy reserves after exposure to AZO_AI and AZO_FP in different time intervals are shown in Fig. 4. In organisms from

Table 1

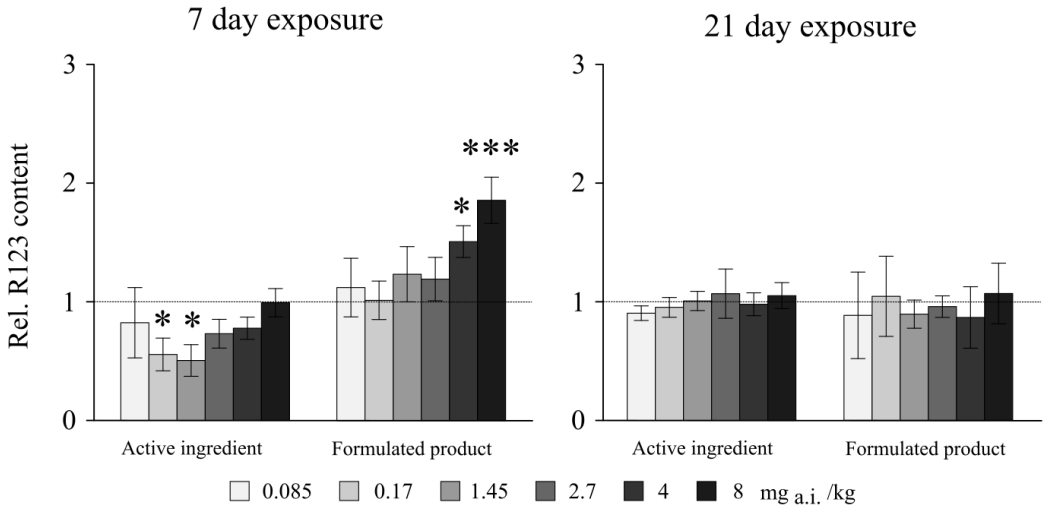


Fig. 2. Fig. 2. Rh123 relative content in *Enchytraeus albidus* after exposure to azoxystrobin as a pure active ingredient (AZO_AI) and formulated product (AZO_FP). Resultsare relative to the corresponding control and expressed as average ± SD.

the control treatment, the ratios of individual energy reserve fractions were similar at different time points. Thus, proteins accounted for about 50% of the total available energy, lipids 40%, and carbohydrates 10%.No changes were observed in the total available energy reserves after exposure to AZO_AI. However, there was a shift in the proportion of each energy reserve fraction. Namely, after seven days of exposure, a signif-
icant decrease (ANOVA, $p < 0.05$) in protein and carbohydrate content was observed, while lipids significantly increased (ANOVA, $p < 0.05$) (up to 54% of total available energy reserves). After 21 days of exposure, lipids decreased significantly (ANOVA, $p < 0.05$) (up to 25% of total available energy), while proteins and carbohydrates were at the control level. The highest concentration of AZO_FP caused a significant increase (ANOVA, $p < 0.05$) in total available energy reserves after seven and 21 days of exposure. Namely, a significant increase (ANOVA, $p < 0.05$) in the amount of energy available occurred after seven days due to the increase in lipid content, while the increase after 21 days was the resultof the increase in protein, lipid and carbohydrate content.

4. Discussion

The effects of AZO on *E. albidus* were evaluated by measuring various endpoints after seven and 21 days of exposure. Although no significant differences were observed between the impact of AZO_AI and AZO_CP on survival, species-specific sensitivity between *E. albidus* and *E. crypticus* was observed. *E. crypticus* has been reported as a more tolerant species to toxicants than *E. albidus* (Pokarzhevskii et al., 2003). Consequently, *E. albidus* (LC_{50} = 15.29 $mg_{a.i.}/kg_{soil}$) showed higher susceptibility to AZO_FP than *E. crypticus* (LC_{50} 150 $mg_{a.i.}/kg_{soil}$) (Kovačević et al., 2021a). Similarly, Gomes et al. (2021) reported an LC_{50} of 39 $mg_{a.i.}/kg_{soil}$ after exposure of *E. crypticus* to AZO_AI, also indicating a higher susceptibility of *E. albidus* (LC_{50} = 20.54 $mg_{a.i.}/kg_{soil}$). The differences in toxicity between the species are attributed to peculiarities in life cycle duration (Pokarzhevskii et al., 2003). Except for survival, both AZO_AI and AZO_FP affected the reproduction rate of *E. albidus*. Although AZO_AI has an impact on reproduction only at the highest tested concentration (8 $mg_{a.i.}/kg_{soil}$), AZO_CP caused a decrease in reproduction at concentrations higher than 0.17 $mg_{a.i.}/kg_{soil}$. The negative impact of the formulated products on *E. crypticus* reproduction has been shown in previous studies with EC_{50} values ranging from 37 $mg_{a.i.}/kg_{soil}$ to 99.2 $mg_{a.i.}/kg_{soil}$ (Gomes et al., 2021; Leitão et al., 2014; Kovačević et al., 2021a). Moreover, the increased number of unhatched cocoons observed in some treatments suggests an effect on embryonic development. The same effect was observed in *E. crypticus* (Kovačević et al., 2021a). Namely, at lower concentrations, AZO_FP caused impairment of embryonic development, while at higher concentrations the reproduction rate was reduced. Zebrafish (*Danio rerio*) embryotox- icity test showed that azoxystrobin alters mitochondrial bioenergetics and causes developmental toxicity (Yang et al., 2021). Moreover, early developmental stages are expected to have less robust and established antioxidant defence systems which may lead to high levels of oxidative stress that result in genotoxicity (Zhang et al., 2020). Additionally, the higher toxicity of AZO_CP may be caused by adjuvants, which increase the bioavailability of active ingredients and allow them to pass more easily through cocoon and bind to target sites and consequently increasing toxicity to non-target organisms (Pereira et al., 2009).

The survival of organisms in an environment with a high concentration of pollutants depends on the MXR mechanism and the efflux of a toxicant from the cell (Epel et al., 2008). The function of the ABC transporters in the MXR mechanism can be affected by pesticides. Namely, some pesticides act as chemosensitizers and can inhibit or induce the activity of the MXR system in enchytraeids (Kovačević et al., 2021b) and earthworms (Velki and Hackenberger, 2013). Furthermore, the activity of the MXR system is extremely sensitive to the concentra- tions of available substrates. Namely, while a small amount of substrate activates the MXR system, higher concentrations inhibit it (Velki and Hackenberger, 2013). Therefore, the opposite response of the MXR mechanism observed after seven days of exposure to AZO_AI and AZO_CP could be associated with the different availability of the active ingredient. In other words, adjuvants in formulated products enhance the absorption and stability of the active ingredient, thus promoting its pesticidal action (Mesnage and Antoniou, 2018). Moreover, the main adjuvants of AZO_FP, 1,2- benzothiazole-3(2H)-one (EU regulation No. 528/2012, 2022; IMAP, 2020; EPA, 2021) and naphthalene (EPA, 2008), have no adverse effects on non-target soil organisms at the concentrations present in this study, but certainly, enhance AZO uptake and stability. Since inhibition of MXR activity causes longer retention of toxicants in the organism, it leads to higher toxicity of AZO_CP.

Changes in antioxidant enzyme activities can indicate the mecha- nism by which organisms protect themselves from toxic effects. Although the influence of AZO on enchytraeid enzyme activity has not been investigated, the research carried out with the earthworm *E. fetida* indicates the importance of the enzymes SOD and GST (Han et al., 2014; Xu et al., 2021). SOD is part of the primary antioxidant defence mechanism and acts in the conversion of the superoxide anion radical (O^{2-}) to hydrogen peroxide (H_2O_2) (Ighodaro and Akinloye, 2018). SOD induc- tion has been reported in earthworms exposed to AZO_AI (Han et al.,

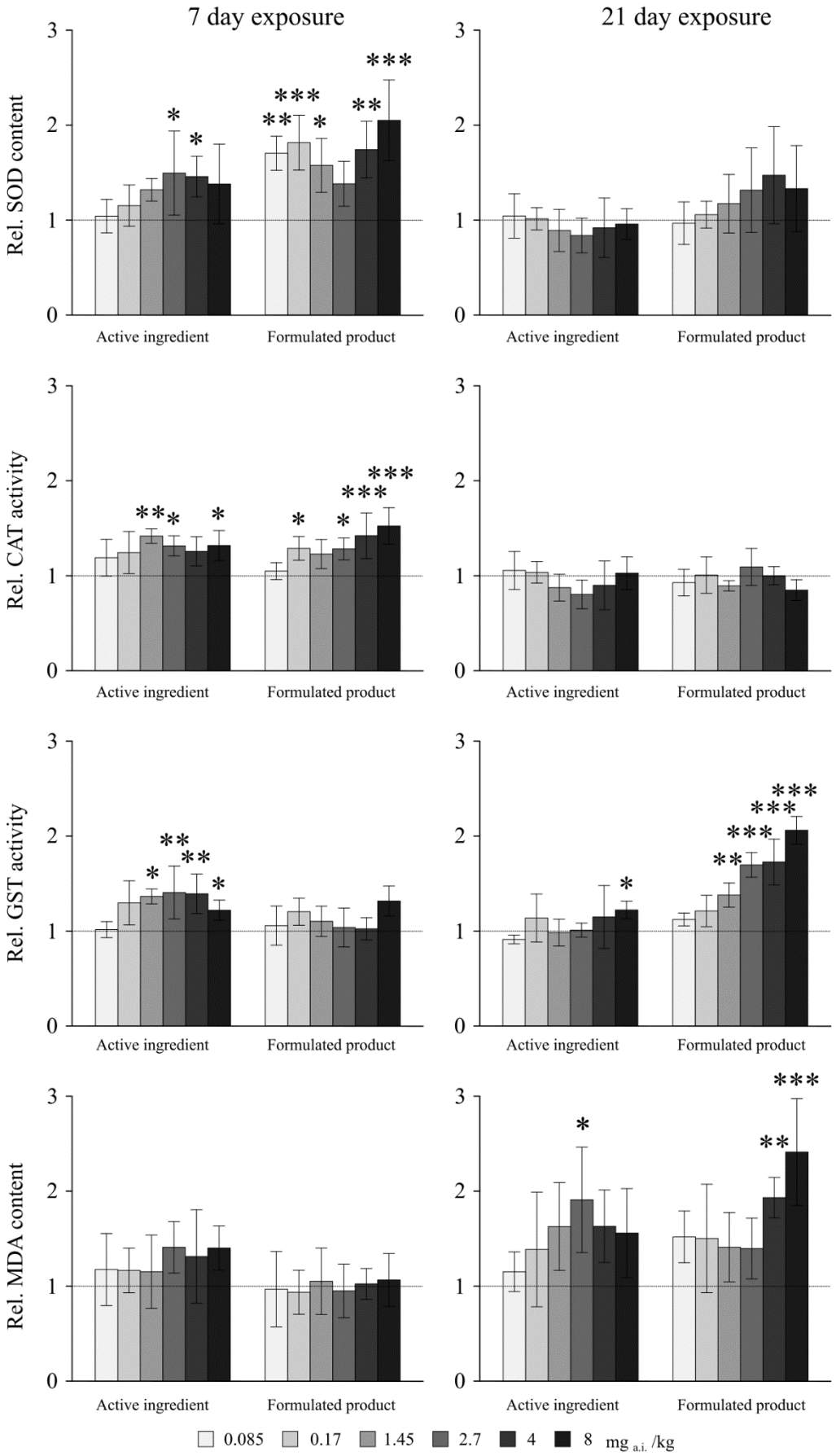


Fig. 3. Differences in biomarker responses of SOD, CAT, GST and MDA in *Enchytraeus albidus* after exposure to azoxystrobin as the pure active ingredient (AZO_AI)and formulated product (AZO_FP) presented as the number of juveniles. Results are relative to the corresponding control and expressed as average ± SD.

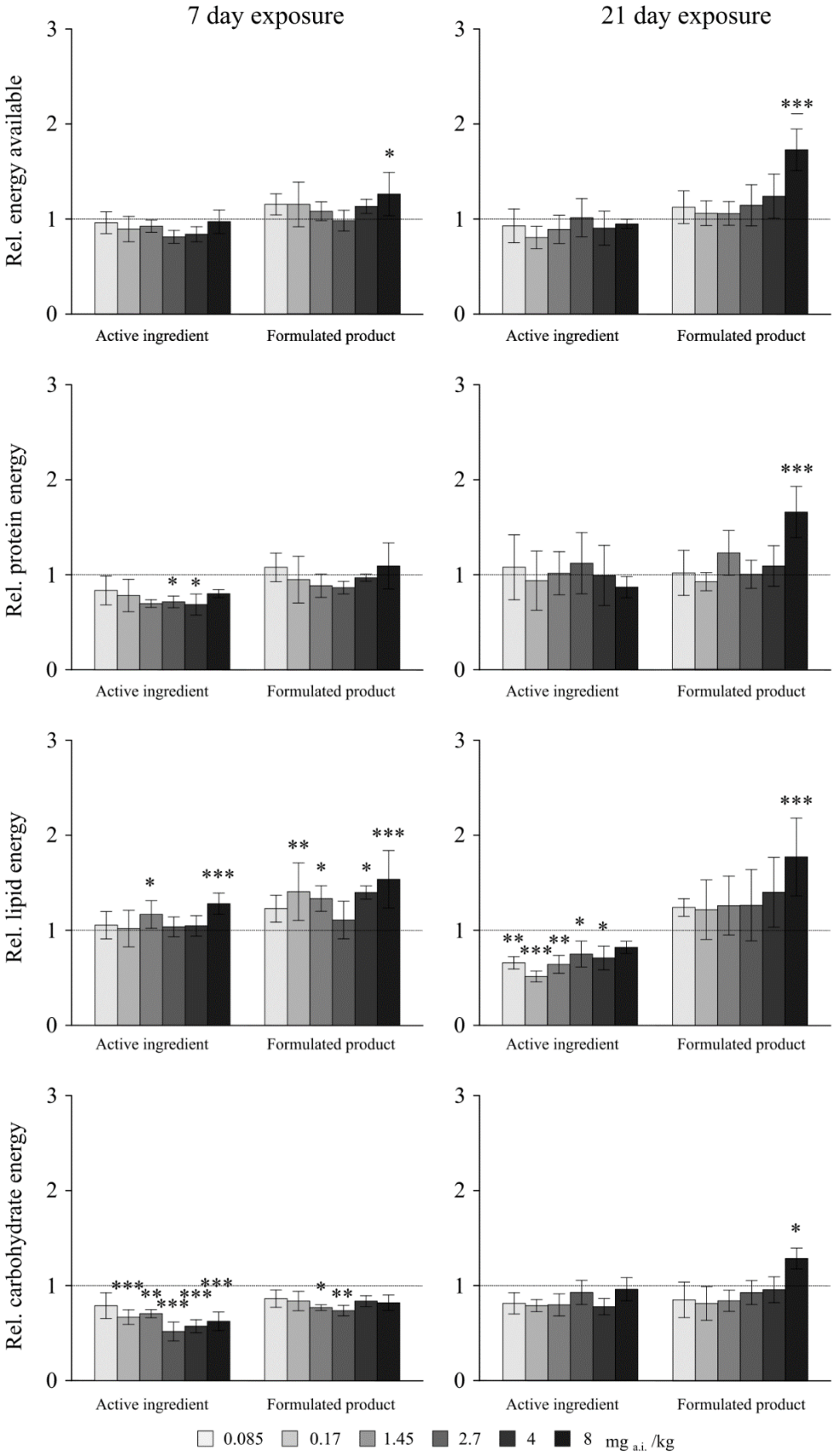


Fig. 4. Available energy reserves (A, B), protein (C, D), lipid (E, F) and carbohydrate (G, H) energy in *Enchytraeus albidus* after exposure to azoxystrobin as the pure active ingredient (AZO_AI) and formulated product (AZO_FP). Energy reserves are expressed as average values ± SD.

2014; Xu et al., 2021). However, while SOD activity in earthworms increased with exposure time, SOD activity in enchytraeids was induced only after seven days of exposure. This response suggests the high efficiency of this enzyme in enchytraeids, which successfully convert O^{2-} after a short exposure time. Although the research suggests different importance and CAT responses in *E. fetida* (Han et al., 2014; Xu et al., 2021), the results obtained indicate that its activity plays a vital role in enchytraeids. Like SOD, CAT showed high efficacy, and after prolonged exposure due to substrate deficiency, its activity returned to the levels observed in control. GST is a crucial second-phase detoxifying enzyme that scavenges lipid hydroperoxides to reduce oxidative damage. GSTwas the most important detoxification enzyme in earthworms after AZO_AI treatment (Han et al., 2014; Xu et al., 2021). Namely, GST ac-tivity increased with increasing concentration and exposure time. Although AZO_AI induced GST activity in enchytraeids after seven daysof exposure, after 21 days, induction was recorded only at the highest concentration tested. Again, this response suggests the success of thedetoxification system in enchytraeids and the lack of substrate for GST after 21 days of exposure. On the contrary, AZO_CP caused GST induc-tion only after 21 days of exposure. Namely, the substrate concentration formed after seven days of exposure was not sufficient for GST induc- tion. However, the higher induction of GST after 21 days of exposure suggests a high substrate production and thus stronger oxidative stress than observed in the AZO_AI treatments. When ROS are formed in sig- nificant amounts, a saturation of antioxidant defence occurs. As a consequence, damage to lipids and other vital molecules may occur.

Most commonly, the degree of lipid peroxidation (LPO) is assessed by measuring the MDA content as a biomarker of oxidative damage (Duryee et al., 2010). ROS production after seven days was insufficientto promote LPO and increase MDA content in *E. fetida* exposed to AZO_AI (Han et al., 2014; Xu et al., 2021). However, after prolonged exposure, LPO was observed. The same pattern was observed in enchytraeids. Moreover, the level of LPO again suggests a higher impact of AZO_CP than AZO_AI.

The impact of AZO on mitochondrial metabolism and induction of oxidative stress suggests a possible imbalance in the energy metabolism of *E. albidus*. Therefore, total protein, lipid, and carbohydrate content were measured to determine the level of available energy reserves. AZO_AI and AZO_FP affected the energy reserves of *E. albidus* differently. Although no changes in total available energy reserves were observed after exposure to AZO_AI, AZO_FP caused an increase in available energy reserves at the highest tested concentration after seven and 21 days of exposure. Moreover, both AZO forms induced a change in the proportion of energy fractions. In control organisms, the proportion of energy reserve fractions was similar to previous studies (50 (proteins): 40 (lipids): 10 (carbohydrates)) (Amorim et al., 2012; Novais et al., 2013). However, after seven days of exposure to AZO_AI, protein and carbo- hydrate content were significantly reduced, due to an intense defence against oxidative stress and increased energy demand. Carbohydratesare commonly used as a primary energy source under stress conditions (Moolman et al., 2007). A reduction in carbohydrate content is a com- mon response in enchytraeids after exposure to pesticides (Novais and Amorim, 2013). On the contrary, lipid content was increased after seven days of exposure to AZO_AI, indicating inflammatory stress (Gomes et al., 2015). After 21-days, proteins and carbohydrates were equivalent to the control, while lipid content was significantly reduced. The reduction in lipid content is associated with increased LPO, which may lead to a reduction in available energy. Exposure to AZO_FP had a different effect on the time course of the energy reserves. After seven days, the carbohydrate content was reduced and lipids increased. However, a significant increase in protein, carbohydrate, and lipid content was observed only at the highest concentration after 21 days of exposure. This increase in protein synthesis, carbohydrate, and lipid accumulation can be explained as a stress response. Namely, Tripathi et al. (2010) reported that an increase in protein content observed in earthworms indicates the possibility of increased synthesis of stress proteins.

After application, formulated products can be absorbed by the plant, deposited on the surface of the soil, or adsorb onto organic matter or clay in the soil (Hildebrandt et al., 2007). Azoxystrobin is mainly applied as a foliar fungicide, and if the treatment is carried out too early, >50% ofthe applied fungicide can end up directly on the soil (Jensen and Spliid, 2003). According to Flury (1996), the active ingredient is released from the formulation after application. However, the release rate depends on various factors such as the type of formulation and environmental conditions. The observed differences between the response of *E. albidus* to AZO_FP and AZO_AI indicate a slow release of azoxystrobin from the tested formulation. Furthermore, low mobility of different forms of azoxystrobin and a higher residue level were observed in the upper layers of the soil (0–20 cm) (Herrero-Hernández et al., 2015; Ghosh and Singh, 2009). Considering that enchytraeids are found in the surface layers of the soil, their exposure to azoxystrobin becomes almost inev- itable. Moreover, the oxidative stress observed at the recommended application dose of AZO_CP (0.17 $mg_{a.i.}/kg_{soil}$) indicates a possible riskto enchytraeids after field application of the formulated products. Although enchytraeids successfully eliminated oxidative stress and reproduced unhindered, higher concentrations may affect embryonic development and reproduction. The leaching behaviour of fungicides plays a major role in the accumulation and impact of fungicides on soil organisms. Khan and Brown (2016) observed differences in leaching between the active ingredient and the formulated products. However, the biggest difference was observed between the emulsifiable concen- trate (EC) and suspension concentrate (SC) formulation, indicating a high impact of adjuvants on the leaching of the active ingredient. ECformulations contains emulsifying agents in a water-insoluble organicsolvent, which is designed to form an oil-in-water emulsion upon dilu- tion that affects the behaviour of pesticide activity. They can restrict the pesticide molecule from dissolving in water or may retard processes controlling sorption to soil with the oily organic solvents surroundingthe pesticide molecule, thus increasing pesticide leaching. On the other hand, SC usually contains suspension agents, wetting agents, and thickeners that may reduce leaching (Khan and Brown, 2016). Hence, adjuvants can affect not only the leaching of fungicides but also the initial and total availability of fungicide. Current maximum concentrations of AZO in European soils reach up to 0.25 $mg_{a.i.}/kg_{soil}$ (Silva et al., 2019), but climate change and agricultural intensification lead to a higher demand for plant protection products. Consequently, this can result in higher concentrations of AZO in the soil, as is already the case in China (>9 $mg_{a.i.}/kg_{soil}$, Xu et al., 2021). Such high concentrations may significantly impact enchytraeid reproduction, and thus the stability of their populations.

5. Conclusion

A detailed insight into the changes in the activity of the measured biomarkers in enchytraeids allows a better assessment of the potential toxicity and the difference between AZO_AI and AZO_FP. The opposite response of the MXR system activity indicates an increased availabilityof the active substance upon exposure to AZO_FP compared to AZO_AI. Although AZO_AI impairs enchytraeid reproduction only at the highest tested concentration, the changes in enzyme activity indicate the occurrence of oxidative stress. The activities of SOD, CAT, and GST were induced during exposure to AZO_AI and AZO_FP, suggesting their involvement in reducing oxidative damage and detoxification. Furthermore, changes in MDA content after 21 days of exposure showed the occurrence of LPO. Therefore, assessment of multiple endpoints is recommended to more accurately predict potential adverse effects and avoid the possibility of underestimating the effects. Since the toxicity ofa formulated product may be higher than that of the active ingredient itself, testing both is crucial to reveal differences in toxicity. Further-more, the differences obtained between the AZO forms imply the importance of evaluating various formulated products. Although the

recommended application dose has not shown an effect on survival and reproduction, such concentrations can cause oxidative stress and affect enchytraeid populations after multiple or multigenerational exposures. Therefore, under the conditions of imminent climate change, it is necessary to determine the most suitable formulated products to establish successful crop protection and ensure the stability of soil communities.

Acknowledgement

This research was conducted in the framework of the DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) funded by the Croatian Science Foundation (HrZZ) under the contract number: IP-09-2014-4459 and "Young Researchers' Career Development Project–Training of Doctoral Students" funded by the Croatian Science Foundation (HrZZ) (DOK-2018-01-4257).

References

Amorim, M.J.B., Gomes, S.I.L., Soares, S.M.V.M., Scott-Fordsmand, J.J., 2012. Energy basal levels and allocation among lipids, proteins, and carbohydrates in *Enchytraeus albidus*: changes related to exposure to cu salt and Cu nanoparticles. Water Air SoilPollut. 223, 477–482.

Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M., Parr-Dobrzanski, B., 2002. The strobilurin fungicides. Pest Manag. Sci. 58, 649–662. https://doi.org/10.1002/ps.520.

Bradford, MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Briones, M.J.I., 2014. Soil fauna and soil functions: a jigsaw puzzle. Front. Environ.Sci. 2. https://doi.org/10.3389/fenvs.2014.00007.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., Amorim, M.J.B., 2012. *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87, 1222–1227.

Christensen, B., Dózsa-Farkas, K., 2006. Invasion of terrestrial enchytraeids into two postglacial tundras: North-eastern Greenland and the Arctic Archipelago of Canada (Enchytraeidae, Oligochaeta). Polar Biol. 29 (454–466), 444.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods of Oxygen Radical Research, pp. 283–284.

De Coen, W., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicitytesting IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress. Recover. 6, 43–55.

De Coen, W., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22,1632–1641.

Didden, W., Römbke, J., 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicol. Environ. Saf. 50, 25e43. https://doi.org/10.1006/eesa.2001.2075.

Duryee, M.J., Klassen, L.W., Schaffert, C.S., Tuma, D.J., Hunter, C.D., Garvin, R.P., Anderson, D.R., Thiele, G.M., 2010. Malondialdehyde-acetaldehyde adduct is the dominant epitope after MDA modification of proteins in atherosclerosis. Free Radic. Biol. Med. 49 (10), 1480–1486. https://doi.org/10.1016/j.freeradbiomed.2010.08.001.

EPA (the United States Environmental Protection Agency), 2008. Reregistration Eligibility Decision for Naphthalene. https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/red_PC-055801_12-Sep-08.pdf.

EPA (the United States Environmental Protection Agency), 2021. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/databases-related-pesticide- risk-assessment.

Epel, D., Luckenbach, T., Stevenson, C.N., MacManus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: newly appreciated roles in protection against pollutants. Environ. Sci. Technol. 42, 3914–3920. https://doi.org/10.1021/ es087187v.

EU regulation No 528/2012, 2022. https://echa.europa.eu/documents/10162/5a180829-e031-777e-db83-477195663762.

Flury, M., 1996. Experimental evidence of transport of pesticides through field soils – a review. J. Environ. Qual. 25, 24–45.

Frings, C.S., Fendley, T.W., Dunn, R.T., Queen, C.A., 1972. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin. Chem. 18 (7), 673–674.

Gagne, F., 2014. Biochemical ecotoxicology: principles and methods. In: Oxidative Stress. Academic Press, New York, pp. 110–111.

Ghosh, R.K., Singh, N., 2009. Leaching behaviour of azoxystrobin and metabolites in soil columns. Pest Manag. Sci. 65 (9), 1009–1014.

Gomes, S.I., Scott-Fordsmand, J.J., Amorim, M.J., 2015. Cellular energy allocation to assess the impact of nanomaterials on soil invertebrates (Enchytraeids): the effect ofCu and Ag. Int. J. Environ. Res. Public Health 12 (6), 6858–6878.

Gomes, S.I.L., Ammendola, A., Casini, S., Amorim, M.J.B., 2021. Toxicity of fungicides to terrestrial non-target fauna – formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – a case study with *Enchytraeus crypticus* (Oligochaeta). Sci. Total Environ. 754, 142098 https://doi.org/10.1016/j.scitotenv.2020.142098.

Habig, W.H., Pabst, M.J., Jakobi, W., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Han, Y., Zhu, L., Wang, Jinhua, Wang, Jun, Xie, H., Zhang, S., 2014. Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin. Ecotoxicol. Environ. Saf. 107, 214–219. https://doi.org/10.1016/j.ecoenv.2014.06.006.

Herrero-Hernández, E., Marín-Benito, J.M., Andrades, M.S., Sánchez-Martín, M.J., Rodríguez-Cruz, M.S., 2015. Field versus laboratory experiments to evaluate the fate of azoxystrobin in an amended vineyard soil. J. Environ. Manag. 163, 78–86.

Hildebrandt, A., Lacorte, S., Barceló, D., 2007. Assessment of priority pesticides, degradation products, and pesticide adjuvants in groundwaters and top soils from agricultural areas of the Ebro river basin. Anal. Bioanal. Chem. 387 (4), 1459–1468.

Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54, 287–293. https://doi.org/10.1016/j.ajme.2017.09.001.

IMAP, 2020. Accelerated Assessment of Industrial Chemicals in Australia. https://www.industrialchemicals.gov.au/sites/default/files/1%2C2-Benzisothiazolinone%20preservatives_%20Environment%20tier%20II%20assessment.pdf.

International Organization for Standardization (ISO), 2004. Soil Quality—Effects of Pollutants on Enchytraeidae (*Enchytraeus* sp.) - Determination of Effects on Reproduction and Survival ISO 16387:2004.

International Organization for Standardization (ISO), 2012. Water Quality Determination of the Inhibition of the Mobility of Daphnia magna Straus (Cladocera,Crustacea) - Acute Toxicity Test. ISO 6341, p. 2012.

Jensen, P.K., Spliid, N.H., 2003. Deposition of spray liquid on the soil below cereal cropsafter applications during the growing season. Weed Res. 43 (5), 362–370.

Jermyn, M.A., 1975. Increasing the sensitivity of the anthrone method for carbohydrate. Anal. Biochem. 68 (1), 332–335.

Khan, M.A., Brown, C.D., 2016. Influence of commercial formulation on leaching of four pesticides through soil. Sci. Total Environ. 573, 1573–1579.

Kovačević, M., Hackenberger, D.K., Hackenberger, B.K., 2021a. Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifloxystrobin) on survival, reproduction and hatching success of *Enchytraeus crypticus*. Sci. Total Environ. 790 https://doi.org/10.1016/j.scitotenv.2021.148143.

Kovačević, M., Hackenberger, D.K., Lončarić, Ž., Hackenberger, B.K., 2021b. Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology. Chemosphere 279. https://doi.org/10.1016/j. chemosphere.2021.130549.

Kurelec, B., 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22, 23–43.

Lam, P.K.S., Gray, J.S., 2003. The use of biomarkers in environmental monitoring programmes. Mar. Pollut. Bull. 46 (2), 182–186.

Leitão, S., Cerejeira, M.J., Van den Brink, P.J., Sousa, J.P., 2014. Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. Appl. Soil Ecol. 76, 124–131. https://doi.org/10.1016/j.apsoil.2013.12.013.

Maraldo, K., Christensen, B., Holmstrup, M., 2011. The excretion of ammonium byenchytraeids (*Cognettia sphagnetorum*). Soil Biol. Biochem. 43, 991–996.

Marques, C., Pereira, R., Gonçalves, F., 2009. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. J. Soils Sediments 9, 137–147. https://doi.org/10.1007/s11368-009-0058-0.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244 (22), 6049–6055.

Mesnage, R., Antoniou, M.N., 2018. Ignoring adjuvant toxicity falsifies the safety profileof commercial pesticides. Public Health Front. 5, 361. https://doi.org/10.3389/fpubh.2017.00361.

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.-E., 2014. Major pesticides are more toxic to human cells than their declared active principles. Biomed. Res. Int. 2014, 1–8. https://doi.org/10.1155/2014/179691.

Moolman, L., Van Vuren, J.H., Wepener, V., 2007. Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. Ecotoxicol. Environ. Saf. 68, 443–450. https://doi.org/10.1016/j.ecoenv.2006.12.017.

Novais, S.C., Amorim, M.J.B., 2013. Changes in cellular energy allocation in Enchytraeus albidus when exposed to dimethoate, atrazine, and carbendazim. Environ. Toxicol. Chem. 32, 2800–2807. https://doi.org/10.1002/etc.2368.

Novais, S.C., Soares, A.M.V.M., De Coen, W., Amorim, M.J.B., 2013. Exposure of *Enchytraeus albidus* to cd and Zn - changes in cellular energy allocation (CEA) andlinkage to transcriptional, enzymatic and reproductive effects. Chemosphere 90, 1305–1309. https://doi.org/10.1016/j.chemosphere.2012.09.030.

Organisation for Economic Cooperation and Development (OECD), 2016. Test no. 220: enchytraeid reproduction test. In: OECD Guidelines for Testing of Chemicals. OECD Publishing, Paris France.

Pelosi, C., Römbke, J., 2016. Are Enchytraeidae (Oligochaeta, Annelida) good indicators of agricultural management practices? Soil Biol. Biochem. 100, 255–263. Pereira, J.L., Antunes, S.C., Castro, B.B., Marques, C.R., Gonçalves, A.M.M., Gonçalves, F., Pereira, R., 2009. Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. Ecotoxicology 18, 455–463. https://doi.org/10.1007/s10646-009-0300-y.

Pokarzhevskii, A., Filimonova, Z.V., Goryachev, O.A., 2003. Life-cycle length determines the differences in sensitivity to toxicants between enchytraeid species. Dokl. Biol.Sci. 390, 256–258. https://doi.org/10.1023/a:1024469919915.
R Studio Team, 2021. RStudio: Integrated Development for R. RStudio, Inc, Boston, MA. http://www.rstudio.com.
RCore Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLoSOne 598 (10), e0146021.
Rodrigues, E.T., Lopes, I., Pardal, M.Â, 2013. Occurrence, fate and effects of azoxystrobin in aquatic ecosystems: a review. Environ. Int. 53, 18–28. https://doi.org/10.1016/j.envint.2012.12.005.
Rõmbke, J., Moser, T., 1999. Organisation and Performance of an International Ring Test for the Validation of the Enchytraeid Reproduction Test. Vols. I and II.; BA-πexte4/ 99.
Silva, V., Mol, H.G.J., Zomer, P., Tienstra, M., Ritsema, C.J., Geissen, V., 2019. Pesticide residues in European agricultural soils – a hidden reality unfolded. Sci. Total Environ. 653, 1532–1545. https://doi.org/10.1016/j.scitotenv.2018.10.441.
Tripathi, G., Kachhwaha, N., Dabi, I., 2010. Comparative studies on carbofuran-induced changes in some cytoplasmic and mitochondrial enzymes and proteins of epigeic, anecic and endogeic earthworms. Pestic. Biochem. Physiol. 96, 30–35. https://doi.org/10.1016/j.pestbp.2009.08.012.
Velki, M., Hackenberger, B.K., 2013. Biomarker responses in earthworm *Eisenia andrei* exposed to pirimiphos-methyl and deltamethrin using different toxicity tests. Chemosphere 90, 1216–1226. https://doi.org/10.1016/j. chemosphere.2012.09.051.
Wang, Y., Wu, S., Chen, L., Wu, C., Yu, R., Wang, Q., Zhao, X., 2012. Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. Chemosphere 88, 484–491. https://doi.org/10.1016/j.chemosphere.2012.02.086.
Wu, R., Zhou, T., Wang, J., Wang, J., Du, Z., Li, B., Juhasz, A., Zhu, L., 2021. Oxidative stress and DNA damage induced by trifloxystrobin on earthworms (*Eisenia fetida*) intwo soils. Sci. Total Environ. 797, 149004 https://doi.org/10.1016/j. scitotenv.2021.149004.
Xu, Y., Li, B., Hou, K., Du, Z., Allen, S.C., Zhu, Lusheng, Li, W., Zhu, Lei, Wang, Jinhua, Wang, Jun 2021. Ecotoxicity evaluation of azoxystrobin on *Eisenia fetida* in different soils. Environ. Res. 194, 110705 https://doi.org/10.1016/j.envres.2020.110705.
Yang, L., Huang, T., Li, R., Souders II, C.L., Rheingold, S., Tischuk, C., Li, N., Zhou, B.,Martyniuk, C.J., 2021. Evaluation and comparison of the mitochondrial and developmental toxicity of three strobilurins in zebrafish embryo/larvae. Environ. Pollut. 270, 116277.
Zhang, C., Zhou, T., Xu, Y., Du, Z., Li, B., Wang, Jinhua, Wang, Jun, Zhu, L., 2020. Ecotoxicology of strobilurin fungicides. Sci. Total Environ. 742, 140611 https://doi.org/10.1016/j.scitotenv.2020.140611.
Zhang, C., Zhu, L., Wang, Jun, Wang, Jinhua, Du, Z., Li, B., Zhou, T., Cheng, C., Wang, Z., 2018. Evaluating subchronic toxicity of fluoxastrobin using earthworms (*Eiseniafetida*). Sci. Total Environ. 642, 567–573. https://doi.org/10.1016/j. scitotenv.2018.06.091.

2.4. Comprehensive study of the effects of strobilurin-based fungicide formulations on *Enchytraeus albidus*

Ecotoxicology (2022) 31:1554–1564
https://doi.org/10.1007/s10646-022-02609-4

Comprehensive study of the effects of strobilurin-based fungicide formulations on *Enchytraeus albidus*

Marija Kovačević[1] • Nikolina Stjepanović[1] • Davorka K. Hackenberger[1] • Željka Lončarić[1] • Branimir K. Hackenberger[1]

Accepted: 28 November 2022 / Published online: 3 December 2022

Abstract

Excessive application of fungicides in crop fields can cause adverse effects on soil organisms and consequently affect soil properties. Existing knowledge on the effects of strobilurin fungicides has been primarily based on toxicity tests with active ingredients, while the effects of fungicide formulations remain unclear. Therefore, this work aims to provide new data on the effects of three commercial formulations of strobilurin fungicides on the soil organism *Enchytraeus albidus*. The tested fungicide formulations were Retengo®(pyraclostrobin—PYR), Zato WG 50®(trifloxystrobin—TRI) and Stroby WG®(kresoxim-methyl- KM). In laboratory experiments, multiple endpoints were considered at different time points. The results showed that PYR had the greatest impact on survival and reproduction (LC_{50} = 7.57 $mg_{a.i.}kg_{soil}^{-1}$, EC_{50} = 0.98 $mg_{a.i.}kg^{-1}$), followed by TRI soil (LC_{50} = 72.98 $mg_{a.i.}kg_{soil}^{-1}$, EC_{50} = 16.93 $mg_{a.i.}kg_{soil}^{-1}$) and KM (LC_{50} = 73.12 $mg_{a.i.}kg_{soil}^{-1}$, $EC_{50} \geq 30$ $mg_{a.i.}kg_{soil}^{-1}$). After 7 days of exposure, MXR activity was inhibited at the highest concentration of all fungicides tested (6 $mg_{PYR}kg_{soil}^{-1}$, 15 $mg_{TRI}kg_{soil}^{-1}$ and 30 $mg_{KM}kg^{-1}$). Furthermore, oxidative stress (induction of SOD, CAT and GST) and lipid peroxidation soil (increase in MDA) were also observed. In addition, there was a decrease in total available energy after exposure to PYR and KM. Exposure to fungicides resulted in a shift in the proportions of carbohydrates, lipids, and proteins affecting the amount of available energy. In addition to the initial findings on the effects of strobilurin formulations on enchytraeids, the observed results suggest that multiple and long-term exposure to strobilurin formulations in the field could have negative consequences on enchytraeidpopulations.

Keywords Strobilurin fungicides · Toxicity · Enchytraeus · Oxidative stress · Energy available

Introduction

The use of pesticides in excessive quantities is of great concern for the environment. According to Nguyen et al. (2016), <0.1% of the pesticides reach their specific targets, while the rest go into ecosystems. Among pesticides, fungicides account for 40% of sales. In the last two decades, strobilurin fungicides have become an indispensable part of agricultural production (Bartlett et al. 2002; Zhang et al. 2020). According to Eurostat data, sales of strobilurin fungicides in Europe almost doubled from 2011 to 2020, reaching ~3 million kg per year (Eurostat). Besides azoxystrobin (AZO), pyraclostrobin (PYR), trifloxystrobin (TRI) and kresoxim-methyl (KRE) are the most commonly used strobilurin fungicides (Zhang et al. 2020; Xu et al. 2021; Wu et al. 2021). PYR, TRI, and KRE are used on various cereal, oil, vegetable, fruit and other crops worldwide (Zhang et al. 2020). Although strobilurin fungicides are expected to be rapidly degraded, other trends have been observed. Namely, the average half-life (DT_{50}) reported for PYR is 28 days, but can extend up to 63 days (Fulcher et al. 2014). In contrast to PYR, the DT_{50} reported for TRI and KM is shorter. However, their metabolites are formed, which may have adverse effects. Although the DT_{50} for TRI ranged from 5 to 25 days, the DT_{50} for the metabolites of TRI ranged from 138 to 231 days (Wang et al. 2015). In addition, KM rapidly dissipates into its acidic metabolites in soil (Khandelwal et al. 2014). The mean DT_{50} values

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10646-022-02609-4.

* Davorka K. Hackenberger
davorka@biologija.unios.hr

[1] Department of Biology, University of Osijek, Cara Hadrijana 8A, HR-31000 Osijek, Croatia

calculated for KM were ~3 days, while its metabolites are present in the soil for more than 90 days. The high-water solubility of strobilurin fungicides can lead to their accumulation in water and soil (Zhang et al. 2020). Previous research has focused mainly on the effects of active ingredients on survival, reproduction, and the occurrence of oxidative stress in aquatic organisms (Li et al. 2016; Jiang et al. 2019; Li et al. 2018; Kumar et al. 2020; Mao et al. 2020; Kim et al. 2021; Li et al. 2021; Yang et al. 2021). However, there are limited data on the impact of strobilurin fungicides on soil ecosystems.

The widespread and extensive application of strobilurin fungicides, where soil ecosystems are the main sink, leads to potential risks for soil organisms and ecosystems (Zhanget al. 2020). Soil processes are mainly influenced by the indispensable part of this ecosystem—the soil fauna (Brussaard 1998). The decline in soil biodiversity and biological activity has been linked to the excessive application of fungicides in agriculture (Pelosi et al. 2014). Enchytraeids are considered indicators of soil quality (Didden and Römbke 2001; Pelosi and Römbke 2016) and perform similar functional role as earthworms, but in a lesser extent (Marinissen and Didden 1997). Namely, they affect the decomposition of organic matter and the circu- lation of nutrients in the soil (Briones et al. 1998; Maraldo et al. 2011). In this way, they create a favourable micro- habitat for the growth and development of microorganisms(Bardgett 2005). Moreover, under tillage pressure in culti- vated soils, a decrease in earthworm populations was observed, while enchytraeids activity increased (Topoliantz et al. 2000). Enchytraeids affect soil structure and porosity in such soils, thus improving their quality. Their role in decomposition and bioturbation together with living close to the surface layers of the soil brings them into direct contact with pollutants and makes them easy targets of environmental pollution.

Enchytraeus albidus is a model organism in soil ecotoxicology (OECD 2016; ISO 2014). However, informationon the effects of strobilurin fungicides on this species is insufficient. Apart from azoxystrobin, which has been extensively studied (Zhang et al. 2020; Xu et al. 2021; Wu et al. 2021), pyraclostrobin (PYR), trifloxystrobin (TRI) and kresoxim-methyl (KM) are the most widely sold fungicidesin this group. PYR and TRI in the form of a pure active ingredient have been shown to inhibit growth and induce oxidative stress and DNA damage in the earthworm *Eisenia fetida* (Ma et al. 2019; Liu et al. 2020; Wu et al. 2021). Furthermore, exposure to formulations containing the active ingredient PYR and TRI impaired the survival and reproduction of *E. crypticus* (Kovačević et al. 2021a), while the effects on other endpoints at the suborganismal level are unknown. To our knowledge, KM has not yet been studied in soil organisms, apart from regulatory risk assessments in which the risk to soil organisms was considered low (EFSA 2010). However, although the risk to aquatic organisms has been classified as low, both KM and its main metabolite BF-490-1 (acid of kresoxim-methyl) are toxic to non-targetaquatic organisms (Li et al. 2021). In addition, strobilurin fungicides have been repeatedly detected in aquatic and terrestrial environments at concentrations above the Reg- ulatory Acceptable Concentration (RAC) and could threatennon-target organisms (Feng et al. 2020; Li et al. 2021).

The ecotoxicological effects of formulations may differ significantly from those of a pure active ingredient (Mesnageet al. 2014; Gomes et al. 2021). Therefore, the need for a detailed assessment of the impacts of formulations has beenhighlighted previously (Marques et al. 2009). However, a comprehensive analysis of different endpoints over differenttime intervals is needed for a better understanding of the mechanisms of action that will allow a more accurate pre- diction of population-level consequences. Standardised testsfocus primarily on endpoints such as survival and reproduc-tion and ignore effects on crucial physiological processes that are important for organisms to adapt to stressful conditions.Therefore, it is important to link subindividual and population biomarkers to elucidate the mechanisms that are important for the survival of organisms and their populations.

This study aimed to comprehensively evaluate the effectsof three commercial formulations containing strobilurin active ingredients (pyraclostrobin, trifloxystrobin, and kre- soxim-methyl) on *E. albidus* at different time points. In addition to survival and reproduction, the effects on MXR activity, oxidative status, and available energy reserves were also investigated. The results obtained will provide valuable information on the effects of different commercialstrobilurin fungicides on enchytraeids.

Material and methods

Test organism and soil

Enchytraeus albidus (Oligochaeta: Enchytraeidae) was usedas the test species. Cultures were maintained in moist soil under controlled conditions according to the prescribed guidelines (OECD 220 2016; ISO 16387 2014) at the University of Osijek (Croatia).

All experiments were conducted in the standard artificialsoil (AS), which consists of 70% air-dried quartz sand, 20%kaolin clay, and 10% sphagnum peat. The AS was preparedaccording to OECD guidelines (2016).

Test materials and spiking procedures

The strobilurin fungicides used were the formulations Retengo® (BASF, 200 gL^{-1} pyraclostrobin—PYR), Zato

WG 50® (Bayer, 500 gkg^{-1} trifloxystrobin—TRI), and Stroby WG® (BASF SE, 500 gkg^{-1} kresoxim-methyl— KM).

The concentrations selected for the range-finding test were 0, 1.87, 3.75, 7.5, 15 and 30 $mg_{a.i.}kg_{soil}^{-1}$ for PYR and 0, 9.38, 18.75, 37.5, 75 and 150 $mg_{a.i.}kg_{soil}^{-1}$ TRI and KM. Based on the recommended application doses (RD) for formulations (RD_{PYR} = 0.16 $mg_{a.i.}$ kg_{soil}^{-1}; RD_{TRI} = 0.1 $mg_{a.i.}kg_{soil}^{-1}$; RD_{KM} = 0.1 $mg_{a.i.}$ kg_{soil}^{-1}) and the results of the range-finding tests, the final test concentrations were Selected. The concentrations selected were 0, 0.08, 0.16, 1.107, 2.053, 3 and 6 $mg_{a.i.}kg_{soil}^{-1}$ for PYR, 0, 0.05, 0.1, 2.57, 5.03, 7.5 and 15 $mg_{a.i.}kg_{soil}^{-1}$ for TRI and 0, 0.05, 0.1, 5.07, 10, 15 and 30 $mg_{a.i.}kg_{soil}^{-1}$ for KM. The fungicides were dissolved in distilled water to obtain the desired concentrations. The solutions were prepared to contain the volume required for 60% of the water holding capacity. After the addition of the pesticide, the soil was homo-geneously mixed and allowed to equilibrate 24 h before the start of the experiment.

Test procedures

As no sufficient information on the effects of PYR, TRI, and KM on *E. albidus* is available, the range-finding test was performed according to the recommended guidelines (OECD 2016) followed by the enchytraeid reproduction test (ERT) (ISO 2014; OECD 2016). Ten adult organisms with well-developed clitellum were used per replicate. Each test vessel contained 20 g of moist soil and a food supply. Forthe range-finding test, the organisms were exposed for 7 days and only survival was assessed. For the final experiment twenty replicates per test concentration were used. In the ERT five replicates were used and adults were exposed for 21 day, with an additional twenty-one day for hatching and growth of juveniles. Five replicates were used for each tested concentration. Simultaneously with the ERT, an additional five replicates per tested concentration were set up for seven-day exposure. Each of the test vessels contained 20 g of soil and ten adult organisms with well-developed clitellum. The 7-day exposure wa s added toallow assessment at an additional time point. Moreover, to determine changes in multixenobiotic resistance mechanism activity (MXR), additional ten replicates were set up, five toassess effects after 7 days of exposure, and five to assess MXR activity after 21 day. During exposure, a constant temperature of 20 ± 1 °C and a photoperiod of 16 h of light and 8 h of darkness were maintained. Organisms were fed once per week with autoclaved oatmeal, while water was replenished weekly, based on weight loss.

After exposure, adults were carefully removed from the soil, counted, pooled per replicate, and weighed. Organisms were homogenised using the IKA RW20 homogeniser in cold 0.1 M potassium phosphate buffer (pH 7.4) at a ratio of 1:15 (w:v). The post-mitochondrial fraction (S9) was obtained after centrifugation of the homogenate (30 min at 9000 *g* and 4 °C). The post-mitochondrial fraction and homogenates were stored at −80 °C before analysis.

Juveniles were counted 21 day after extraction of the adults. To enable counting, juveniles were fixed with ethanol and stained with Bengal rose (1% in ethanol). The soil samples were sieved through the mesh (63 µm) to prevent blurring and to facilitate counting with a stereomicroscope.

Organisms used for the MXR system analyses were isolated, washed, and transferred to a 5 mM solution of Rh123 in ISO water. Exposure to the fluorescent substrate took place in the dark at 20 °C and lasted for 1 h. MXRactivity was measured according to Kovačević et al. (2021b). The concentration of Rh123 in the organisms was expressed as µM of Rh123 per g of wet tissue and nor- malised to the control.

To determine the oxidative status of the organisms, the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) were measured in the S9 fraction. The method described byMcCord and Fidovich (1969) was used to measure SOD activity. The CAT activity was measured according to Claiborne (1985) and the GST according to Habig et al. (1974). Malondialdehyde (MDA) content was measured as described by Gagne (2014) and used to determine LPO. Enzyme activities and LPO were calculated per protein content and expressed as relative values compared to the control. Lipid and carbohydrate content were measured in the homogenate using the method described by Frings et al.(1972) and Jermyn (1975). The protein content in the S9 fraction was determined according to the method of Brad- ford (1976). To allow calculation of available energy reserves, the determined content of carbohydrates, lipids, and proteins was expressed in energetic equivalents using the enthalpy of combustion (17.5 kJg^{-1} carbohydrates, 39.5 kJg^{-1} lipids, and 24 kJg^{-1} protein) as described in De Coen and Janssen (1997); De Coen and Janssen (2003).

Statistical analysis

Data analysis was performed using R software version 4.3.0(R Development Core Team 2022) and RStudio (RStudio Team 2022). The Shapiro–Wilk test was used to test the distribution of data and Bartlett's test was used to test the homogeneity of variances. As the data did not deviate from the normal distribution, one-way ANOVA followed by Dunnett's post hoc test ($p \leq 0.05$) was used to determine the difference between the control group and the tested con-centrations. The package drc (Ritz et al. 2015) was used to calculate the lethal (LC_X) and effect concentrations (EC_X).

Results & discussion

Survival and reproduction

Our results showed a higher impact of PYR (LC_{50} = 7.6 $mg_{a.i.}kg_{soil}^{-1}$) on the survival of *E. albidus* compared to TRI (LC_{50} = 73 $mg_{a.i.}kg_{soil}^{-1}$) and KM (LC_{50} = 73 $mg_{a.i.}kg_{soil}^{-1}$) (Table 1). Reported LC_{50} for *E. crypticus* exposed to PYR (LC_{50} = 4.26 $mg_{a.i.}kg_{soil}^{-1}$) and TRI (LC_{50} = 2.34 $mg_{a.i.}kg_{soil}^{-1}$) suggest a higher resistance of *E. albidus* to PYR and, in particular, to TRI (Kovačević et al. 2021a).
Consistent with the effects on survival, PYR was most detrimental to reproduction (Table 1). While PYR showed the strongest effect on reproduction, the effects of TRI (EC_{50} = 17 $mg_{a.i.}kg_{soil}^{-1}$) and KM (EC_{50} ≥ 30 $mg_{a.i.}kg_{soil}^{-1}$) were lower (Fig. 1). Unlike the other two strobilurins, KM significantly impaired reproduction only at the highest concentration tested. Although *E. albidus* showed the ability to survive exposure to higher concentrations of PYR than *E. crypticus*, the effect on reproduction was the opposite. With an EC_{50} of 0.98 $mg_{a.i.}kg_{soil}^{-1}$, *E. albidus* appears to be more sensitive to PYR than *E. crypticus* (EC_{50} = 1.85 $mg_{a.i.}kg_{soil}^{-1}$). The recommended application dose prescribed for PYR exceeds the EC_{50} value calculated for *E. albidus*. Additionally, soil analysis of some agricultural land revealed the presence of PYR in the range of 0.0128–1.5 $mg_{a.i.}kg_{soi}^{-1}$ (Zhao et al. 2020), which also exceeds the EC_{50} value. Therefore, the concentrations of PYR present in the environment may pose a threat to the stability of enchytraeid populations and the function of soil ecosystems.

MXR

The negative effects of strobilurin fungicides were also evidentat the sub-organismal level, causing impairment of MXR and induction of oxidative stress. A signifiant inhibition (ANOVA, $p < 0.001$, $F = 44.675$) of the MXR system was observed after exposure to higher concentrations of the three fungicides (Fig. 2). The strongest inhibition of the MXR system (up to7-fold) was observed after a seven-day exposure to PYR (6 $mg_{a.i.}kg_{soi}^{-1}$), followed by a 4-fold increase at the same concentration after twenty-one day of exposure. TRI caused a significant dose-dependent increase in Rh123 levels after 21 day of exposure. As with reproductive success, exposure toKM inhibited the MXR system only at the highest concentra- tion (30 $mg_{a.i.}kg_{soil}^{-1}$) after 7 and 21 day. The dye efflux assay is a commonly used method to quantify the effects of environmental contaminants on MXR activity (Luckenbach et al. 2014). Few studies have reported the effects of fungicides on the MXR system of enchytraeids (Kovačević et al. 2021b, Kovačević et al. 2022), but such a response has also been observed in earthworms (Velki et al. 2018). The MXR mechanism removes harmful substances from cells immediately after exposure and is therefore considered the first line of defence (Ferreira et al. 2014). Furthermore, changes in MXR activity are used as biomarkers of exposure and can provide additional information on the effects and toxicity of fungicides. Indeed, inhibition of MXR activity may lead to a longer residence time of fungicides in cells, which consequently influences their toxicity (Hackenberger et al. 2012). Although there is no available information on the accumulation of PYR in soil organisms, studies with zebrafish (*Danio rerio*) show that mostof PYR enters the body through the gills and intestines - epi-thelial tissues rich in MXR transporters (Huang et al., 2021). Furthermore, the toxicity of PYR in zebrafish was related to its accumulation in the organism. Therefore, the higher toxicity of PYR could be related to the strong inhibition of the MXR system, which consequently led to the accumulation of fungicides in the organism. Furthermore, the accumulation of fungicides in cells can lead to oxidative stress, as antioxidant defence may be overwhelmed.

Table 1 Lethal (LC_x) and effect (EC_x) concentrations calculated for *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI), and kresoxym-metil (KM)

		LC_{10}	LC_{50}	LC_{90}
Survival	PYR	4 (3–5)	7.6 (6–8)	10 (9–11)
	TRI	15 (9–20)	73 (67–81)	130 (110–152)
	KM	21 (19–24)	73 (47–91)	98 (90–109)
		EC_{10}	EC_{50}	EC_{90}
Reproduction	PYR	0.12 (0.025–0.26)	0.98 (0.51–1.5)	8 (7–12)
	TRI	3 (1.8–4.5)	17 (12–18)	31 (28–>30)
	KM	17 (10–24)	>30	>30

Concentrations are presented as $mg_{a.i.}kg_{soil}^{-1}$ and show LC/EC and the 95% confidence intervals

Oxidative stress

Changes in the activity of enzymes of the antioxidant system (SOD, CAT, GST) and the observed LPO indicate the appearance of oxidative stress. Although data on antioxidant enzymes in enchytraeids after exposure to strobilurin fungicides are lacking, there is information on the effect of the pure active ingredient on the earthworm *E. fetida* (Ma et al. 2019; Liu et al. 2020; Wu et al. 2021). Listed studies have shown that strobilurin fungicides in the form of pure active ingredients cause oxidative stress, suggesting similar effects in other non-target soil organisms. One of the enzymes that respond first to the onset of oxidative stress is SOD. As part of the primary antioxidant defence mechanism, SOD converts the superoxide anion radical (O_2^-) into hydrogen peroxide (H_2O_2) (Ighodaro and Akinloye 2018). In this study, there was a significant induction of SOD activity after a seven-day exposure to lower concentrations of PYR and almost all

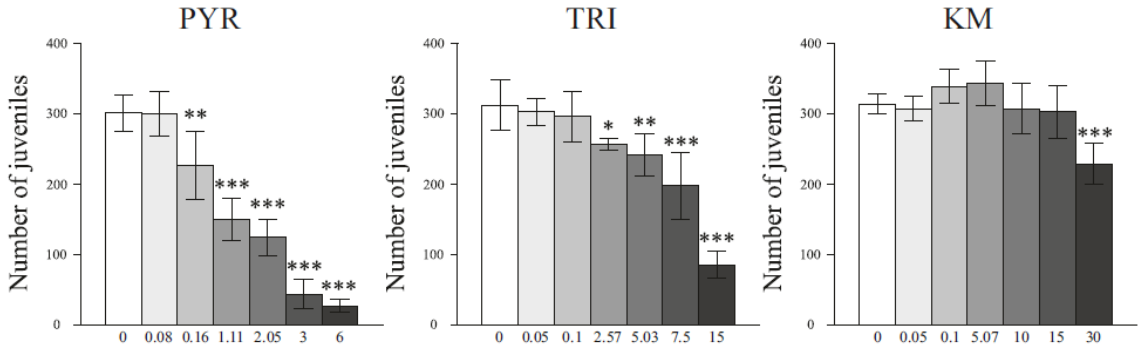


Fig. 1 Reproduction of *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI), and kresoxym-metil (KM) presented as the number of juveniles. Results express average values ± SD. Significant differences compared to control are labeled with *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$)

concentrations of TRI (Fig. 2). The induction of SOD indicates the presence of O_2^-, which the enzyme attempts to remove. SOD induction has been observed after 7 days of exposure to PYR (0.08 $mg_{a.i.}kg_{soil}^{-1}$ and 16 $mg_{a.i.}kg_{soil}^{-1}$) and TRI (0.1 $mg_{a.i.}kg_{soil}^{-1}$ to 15 $mg_{a.i.}kg_{soil}^{-1}$). The activity of SOD remained induced at lower concentrations of PYR(0.16 $mg_{a.i.}kg_{soil}^{1}$ and 1.107 $mg_{a.i}kg_{soil}^{-1}$) and was induced at the three highest concentrations of KM after 21 days of exposure ($p < 0.05$). However, at the highest tested concentrations of PYR (6 $mg_{a.i.}kg_{soil}^{-1}$) and TRI (7.5 $mg_{a.i.}kg_{soil}^{-1}$, 15 $mg_{a.i.}kg_{soil}^{-1}$), the amount of substrate exceeds the enzyme capacity, and its depletion and degradation occur. Since no new enzyme is synthesised, this reaction leads to a significant reduction in the amount of enzyme. A similar response to PYR and TRI has been observed in the earthworm *E. fetida*. Namely, Ma et al. (2019) reported induction of SOD after 7 and 14 days of exposure to PYR, while inhibition was observed after 21 and 28 days. Furthermore, Liu et al. (2020) observed induction only at the highest TRI concentration tested (10 $mg_{a.i.}kg_{soil}^{-1}$), while Wu et al. (2021) observed induction of SOD activity after 7 and inhibition after28 and 56 days. SOD scavenges excess O^- and converts it toH_2O_2 (Shao et al. 2019). As H_2O_2 is a substrate for CAT, itsactivity was induced in all treatments after 7 days (ANOVA, p== 0.0058, $F = 9.4$) (Fig. 2). However, after 21 day, the activity of CAT returned to the control level, indicating that the elimination of H_2O_2 was successful at lower concentra-tions. Only at the highest concentrations of PYR and KM, theactivity of CAT was significantly inhibited (ANOVA, $p = 0.0092$, $F = 3.585$), indicating that the enzyme capacity was exceeded and degraded. Similarly, in *E. fetida*, inductionof CAT activity was observed after shorter exposures (7, 14,and 21 day) to PYR and TRI (7 and 28 days) and without change or inhibition after longer exposures (Ma et al. 2019;Wu et al. 2021). GST is a crucial enzyme in the second biotransformation phase. Unlike SOD and CAT, which are activated immediately after exposure to free radicals, the effect of GST is most pronounced after prolonged exposure. Indeed, in this study, no significant changes in GST activity were observed after a seven-day exposure (Fig. 2). However, after twenty-one day of exposure to TRI and KM, there was a dose-dependent induction (ANOVA, $p < 0.0001$, $F = 27.46$). GST was also induced at two lower concentrations of PYR, but at higher concentrations, its activity was significantly inhibited. This result suggests a higher ROS production caused by exposure to PYR and/or glutathione depletion (a substrate for GST). Earthworms appeared to cope better with PYR since only induction was detected (Ma et al. 2019), although the duration of exposure may not have been long enough to detect GST inhibition. Indeed, exposure to TRI induced GST in earthworms after 7 days but inhibited it after56 days (Liu et al. 2020; Wu et al. 2021). This also suggests a prolonged effect of strobilurins on soil invertebrates and urges caution, as multiple and mixed applications of pesticides regularly occur in this time frame under the given environmental conditions. Excessive long-term ROS production can cause LPO. LPO is a sign of severe oxidative stress and the inability of an organism to defend itself. Measurement of MDA content is often used to determine the extent of LPO (Gaweł et al. 2004). Normally, LPO does not occur after a short exposure. However, after only 7 days, the MDA content increased significantly at the highest concentrations of PYR (6 $mg_{a.i.}kg_{soil}^{-1}$) and TRI (30 $mg_{a.i.}kg_{soil}^{-1}$) (ANOVA, $p = 0.002$, $F = 4514$) (Fig. 2). After 21 day of exposure to thethree fungicides, a significant increase in MDA was observed at higher concentrations. These results are consistent with thepreviously reported increase in MDA after exposure of *E. fetida* to PYR and TRI (Ma et al. 2019; Liu et al. 2020; Wuet al. 2021).

Available energy reserves

Detoxification processes are energetically costly for organisms, which can lead to a reduction in the energy used for basic processes such as growth and reproduction (Świątek and Bednarska 2019). The total available energy of the

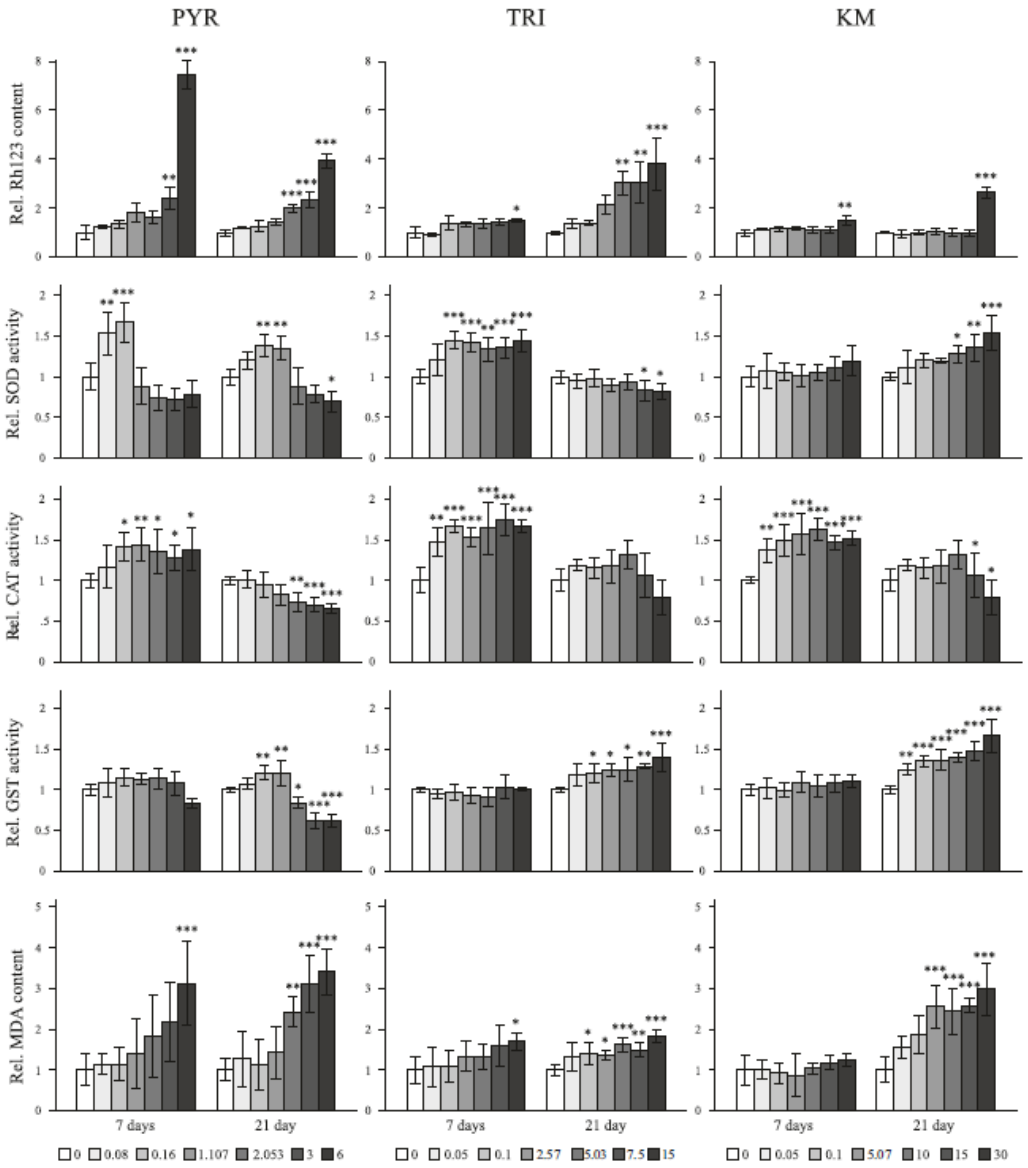


Fig. 2 Responses of different endpoints (MXR (Rh123 content), SOD, CAT, and GST activity, and lipid peroxidation (MDA content)) in *Enchytraeus albidus* after exposure to formulated products of strobi- lurin fungicides based on pyraclostrobin (PYR), trifloxystrobin (TRI) and kresoxym-metil (KM). Results are expressed as average ± SD and relative to the corresponding control. Significant differences comparedto control are labeled with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

organisms in the control groups was approximately 1700 $mJmg_{wt}^{-1}$ while the ratio of energy reserves was about 10:35:55 (carbohydrates:lipids:proteins). These results agree with previous studies on enchytraeids (Amorim et al.2012; Novais et al. 2013).

Exposure to commercial l strobilurin fungicides resulted in the available energy changes in *E. albidus* (Table 2). A significant decrease in carbohydrate fraction was observed after a seven-day exposure to the two highest concentrations of PYR and after a twenty-one day exposure to all fungi- cides tested. The decrease in the carbohydrate fraction is a result of oxidative stress, a condition in which carbohy- drates are used as the primary source of energy (Moolman et al. 2007). The reduction of carbohydrate content has beenobserved after exposure of enchytraeids to the pesticides dimethoate, anthrazine, and carbendazim (Novais and Amorim 2013). Since lipids are extremely efficient as energy storage, they are usually mobilised simultaneously

Table 2 Total energy available and proportions of each energy reserve (carbohydrates, lipids, and proteins) in *Enchytraeus albidus* after exposure to formulated products of strobilurin fungicidesbased on pyraclostrobin (PYR), trifloxystrobin (TRI), and kresoxym-metil (KM)

	PYR				TRY				KM			
	Energy available	Proportion of energy reserve			Energy available	Proportion of energy reserve			Energy available	Proportion of energy reserve		
		CH	Lipid	Protein		CH	Lipid	Protein		CH	Lipid	Protein
7-day exposure												
Ctrl.	1697 ± 88	12 ± 2.1	37 ± 3.7	50 ± 2.8	1673 ± 181	11 ± 1.9	34 ± 5	54 ± 3.8	1735 ± 98	11 ± 1.4	35 ± 3.7	54 ± 2.7
C1	1692 ± 112	12 ± 1.4	38 ± 1.8	50 ± 2.7	1591 ± 128	10 ± 0.9	32 ± 4.9	58 ± 4.1	1825 ± 114	11 ± 0.3	35 ± 3.5	53 ± 3.4
C2	1750 ± 23	12 ± 2.5	42 ± 5.7	46 ± 3.8	1755 ± 87	11 ± 1.4	39 ± 2.5	50 ± 1.5	1905 ± 82	10 ± 1.4	37 ± 1.9	53 ± 2.8
C3	1782 ± 82	12 ± 1	41 ± 2.9	47 ± 2.8	1796 ± 139	13 ± 2.3	36 ± 5.5	50 ± 3.4	1977 ± 168**	10 ± 1.4	45 ± 3***	45 ± 4
C4	1696 ± 15	9.3 ± 2	40 ± 6.4	50 ± 6.3	1863 ± 178	12 ± 1.9	40 ± 5.1	48 ± 5.3	1997 ± 48**	11 ± 2	45 ± 1.8***	43 ± 2.2
C5	1719 ± 86	5.2 ± 1.3***	44 ± 2.8	50 ± 2.4	1795 ± 143	9 ± 2.5	41 ± 2.4	50 ± 1.3	1957 ± 65*	10 ± 1.8	41 ± 2.9**	49 ± 2.7
C6	1624 ± 170	3 ± 0.66***	44 ± 4.2	51 ± 4.7	1796 ± 43	10 ± 1.3	41 ± 2.7	48 ± 2	1864 ± 111	10 ± 1.5	43 ± 3**	47 ± 2.4
21-day exposure												
Ctrl.	1632 ± 99	12 ± 1.6	35 ± 1.7	52 ± 1.9	1613 ± 62	10 ± 1.4	36 ± 2.5	53 ± 3.6	1753 ± 166	10 ± 0.9	35 ± 2.3	54 ± 1.7
C1	1622 ± 80	12 ± 2.8	30 ± 1.8	58 ± 4.4	1335 ± 38	7 ± 1.5***	30 ± 3.6***	63 ± 3	1737 ± 120	8 ± 0.9**	35 ± 1.1	57 ± 0.8
C2	1553 ± 51	10 ± 3.1	28 ± 1.4*	62 ± 2.6*	1507 ± 130	6 ± 1***	27 ± 1.2***	67 ± 2*	1706 ± 73	8 ± 1.9**	33 ± 3.5	59 ± 2.9
C3	1540 ± 38	8 ± 3**	27 ± 4***	65 ± 1.8**	1784 ± 103	6 ± 0.35***	24 ± 1.9***	70 ± 2***	1599 ± 86	8 ± 1*	31 ± 2.3*	61 ± 2.2
C4	1423 ± 99**	6 ± 1.6***	22 ± 4.5***	71 ± 4.9**	1634 ± 160	6 ± 0.8***	22 ± 4.6***	72 ± 4.6***	1582 ± 45	8 ± 1.7**	30 ± 2.3**	62 ± 1.1
C5	1343 ± 100***	5 ± 0.75***	18 ± 4***	77 ± 3.3**	1741 ± 56	7 ± 1.5*	22 ± 2***	71 ± 3.1***	1546 ± 75*	8 ± 1.5**	29 ± 3.7**	63 ± 4.1
C6	1263 ± 58***	3 ± 0.78***	17 ± 1.4***	80 ± 2**	1789 ± 91	6 ± 0.76***	24 ± 1.8***	70 ± 1.3***	1528 ± 120*	7 ± 1.2***	25 ± 2.9***	67 ± 4

Results are expressed as average ± standard deviation. Significant differences compared to control are labeled with *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$)

with carbohydrates under stress conditions (Smolders et al., 2003). However, after a 7-day exposure to KM (2.57, 10, 15 and 30 $mg_{a.i.}kg_{soil}^{-1}$), lipid content increased. An increase in lipid content has previously been associated with inflammatory stress (Gomes et al. 2015). The tendency to accumulate lipids was also observed after 8 days of exposure of *E. albidus* to the fungicide carbendazim (Novais and Amorim 2013). The lipid content decreased after 21 day ofexposure to all tested fungicides. Lipid depletion could be a consequence of severe oxidative stress and damage to the cell membrane. Increased levels of MDA and changes in activities of SOD, CAT, MXR, and GST were measured in all treatments in our studies. Similar changes in lipids havebeen reported after exposure of *E. albidus* to Cd and Zn (Novais et al. 2011; Novais et al. 2013). As anabolic components, proteins are only used as an energy source during extreme energy deficiency (Świątek and Bednarska 2019). Sokolova et al. (2012) reported that an increase in protein levels under intermediate stress indicates a higher expression of stress response proteins. In our research proteins increased significantly after 21 day of exposure to higher concentrations of PYR and TRI (Table 2) and no decrease was observed. These shifts in carbohydrate, lipid, and protein content affected total available energy, which increased after 7 days of exposure to KM and significantly decreased after 21 day of exposure to higher concentrationsof PYR and KM. Exposure to TRI did not affect the total available energy. Since an increase in lipid content strongly influences total available energy, an increase in lipid contentafter exposure to different pesticides led to an increase in available energy (Novais and Amorim 2013). According to the general prediction of metabolic models, the metabolic rate should increase with intoxication (Calow and Sibly 1990), which may be related to the results of this research. Although in KM treatments, the decrease in available energy may be a consequence of excessive energy con- sumption as a result of the simultaneous defence against oxidative stress and reproduction, in the case of PYR, it wasdue to the higher toxicity and the need for a more intense defence against stress. Furthermore, although oxidative stress was observed after TRI treatment, the lack of repro- duction compensated for the loss of energy, and the decrease in total available energy, was not observed.

Strobilurin fungicides are known for their ability to act on the mitochondrial respiratory chain and consequently reduce the available energy of organisms. Furthermore, the breakdown of the electron transfer chain and mitochondrial membrane protein leads to the formation of ROS and, consequently, oxidative stress. Although changes in apical endpoints, such as growth and behaviour, may not be observed, even extremely low concentrations of strobilurin fungicides can cause oxidative stress in aquatic organisms (Wang et al. 2021). Consequently, although exposure to some formulations did not affect survival and reproduction, changes in CAT and GST activity when exposed to the recommended application doses indicate a negative effect. In addition to oxidative stress, the reduction in available energy observed after prolonged exposure suggests a negative effect on mitochondrial respiration. Similar con- clusions about the strobilurin mechanism were made for different non-target organisms (Wang et al. 2021). Fur- thermore, a high concentration of strobilurin fungicides suppresses mitochondrial respiration, and consequently, cell apoptosis occurs (Rodrigues et al. 2015). Therefore, inhi- bition of the MXR system and accumulation of fungicides within the cell, observed in PYR treatments, increase the possibility of fungicide binding to the mitochondria, caus- ing apoptosis and enchytraeid mortality.

Most laboratory studies with suborganismic biomarkers are based on short-term exposure to high concentrations of pollutants (Rodríguez-Castellanos and Sanchez-Hernandez, 2007). However, in the environment, with soil as the main sink, long-term exposure to low concentrations is a more common scenario. Although environmentally relevant concentrations did not show effects on enchytraeid reproduction, the occurrence of oxidative stress and reduction in available energy showed that the adverse impact of strobilurin formulations begins much earlier than is evident from population biomarkers. The inclusion of the suborganismic biomarker approach showed a marked adaptability of enchytraeids to exposure. Although activation of the antioxidative system has been shown as a successful defence method, changes in the amount of available energy suggest that this strategy is not sustainable in long-term exposure. Namely, in such situations, there is a certain reduced rate of reproduction or its absence, which can ultimately lead to the collapse of populations. Moreover, formulated products often exhibited stronger adverse effects on non-target organisms than a pure active ingredient commonly used in research (Marques et al. 2009; Mesnage et al. 2014; Gomes et al. 2021). Therefore, research carried out with formulated products provides results more relevant to environmental conditions. Adjuvants present in commercial products increase the absorption rate and stability of the active ingredient, consequently affecting its bioavailability and causing higher toxicity to non-target organisms (Pereira et al. 2009; Mesnage and Antoniou 2018). PYR applied in the form of suspension concentrate (SC) showed the strongest effect on enchytraeids compared to TRI and KM applied in the form of water-dispersible granules (WG). SC formulations contain small particles of the active substance dissolved in a liquid medium, usually an aqueous solution with various additives. Among the additives are various suspension agents, wetting agents, and thickeners that increase the availability of the active substance and can affect the behaviour of the formulation in the environment

(Khan and Brown, 2016). Compared to other formulations,SC formulations have reduced leaching, which results in increased persistence and accumulation in the environment, which can result in a stronger effect on non-target organ- isms compared to other forms of formulations.

Conclusion

This study highlights the importance of assessing effects on multiple endpoints over different periods. Therefore, comprehensively considering the changes in multiple endpoints over different periods provided better insight into the impact of strobilurin fungicides. The results obtained indicate differences in the response of *E. albidus* to three commercial formulations of strobilurin fungicides. Overall, when all measured endpoints, are assessed PYR was the most toxic, followed by TRI and KM. Although with different intensities, all three strobilurins affected reproduction and induced oxidative stress in *E. albidus*. The analyses ofavailable energy showed that the exposure also representeda high energetic cost for the organisms. While these costs after exposure to PYR and TRI were mainly related to antioxidant defence, the energetic costs after KM exposure,which was the least toxic, were associated with both costly defence and reproduction processes. Research like this is important to determine the sublethal toxicity of these fun- gicides because the frequent detection of strobilurins in theterrestrial environment suggests the potentiality for adverseeffects on soil-dwelling organisms. Furthermore, like other pesticides, strobilurins are used indiscriminately in agri- culture and predicted climate changes indicate an increase in fungicide use. The EC_{50} for PYR determined in this studyis already within the range of concentrations observed in theenvironment. Future research should consider several aspects: exposure to formulations under realistic conditionsin a native soil, exposure to pesticide mixtures or for-mulations with different combinations of strobilurins, which are commonly used, and multigenerational studies in whichthe consequences of oxidative stress and higher energy expenditure could be investigated at the population level.

Acknowledgements This research was conducted in the framework of the DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) funded by the Croatian Science Foundation (HrZZ) under the contract number: IP-09-2014-4459 and "Young Researchers' Career Development Project−Training of Doctoral Students" funded by the Croatian Science Foundation (HrZZ) (DOK-2018-01-4257).

Author contributions MK—Conceptualization, investigation, data curation and interpretation, processing of figures and tables in the paper, original draft writing, revising the draft; NS—Investigation, review and editing of draft paper; ŽL—Investigation, review and editing of draft paper; DKHr—Conceptualization, supervision, reviewand editing of draft paper; BKH—Conceptualization, supervision, funding acquisition, review and editing of draft paper.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

References

Amorim MJB, Gomes SIL, Soares AMVM, Scott-Fordsmand JJ (2012) Energy Basal Levels and Allocation among Lipids, Pro- teins, and Carbohydrates in *Enchytraeus albidus*: Changes Related to Exposure to Cu Salt and Cu Nanoparticles. Water Air Soil Pollut 223:477−482.

Bardgett RD (2005) The Biology of Soil. Oxford University Press Inc., New York

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B (2002) The strobilurin fungicides. Pest Manag Sci 58:649−662. https://doi.org/10.1002/ps.520

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle ofprotein-dye binding. Anal Biochem 72:248−254

Organization for Economic Cooperation and Development (OECD) (2016) Test no. 220: enchytraeid reproduction test. OECD Guidelines for Testing of Chemicals. OECD Publishing, Paris France

RCore Team (2022) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/

R Studio Team (2022) RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, http://www.rstudio.com

Briones MJI, Carreira J, Ineson P (1998) *Cognettia sphagnetorum* (Enchytraeidae) and nutrient cycling in organic soils: a micro- cosm experiment. Appl Soil Ecol 9:289−294

Brussaard L (1998) Soil fauna, guilds, functional groups, and ecosystem processes. Appl Soil Ecol 9(1−3):123−135

Calow P, Sibly RM (1990) A physiological basis of population processes: ecotoxicological implications. Funct Ecol 4:283−288

Claiborne A (1985) Catalase activity. In: Greenwald RA, Ed., CRC Handbook of Methods for Oxygen Radical Research, CRC Press,Boca Raton, 283-284.

De Coen W, Janssen CR (1997) The use of biomarkers in *Daphnia magna* toxicity testing IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J Aquat Ecosyst Stress Recovery 6:43−55

De Coen W, Janssen CR (2003) The missing biomarker link: relationships between effects on the cellular energy allocation bio-marker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ Toxicol Chem 22:1632−1641

Didden W, Römbke J (2001) Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicol Environ Saf50:25−43

European Food Safety Authority, 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance kresoxim-methyl. EFSA J 8(11):1891. https://doi.org/10.2903/j.efsa.2010. 1891. Available online: www.efsa.europa.eu/efsajournal.htm

Feng Y, Huang Y, Zhan H, Bhatt P, Chen S (2020) An overview of strobilurin fungicide degradation: current status and future per- spective. Front Microbiol 11:389

Ferreira M, Costa J, Reis-Henriques MA (2014) ABC transporters in fish species: a review. Front Physiol 5:266. https://doi.org/10.3389/fphys.2014.00266

Frings CS, Fendley TW, Dunn RT, Queen CA (1972) Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin Chem 18(7):673–674

Fulcher JM, Wayment DG, White Jr PM, Webber CL (2014) Pyraclostrobin wash-off from sugarcane leaves and aerobic dissipa- tion in agricultural soil. J Agric Food Chem 62(10):2141–2146.

Gagne F (2014) Biochemical Ecotoxicology: Principles and Methods. Oxidative stress Academic Press, New York, p110e111

Gaweł S, Wardas M, Niedworok E, Wardas P (2004) Mal-ondialdehyde (MDA) as a lipid peroxidation marker. Wiadomoscilekarskie (Warsaw, Poland: 1960) 57(9–10):453–455

Gomes SI, Scott-Fordsmand JJ, Amorim MJ (2015) Cellular energy allocation to assess the impact of nanomaterials on soil inverte- brates (Enchytraeids): the effect of Cu and Ag. Int J Environ Res12(6):6858–6878

Gomes SIL, Ammendola A, Casini S, Amorim MJB (2021) Toxicity of fungicides to terrestrial non-target fauna – Formulated products versus active ingredients (azoxystrobin, cyproconazole, prothio- conazole, tebuconazole) – A case study with *Enchytraeus crypticus* (Oligochaeta). Sci Total Environ 754:142098.

Habig WH, Pabst MJ, Jakobi W (1974) Glutathione S-Transferases the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Hackenberger BK, Velki M, Stepić S, Hackenberger DK (2012) First evidence for the presence of efflux pump in the earthworm *Eisenia andrei*. Ecotoxicol Environ Saf 75:40–45. https://doi.org/10.1016/j.ecoenv.2011.06.024

Huang T, Souders CL, Wang S, Ganter J, He J, Zhao YH, Cheng H, Martyniuk CJ (2021) Behavioral and developmental toxicity assessment of the strobilurin fungicide fenamidone in zebrafish embryos/larvae (Danio rerio). Ecotox Environ Safety 228112966-S0147651321010782:112966.

Ighodaro OM, Akinloye OA (2018) First line defense antioxidants-superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX): Their fundamental role in the entire anti-oxidant defense grid. Alexandria. J Med 54:287–293. https://doi.org/10.1016/j.ajme.2017.09.001

International Organization for Standardization (ISO) (2014) Soil quality—effects of pollutants on Enchytraeidae (*Enchytraeus* sp.) - determination of effects on reproduction and survival. ISO 16387, Switzerland, Geneva

Jermyn MA (1975) Increasing the sensitivity of the anthrone method for carbohydrates. Analytical Biochem 68(1):332–335

Jiang J, Lv L, Wu S, An X, Wang F, Liu X, Zhao X (2019) Developmental toxicity of kresoxim-methyl during zebrafish (*Danio rerio*) larval development. Chemosphere 219:517–525. https://doi.org/10.1016/j.chemosphere.2018.12.061

Khan MA, Brown CD (2016) Influence of commercial formulation on leaching of four pesticides through soil. Sci Total Environ 5731573-1579S0048969716319957. https://doi.org/10.1016/j.scitotenv.2016.09.076

Khandelwal A, Gupta S, Gajbhiye VT, Varghese E (2014) Degrada- tion of kresoxim-methyl in soil: Impact of varying moisture, organic matter, soil sterilization, soil type, light and atmospheric CO_2 level. Chemosphere 111:209–217.

Kim C, Choe H, Park J, Kim G, Kim K, Jeon HJ, Moon JK, Kim MJ, Lee SE (2021) Molecular mechanisms of developmental toxi- cities of azoxystrobin and pyraclostrobin toward zebrafish (*Daniorerio*) embryos: Visualization of abnormal development using two transgenic lines. Environ Pollut. 270. https://doi.org/10.1016/j.envpol.2020.116087

Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product. Pestic Biochem Physiol 187(454–466):105198. https://doi.org/10.1016/j.pestbp.2022.105198

Kovačević M, Hackenberger DK, Hackenberger BK (2021a) Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and tri- floxystrobin) on survival, reproduction, and hatching success of *Enchytraeus crypticus*. Sci Total Environ. 790. https://doi.org/10.1016/j.scitotenv.2021.148143

Kovačević M, Hackenberger DK, Lončarić Ž, Hackenberger BK (2021b) Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology. Chemosphere. 279. https://doi.org/10.1016/j.chemosphere.2021.130549

Kumar N, Willis A, Satbhai K, Ramalingam L, Schmitt C, Moustaid-Moussa N, Crago J (2020) Developmental toxicity in embryo- larval zebrafish (*Danio rerio*) exposed to strobilurin fungicides (azoxystrobin and pyraclostrobin). Chemosphere. 241:124980. https://doi.org/10.1016/j.chemosphere.2019.124980

Li D, Liu M, Yang Y, Shi H, Zhou J, He D (2016) Strong lethality and teratogenicity of strobilurins on *Xenopus tropicalis* embryos:Basing on ten agricultural fungicides. Environ Pollut 208:868e874. https://doi.org/10.1016/j.envpol.2015.11.010

Li H, Cao F, Zhao F, Yang Y, Teng M, Wang C, Qiu L (2018) Developmental toxicity, oxidative stress, and immunotoxicity areinduced by three strobilurins (pyraclostrobin, trifloxystrobin, and picoxystrobin) in zebrafish embryos. Chemosphere 207:781–790. https://doi.org/10.1016/j.chemosphere.2018.05.146

Li H, Jing T, Li T, Huang X, Gao Y, Zhu J, Lin J, Zhang P, Li B, Mu W (2021) Ecotoxicological effects of pyraclostrobin on tilapia (*Oreochromis niloticus*) via various exposure routes. Environ Pollut 285:117188. https://doi.org/10.1016/j.envpol.2021.117188

Li XY, Qin YJ, Wang Y, Huang T, Zhao YH, Wang XH, Martyniuk CJ, Yan B (2021) Relative comparison of strobilurin fungicides at environmental levels: Focus on mitochondrial function and larvalactivity in early staged zebrafish (*Danio rerio*). Toxicology. 452:152706

Liu T, Liu Y, Fang K, Zhang X, Wang X (2020) Transcriptome, bioaccumulation, and toxicity analyses of earthworms (*Eisenia fetida*) affected by trifloxystrobin and trifloxystrobin acid. Environ Pollut 265:115100. https://doi.org/10.1016/j.envpol.2020.115100

Luckenbach T, Fischer S, Sturm A (2014) Current advances on ABC drug transporters in fish. Comp Biochem Physiol C Toxicol Pharmacol 165:28e52. https://doi.org/10.1016/j.cbpc.2014.05.002

Ma J, Cheng C, Du Z, Li B, Wang J, Wang J, Wang Z, Zhu L (2019) Toxicological effects of pyraclostrobin on the antioxidant defense system and DNA damage in earthworms (*Eisenia fetida*). Ecol Indic 101:111–116. https://doi.org/10.1016/j.ecolind.2019.01.015 Mao L, Jia W, Zhang L, Zhang Y, Zhu L, Sial MU, Jiang H (2020) Embryonic development and oxidative stress effects in the larvaeand adult fish livers of zebrafish (*Danio rerio*) exposed to the strobilurin fungicides, kresoxim-methyl, and pyraclostrobin. Sci Total Environ 729:139031. https://doi.org/10.1016/j.scitotenv. 2020.139031

Maraldo K, Christensen B, Holmstrup M (2011) The excretion of ammonium byenchytraeids (*Cognettia sphagnetorum*). Soil Biol Biochem 43:991–996

Marinissen JCY, Didden WAM (1997) Influence of the Enchytraeid worm *Buchholzia appendiculate* aggregate formation and organicmatter decomposition. Soil Biol Biochem 29:387–390

Marques C, Pereira R, Goncalves F (2009) Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and

their active ingredients on natural soils. J. Soils Sediment 9:137–147

Marques C, Pereira R, Gonçalves F (2009) Using earthworm avoidance behaviour to assess the toxcity of formulated herbicides andtheir active ingredients on natural soils. J. Soils Sediments 9:137–147. https://doi.org/10.1007/s11368-009-0058-0

McCord JM, Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244(22):6049–6055

Mesnage R, Antoniou MN (2018) Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Public Health Front 5:361. https://doi.org/10.3389/fpubh.2017.00361

Mesnage R, Defarge N, Spiroux de Vendômois J, Séralini GE (2014) Major pesticides are more toxic to human cells than their declaredactive principles. Biomed Res Int 2014:179691. https://doi.org/10.1155/2014/179691

Mesnage R, Defarge N, Spiroux de Vendomois J, Seralini G-E (2014) Major pesticides are more toxic to human cells than their declaredactive principles. Biomed Res Int 1–8. https://doi.org/10.1155/ 2014/179691

Moolman L, Van Vuren JH, Wepener V (2007) Comparative studies onthe uptake and effects of cadmium and zinc on the cellular energyallocation of two freshwater gastropods. Ecotoxicol Environ Saf 68:443–450. https://doi.org/10.1016/j.ecoenv.2006.12.017

Nguyen DB, Rose MT, Rose TJ, Morris SG, Van Zwieten L (2016) Impact of glyphosate on soil microbial biomass and respiration: ameta-analysis. Soil Biol Biochem 92:50e57. https://doi.org/10.1016/j.soilbio.2015.09.014

Novais SC, Amorim MJB (2013) Changes in cellular energy allocationin *Enchytraeus albidus* when exposed to dimethoate, atrazine, and carbendazim. Environ Toxicol Chem 32:2800–2807. https://doi.org/10.1002/etc.2368

Novais SC, Soares AMVM, de Coen W, Amorim MJB (2013) Exposure of *Enchytraeus albidus* to Cd and Zn - Changes in cellular energy allocation (CEA) and linkage to transcriptional, enzymatic, and reproductive effects. Chemosphere 90:1305–1309. https://doi.org/10.1016/j.chemosphere.2012.09.030

Novais SC, Gomes SIL, Gravato C, Guilhermino L, de Coen W, Soares AMVM, Amorim MJB (2011) Reproduction and bio- chemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ Pollut 159:1836–1843

Pelosi C, Römbke J (2016) Are Enchytraeidae (Oligochaeta, Annelida) good indicators of agricultural management practices? Soil Biol Biochem 100:255–263

Pelosi C, Barot S, Capowiez Y, Hedde M, Vandenbulcke F (2014) Pesticides and earthworms. A review. Agron Sustain Dev34:199–228

Pereira JL, Antunes SC, Castro BB, Marques CR, Gonçalves AMM, Gonçalves F, Pereira R (2009) Toxicity evaluation of three pes- ticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. Ecotoxicology 18:455–463. https://doi.org/10.1007/s10646-009-0300-y

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLOs ONE 598(10):e0146021

Rodrigues ET, Pardal MA, Laize V, Cancela ML, Oliveira PJ, SerafimTL (2015) Cardiomyocyte H9c2 cells present a valuable alter- native to fish lethal testing for azoxystrobin. Environ Pollut 206:619e626

Rodríguez-Castellanos L, Sanchez-Hernandez JC (2007) Earthworm biomarkers of pesticide contamination: Current status and perspectives. J Pest Sci 32(4):360–371. https://doi.org/10.1584/jpestics.R07-14

Shao Y, Hou K, Du Z, Li B, Wang J, Juhasz AL, Zhu L (2019) Evaluation of the toxicity of 1-butyl-3-methyl imidazolium tet- rafluoroborate using earthworms (*Eisenia fetida*) in two soils. SciTotal Environ 686:946e958

Smolders R, De Boeck G, Blust R (2003) Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Daniorerio*). Environ Toxicol Chem 22(4):890–899. https://doi.org/10.1002/etc.5620220429

Sokolova IM, Frederich M, Bagwe R, Lannig G, Sukhotin AA (2012) Energy homeostasis as an integrative tool for assessing the limits of environmental stress tolerance in aquatic invertebrates. Environ Res79:1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Świątek ZM, Bednarska AJ (2019) Energy reserves and respiration rate in the earthworm Eisenia andrei after exposure to zinc in nanoparticle or ionic form. Environ Sci Pollut Res 26:24933–24945. https://doi.org/10.1007/s11356-019-05753-3

Topoliantz S, Ponge JF, Viaux P (2000) Earthworm and enchytraeid activity underdifferentarable farming systems, as exemplified bybiogenic structures. Plant Soil 225:39–51

Velki M, Weltmeyer A, Seiler TB, Hollert H (2018) Acute toxicities and effects on multixenobiotic resistance activity of eight pesti- cides to the earthworm *Eisenia andrei*. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-018-3959-x

Wang C, Wu J, Zhang Y, Wang K, Zhang H (2015) Field dissipation of trifloxystrobin and its metabolite trifloxystrobin acid in soil andapples. Environ Monit Assess 187:4100. https://doi.org/10.1007/ s10661-014-4100-3

Wang Y, Qin Y, Bing Y, Martyniuk CJ (2021) A comprehensive review of strobilurin fungicide toxicity in aquatic species: Emphasis on mode of action from the zebrafish model. Environ Poll 275116671-S0269749121002499:116671. https://doi.org/10.1016/j.envpol.2021.116671

Wu R, Zhou T, Wang J, Wang J, Du Z, Li B, Juhasz A, Zhu L (2021) Oxidative stress and DNA damage induced by trifloxystrobin on earthworms (*Eisenia fetida*) in two soils. Sci Total Environ 797:149004. https://doi.org/10.1016/j.scitotenv.2021.149004

Xu Y, Li B, Hou K, Du Z, Allen SC, Zhu L, Li W, Zhu L, Wang J, Wang J (2021) Ecotoxicity evaluation of azoxystrobin on *Eisenia fetida* in different soils. Environ Res 194:110705. https://doi.org/10.1016/j.envres.2020.110705

Yang L, Houang T, Li R, Souders CL, Rheingold S, Tischuk C, ZhouB, Martyniuk CJ (2021) Evaluation and comparison of the mitochondrial and developmental toxicity of three strobilurins inzebrafish embryo/larvae. Environ Pollut 270:116277. https://doi.org/10.1016/j.envpol.2020.116277

Zhang C, Zhou T, Xu Y, Du Z, Li B, Wang J, Wang J, Zhu L (2020) Ecotoxicology of strobilurin fungicides. Sci Total Environ 742:140611. https://doi.org/10.1016/j.scitotenv.2020.140611

Zhao L, Li Y, Ren W, Huang Y, Wang X, Fu Z, Ma W, Teng Y, Luo Y (2020) Pesticide residues in soils planted with *Panax noto- ginseng* in south China, and their relationships in *Panax noto- ginseng* and soil. Ecotoxicol Environ Saf. 201. https://doi.org/10.1016/j.ecoenv.2020.110783

3. RASPRAVA

Provedena istraživanja predstavljaju prvi sveobuhvatni prikaz djelovanja strobilurinskih fungicida na enhitreje vrsta *E. albidus* i *E. crypticus*, a dobiveni rezultati doprinose boljem razumijevanju mehanizma djelovanja ovih fungicida. Dokazano je kako testirani fungicidi pri višim koncentracijama mogu negativno djelovati na preživljavanje i reprodukciju enhitreja. Međutim, niže koncentracije strobilurinskih fungicida, mogu uzrokovati odgodu izlijeganja, promjene aktivnosti MXR mehanizma, ali i disbalans oksidativnog sustava koji vodi ka pojavi oksidativnog stresa, lipidne peroksidacije i promjena u količinama dostupne energije.

Gotovo je nemoguće spriječiti ulazak strobilurinskih fungicida i njihovih pripravaka u ekološke sustave, a iako kemijska i fizikalna svojstva strobilurinskih fungicida ukazuju na brzu razgradnju (Bartlett i sur., 2002) sve se češće detektiraju u vodi i tlu (Feng i sur., 2020; Li i sur., 2006, 2021; Silva i sur., 2009; Xu i sur 2021). Upotreba strobilurinskih fungicida nužna je za uspješnu poljoprivredu, a zbog neizbježnih klimatskih promjena, razvoja rezistentnosti gljivičnih vrsta, ali i pojave novih, invazivnih vrsta, neizbježno je povećanje količine primijenih fungicida (Nnadi i Carter, 2021; Fisher i sur., 2012; Hakala i sur., 2011; Boxall i sur., 2009). Stoga je potrebno istražiti djelovanje strobilurinskih fungicida na ključne skupine organizama tla, ali i odrediti aktivne tvari te komercijalne pripravke koji pokazuju najmanji utjecaj na neciljne organizme. Rezultati velikog broja istraživanja ukazuje na negativan utjecaj strobilurinskih fungicida na akvatičke organizme (Rodrigues i sur., 2013). Među neciljnim organizmima tla, glavnina istraživanja usmjerena je na gujavice vrste *E. fetid*a. Tako je uočena visoka toksičnost azoksistrobin primljenog u obliku čiste aktivne tvari, koja je izraženija u prirodnom nego umjetnom tlu (Xu i sur., 2021). Nadalje, uočeno je negativno djelovanje piraklostrobin i trifloksistrobin na rast gujavica, dok su niže koncentracije uzrokovale pojavu oksidativnog stresa (Wu i sur., 2021; Liu i sur., 2020; Ma i sur., 2019). Iako postojeće informacije govore u prilog toksičnosti strobilurinskih fungicida za organizme tla, upitna osjetljivost i prikladnost vrsta *E. fetida* i *E. andrei* umanjuje njihovu relevantnost. Naime, ove vrste gujavica ne nalazimo u poljoprivrednim tlima, te su često manje osjetljive na pesticide u usporedbi s ostalim organizmima tla što može utjecati na relevantnost rezultata. Kako je korištenje većine nativnih vrsta gujvica praćeno teškoćama u njihovom uzgoju i korištenju u laboratorijskim uvijetima, nameće se potreba za korištenjem drugih tj. relevantnijih vrsta organizama (Bart i sur., 2018). Zbog toga se sve veća pažnja ekotoksikoloških istraživanja posvećuje enhitrejama. Enhitreje su organizmi tla koji imaju sličnu funkcionalnu ulogu poput gujavica, prisutne su u tlima diljem svijeta, te odolijevaju intenzivnoj mehaničkoj obradi tla. Osim toga, zbog svoje veličine, duljine životnog ciklusa i njegovih specifičnosti enhitreje su

prikladne za različite modifikacije standardiziranih testova koje omogućuju procjenu dodatnih krajnjih točaka. Tako se u današnjim istraživanjima koristi pet vrsta enhitreja: *E. albidus, E. buchholzi, E. bulbosus, E. crypticus* i *E. luxuriousus*. Međutim, potreba za standardizacijom testova i njihovom interkalibracijom rezultirala je masovnom uporabom vrsta *E. albidus* i *E. crypticus*. Upravo su ove vrste pokazale dovoljnu osjetljivost i najbolje osobine za uzgoj u laboratoriju i provođenje ekotoksikoloških testova što je rezultiralo ponavljanjem slične situacije kao u slučaju gujavica vrste *E. fetida* i *E. andrei*. Intenzivno korištenje novih ili slabije korištenih vrsta zahtjevalo bi dugogodišnju i zahtjevnu selekciju do vrsta koje bi zaista mogle zadovoljiti sve potrebe suvremene ekotoksikologije.

Iako su enhitreje priznati indikatorski organizmi ekotoksikologije tla, te je razvijen čitav niz testova koji omogućava procjenu različitih krajnjih točaka, neki mehanizmi i dalje nisu potvrđeni. Skupini neistraženih mehanizama pripada i MXR mehanizam koji je ključan za opstanak organizama u okolišu u kojemu su prisutna različita zagađivala. MXR mehanizam prva je linija obrane organizama nakon dodira s okolišnim zagađivalima, a promjene aktivnosti ovog mehanizma učestalo se koriste kao rani biomarker upozorenja (Ács i sur., 2015). Prisutnost MXR mehanizma nije ranije dokazana kod enhitreja, te nije ustanovljena metodologija specifična za ovu skupinu organizama. Prilagodba metoda koje se koriste za procjenu promjena aktivnosti MXR mehanizma gujavica (Bošnjak i sur., 2014; Hackenberger i sur., 2012) dovela je do razvoja nove metode koja se može uspješno koristiti za mjerenje promjena aktivnosti MXR mehanizma enhitreja. Kao i kod ostalih organizama, sam test temelji se na akumulaciji fluorescentnih supstrata. Izlaganje organizama fluorescentnim supstratima prilikom testa akumulacije uglavnom se provodi na filter papiru, vodenom mediju ili tlu. Kako zbog veličine enhitreja i velike mogućnosti isušivanja izlaganje na filter papiru nije prihvatljiva opcija, kao optimalno rješenje odabrano je izlaganje u ISO vodi. Ovakav način osigurava ravnomjerno izlaganje organizama, a mogućnost oštećenja tijekom rukovanja svedena je na minimum. Niz eksperimenata provedenih u ISO vodi omogućio je određivanje optimalnog vremena izlaganja, te odabir najprikladnijeg fluorescentnog supstrata i njegove koncentracije. Rodamin 123 smatra se supstratom izbora prilikom mjerenja promjena aktivnosti MXR mehanizma gujavica (Bošnjak i sur., 2014), a provedena istraživanja na enhitrejama pokazala su njegove prednosti u odnosu na rodamin B. Nadalje, kako bi se dokazale promjene aktivnosti MXR sustava pod djelovanjem različitih okolišnih zagađivala nužno je odrediti modelne inhibitore i inducere koji se koriste kao pozitivna i negativna kontrola. Izlaganje različitim koncentracijama modelnih inhibitora i inducera izazvalo je očekivano djelovanje sugerirajući

sličnosti MXR mehanizma gujavica i enhitreja, čime je potvrđena prva hipoteza ovog rada. Naime, dokazana je prisutnost MXR mehanizma kod enhitreja i uspostavljena metodologija koja omogućava mjerenje ovog biomarkera tijekom standardiziranih testova. Ciklosporin A, poznati ne kompetitivni inhibitor P-gp transportera (Twentyman, 1992), pokazao se kao najsnažniji inhibitor MXR mehanizma kod obije vrste enhitreja. Iako je verapamil modelni inhibitor, u kombinaciji s rodaminom B uzrokovao je indukciju MXR mehanizma vrste *E. albidus*. Ovakav odgovor posljedica je razlika u veznim mjestima i omjeru P-gp i MRP proteina između vrsta *E. albidus* i *E. crypticus*, te interakcije između fluorescentnog supstrata i inhibitora ili veznog mjesta, a uočen je i kod gujavica vrste *E. fetida* (Bošnjak i sur., 2014). Aktivnost MXR mehanizma igra važnu ulogu u odgovoru organizama na prisutnost zagađivala u okolišu, stoga je važno proučiti njihove interakcije i utjecaj na promjene aktivnosti MXR sustava. Kemosenzitizatorima pripada i veliki broj fungicida, koji su prepoznati kao inhibitori MXR mehanizma (Mazur i sur 2015; Velki i Hackenberger, 2013a; Velki i Hackenberger, 2013b; Velki i Hackenberger 2012). Inhibitorna svojstva propikonazola dokazana su validacijskim testom provedenim s obije vrste enhitreja nakon izlaganja u ISO vodi i tlu. Ovim eksperimentom dokazana je i druga hipoteza rada. Naime, potvrđena je mogućnost da ksenobiotici prisutni u okolišu, poput fungicida propikonazola, mogu djelovati na aktivnost MXR sustava enhitreja i uzrokovati njegovu inhibiciju ili indukciju. Nadalje, svim provedenim eksperimentima pokazana je primjenjivost i točnost nove metode koja se može koristiti kao vrijedan komplementarni biomarker u ekotoksikološkim istraživanjima koja se provode s enhitrejama, čime je dokazana i treća hipoteza ovoga rada. Osim toga, dobiveni rezultati ukazali su na potrebu za daljnjim istraživanjem djelovanja fungicida na promjene aktivnosti MXR mehanizma.

Kako su strobilurini relativno nova skupina fungicida koja je tek nedavno privukla pažnju šire znanstvene zajednice, informacije o njihovom djelovanju na neciljne organizme tla, posebice enhitreje su nedostatne. Stoga je cilj drugog istraživanja bio prikupiti osnovne informacije o djelovanja tri komercijalna pripravaka na preživljavanje i reprodukciju vrste *E. crypticus*. Standardizirani reprodukcijski test pokazao je negativno djelovanje tri komercijalna pripravka temeljena na azoksistrobinu, piraklostrobinu i trifloksistrobinu na preživljavanje i reprodukciju vrste *E. crypticus* potvrdivši četvrtu hipotezu rada. Komercijalni pripravak temeljen na aktivnoj tvari trifloksistrobin pokazao je najjače djelovanje na preživljavanje i reprodukciju, dok je nešto blaže djelovanje uočeno pri tretmanima provedenima s komercijalnim pripravcima temeljenima na piraklostrobinu i azoksistrobinu. Opažene razlike u

djelovanju pojedinih komercijalnih pripravaka potvrdile su petu hipotezu ovoga rada. Nadalje, proširena su postojeća znanja o negativno djelovanje azoksistrobina na vrstu *E. crypticus*, dok rezultati o djelovanju piraklostrobina i trifloksistrobina predstavljaju temeljne informacije o djelovanju ovih pripravaka i aktivnih tvari na enhitreje. Temeljem standardiziranih reprodukcijskih testova uočeno je povećanje broja ne izleženih kokona pri pojedinim koncentracijama koje se može povezati s pojavom oštećenja embrionalnog razvoja ranije zabilježenog kod akvatičkih organizama (Ma i sur., 2020; Zhang i sur., 2020c). Dok su tretmani višim koncentracijama komercijalnih pripravaka uzrokovali inhibiciju reprodukcije, ona se pri nižim koncentracijama odvijala neometano. Međutim, niže koncentracije fungicida uzrokuju promjene embrionalnog razvoja koje za posljedicu imaju odgodu ili potpuni izostanak izlijeganja. Prijašnja istraživanja potvrdila su razliku u osjetljivosti između životnih faza organizama tla (Gomes i sur., 2018; Castro-Ferreira i sur., 2012), te moguće djelovanje strobilurinskih fungicida na embrionalni razvoj akvatičkih organizama (Kim i sur., 2020; Jiang i sur., 2019a). Međutim, informacije o djelovanju strobilurinskih fungicida na embrionalni razvoj organizama tla nisu bile dostupne. Tlo kao medij čini embriološka istraživanja složenima, a životni ciklusi organizama tla često su dugotrajni i nedovoljno istraženi. Tako je primjerice vrijeme potrebno za provođenje testova izlijeganja gujavica 60 do 70 dana (Bart i sur., 2018; Jensen i Holmstrup 1997). Iako postoje dokazi kako primjena pojedinih pesticida poput glifosata pri polovici preporučene doze može smanjiti stopu izlijeganja gujavica za 50% (Gaupp-Berghausen i sur., 2015) informacije o djelovanju strobilurinskih fungicida nisu dostupne. Međutim, kratak životni ciklus, visoka stopa reprodukcije i prozirni kokoni vrste *E. crypticus* (Castro-Ferreira i sur., 2012) omogućili su provođenje testova izlijeganja u puno kraćem vremenskom razdoblju. Naime, sam test izlijeganja traje 11 dana, dok je prije testa potrebno provesti sinkronizaciju organizama, čime se cjelokupno trajanje testa povećava na 14 dana (Bicho i sur., 2015). Osim toga, testove izlijeganja moguće je modificirati čime se omogućuje praćenje dinamiku izlijeganja. Nedostatak testova izlijeganja vrste *E. crypticus* je destruktivno uzorkovanje čime se stvara potreba za velikim brojem replika. Međutim, veličina kokona omogućava provođenje ovog testa u 5 g tla, dok visoka stopa reprodukcije vrste *E. crypticus* olakšava prikupljanje velikog broja sinkroniziranih kokona. Sinkronizacija kokona obavlja se na novim pločama agara na koje se prenose odrasle jedinke s vidljivim klitelumom. Prilikom sinkronizacije potrebno je prikupiti 2.5 puta više odraslih jedinki od željenog broja kokona, a sinkronizirani kokoni se kistom prenose u tlo (Bicho i sur., 2015). Zbog mogućnosti isušivanja kokona iznimno je važno voditi brigu o vlažnosti tla, te kokone u cijelosti prekriti

tlom za vrijeme izlaganja. Produljenje testa izlijeganja s 11 na 19 dana uz povećanje broja replika omogućilo je kontinuirano praćenje dinamike izlijeganja vrste *E. crypticus* i izračunavanje vremena potrebnog za izlijeganje. Koncentracije azoksistrobina na kojima je uočen utjecaj na odgodu izlijeganja bile su poprilično visoke i u normalnim okolnostima se ne očekuju u okolišu. Međutim, piraklostrobin je uzrokovao odgodu izlijeganja pri koncentracijama koje su okolišno relevantne (≤ 2.5 mga.i./kg). Prikupljeni rezultati pokazali su da embriotoksičnost strobilurinskih fungicida nije karakteristična samo za akvatičke, već i za organizme tla, a niske koncentracije strobilurinskih fungicida u okolišu mogu utjecati na dinamiku izlijeganja i posljedično djelovati na populacije enhitreja.

Postojeće informacije o djelovanju strobilurinskih fungicida uglavnom su rezultat ekotoksikoloških testova provedenih sa čistom aktivnom tvari, a ne komercijalnim pripravcima kojima su izloženi organizmi u okolišu. Osim toga djelovanje komercijalnih pripravaka često je snažnije od djelovanja čiste aktivne tvari (Mesnage i sur., 2014; Marques i sur., 2009). Upravo zbog toga naglašena je potreba za istraživanjima koja bi proučavala djelovanje različitih komercijalnih pripravaka ili usporedila djelovanje čiste aktivne tvari i komercijalnih pripravaka (Gomes i sur., 2021). Iako je vrsta *E. crypticus* zbog trajanja životnog ciklusa i specifičnosti kokona pogodna za provođenje testova preživljavanja, reprodukcijskih testova i testova izlijeganja, njen glavni nedostatak je veličina. Izrazito male i nježne jedinke čine mjerenje promjena aktivnosti MXR sustava dugotrajnima, a mjerenje različitih molekularnih biomarkera nemogućima zbog nedostatka potrebne količine tkiva. Iz tog razloga jedinke vrste *E. albidus* izložene su čistoj aktivnoj tvari azoksistrobin i komercijalnom pripravku temeljenom na istoj aktivnoj tvari. Kako bi se dobile što točnije informacije o djelovanju testiranih fungicida, mjerenja različitih biomarkera provedena su nakon 7 i 21 dana izlaganja. Prikupljeni rezultati pokazali su kako istraživanja toksičnosti čiste aktivne tvari nisu dostatna za procjenu okolišnog rizika jer se dodatcima u komercijalnim pripravcima bitno mijenjaju toksikološka svojstva aktivne tvari, potvrdivši šestu hipotezu rada. Iako je utjecaj na preživljavanje bio sličan, uočena je značajna razlika u djelovanju na reprodukciju. Naime, komercijalni pripravak pokazao je puno veću toksičnost i uzrokovao značajno smanjenje reprodukcije. Ovakva razlika u odgovoru povezuje se s dodatcima prisutnima u komercijalnom pripravku. Upravo dodatci mogu povećati bioraspoloživost aktivne tvari i omogućiti joj lakši prolazak kroz membranu kokona što rezultira većom toksičnosti za enhitreje (Mesnage i Antoniou, 2018; Pereira i sur., 2009). U prilog tome govori i odgovor MXR mehanizma enhitreja. Indukcija MXR mehanizma zabilježena nakon izlaganja čistoj aktivnoj tvari, a inhibicija nakon izlaganja komercijalnom

pripravku. Aktivnost MXR mehanizma izrazito je osjetljiva na koncentracije dostupnog supstrata, te niske koncentracije uzrokuju indukciju MXR mehanizma, dok pri višim koncentracijama dolazi do njegove inhibicije (Velki i Hackenberger, 2013a). Aktivna tvar se gotovo nikada ne primjenjuje kao pojedinačni pripravak, već se miješa s raznim dodatcima koji poboljšavaju njezina svojstva miješanja, razrjeđivanja, primjene i stabilnosti (Tominack, 2000). Iako sami dodatci zbog svoje koncentracije i svojstava ne uzrokuju toksične učinke, povećavaju bioraspoloživost aktivne tvari, njenu stabilnost i osiguravaju lakši ulazak u stanice organizama čime uzrokuju veću efikasnost, ali i snažnije negativno djelovanje (Nagy i sur., 2020). Osim odgovora MXR mehanizma, uočene su promjene u aktivnosti ključnih enzima antioksidativnog sustava, te promjene u količini dostupne energije. Mjerenje promjena aktivnosti enzima prve faze antioksidativnog sustava, SOD i CAT, pokazalo je njihovu izrazitu važnost nakon 7 dana izlaganja. Dok nakon 21 dana izlaganja, glavnu ulogu ima GST. Iako su istraživanja provedena na gujavicama *E. fetida* pokazala kako SOD i GST imaju ključnu ulogu u smanjenju negativnog djelovanja strobilurinskih fungicida (Xu i sur., 2021; Han i sur., 2014), istraživanje provedeno s vrstom *E. albidus* pokazalo je važna i uloga CAT u metabolizmu enhitreja. Nakon 21 dana izlaganja većina enzima antioksidativnog sustava jedinki izloženih čistoj aktivnoj tvari vratila se na kontrolnu razinu sugerirajući uspješno uklanjanje ROS-a. Međutim, tretman komercijalnim pripravkom pokazao je snažniji utjecaj i povećanu aktivnost GST i nakon 21 dana izlaganja. Osim toga uočena je povećana koncentracija MDA koja sugerira pojavu lipidne peroksidacije. Kao i kod gujavica, produkcija ROS-a nakon 7 dana izlaganja bila je nedovoljna za uzrokovanje lipidne peroksidacije, međutim nakon 21 dana došlo je do njene pojave. Izlaganje aktivnoj tvari i komercijalnom pripravku temeljeni na azoksistrobinu nije uzrokovalo promjene u količini dostupne energije, ali su uočene promjene u omjeru pojedinih komponenti (ugljikohidrati, lipidi i proteini). Intenzivna obrana od oksidativnog stresa uzrokovala je smanjenje koncentracije ugljikohidrata nakon 7 dana izlaganja. Upravo je redukcija ugljikohidrata, primarnog izvora energije (Moolman i sur., 2007), poznat odgovor enhitreja nakon izlaganja pesticidima (Novais i Amorim, 2013). Povećana koncentracija proteina nakon 21 dana izlaganja komercijalnom pripravku sugerira povećanu sintezu proteina kao odgovor na stresne uvijete (Tripathi i sur., 2010). Opaženi rezultati su osim potrebe za testiranjem komercijalnih pripravaka ukazali na važnost sveobuhvatne analize različitih krajnjih točaka u cilju dobivanja što detaljnijih, ali i relevantnijih rezultata.

Zbog razlike u djelovanju komercijalnog pripravka i čiste aktivne tvari, te u svrhu nadopunjavanja postojećih znanja provedeno je i četvrto istraživanje kojim je uspoređeno

djelovanje različitih komercijalnih pripravaka na vrstu *E. albidus*. Dobiveni rezultati pokazali su kako je za opažanja promjena krajnjih točaka na višim razinama organizacije, poput preživljavanja i reprodukcije, ponekad potrebno izlaganje izrazito visokim koncentracijama strobilurinskih fungicida. Međutim, niže koncentracije prisutne u okolišu mogu uzrokovati oksidativni stres i promjene energetskog statusa enhitreja. Na djelovanje komercijalnih pripravaka uvelike utječu promjene aktivnosti MXR mehanizma. Krezoksim-metil koji je pokazao najslabije djelovanje na preživljavanje i reprodukciju enhitreja, te ujedno izazvao najmanje promjene aktivnosti MXR mehanizma. Naime, pri tretmanima krezoksim-metilom inhibicija MXR mehanizma zabilježena je samo pri najvišoj testiranoj koncentraciji. Sukladno tome, piraklostrobin koji je pokazao najveće djelovanje na preživljavanje i reprodukciju uzrokovao je najsnažniju inhibiciju MXR mehanizma. Svi testirani komercijalni pripravci uzrokovali su pojavu oksidativnog stresa koja je karakterističan odgovor na strobilurinske fungicide kod organizama tla (Xu i sur., 2021; Wu i sur 2021). Osim toga duže izlaganje komercijalnim pripravcima (21 dan) uzrokuje promjene u količinama dostupne energije organizma.

Procjena djelovanja čiste aktivne tvari i različitih pripravaka strobilurinskih fungicida na aktivnosti MXR mehanizma, preživljavanje, reprodukciju, uspjeh izlijeganja, te oksidativni i energetski status dala je informacije o mogućim posljedicama izlaganja organizama različitim strobilurinskim fungicidima, ali i omogućila bolje razumijevanje mehanizma djelovanja čime je potvrđena i sedma hipoteza ovog rada. Iako izlaganje enhitreja okolišno relevantnim koncentracijama aktivne tvari ne bi uzrokovalo vidljive utjecaje na preživljavanje i reprodukciju, korištenje nižih koncentracija komercijalnih pripravaka i sveobuhvatnog pristupa spriječilo je moguće podcjenjivanje negativnog djelovanja ove skupine fungicida. Biomarkeri na nižim razinama organizacije nezaobilazan su i iznimno važan pristup u modernoj ekotoksikologiji, međutim i dalje se preispituje njihova uloga i točnost prilikom povezivanja s pojavama na višim razinama organizacije (Forbes 2006). Tako se krajnje točke poput preživljavanja i reprodukcije smatraju ekološki relevantnijima od biomarkera na nižim razinama organizacije. Međutim, odgovori na molekularnoj, staničnoj ili fiziološkoj razini ključni su za rasvjetljavanje točnog mehanizma djelovanja zagađivala (Shi i Wang, 2017). Stoga je prilikom istraživanja koja prikupljaju inicijalna znanja nužno provesti sveobuhvatnu analizu različitih krajnjih točaka i biomarkera. Sveobuhvatni pristup proveden tijekom izrade ove disertacije omogućio je detekciju najosjetljivijih točaka životnog ciklusa enhitreja, ali i ključnih mehanizama koji pomažu pri sprječavanju pojave negativnog djelovanja, čime je

potvrđena i posljednja hipoteza ovog rada. Dokazano je kako strobilurinski fungicidi prvenstveno izazivaju oksidativni stres. Oksidativi stres je najvjerovatnije posljedica djelovanja na mitohondrijsku respiraciju. Na taj način strobilurinski fungicidi utječu na transportni lanac elektrona, nastanak energije i posljedično povećanje brojnosti ROS-a. Osim toga, dokazano je kako MXR mehanizam ima iznimnu važnost pri sprječavanju štetnog djelovanja strobilurinskih fungicida. Naime, funkcionalni MXR mehanizam sprječava akumulaciju aktivne tvari unutar organizma i djelovanje na antioksidativni sustav. Antioksidativni sustav odraslih jedinki dobro je razvijen i može uspješno odolijevati nižim koncentracijama komercijalnih pripravaka strobilurinskih fungicida. Međutim, embrionalni razvoj pokazao se kao najosjetljivija točka životnog ciklusa enhitreja. Odrasle jedinke pokazale su izrazitu prilagodljivost na štetno djelovanje strobilurinskih fungicida. Tako se pri nekim tretmanima smanjenje dostupne energije kompenziralo smanjenjem ili potpunom odgodom reprodukcije, te usmjeravanjem energije na obranu od oksidativnog stresa. Iako se ovakva strategija pokazala uspješna za preživljavanje odraslih jedinki, dugoročno bi mogla predstavljati opasnost za stabilnost populacija. Na samo trajanje izlaganja u poljoprivrednim tlima utječe učestalost primjene, ali i mobilnost fungicida u tlu i njihovo ispiranje. Zbog slabe mobilnosti, azoksistrobin i krezoksim-metil ostaju u površinskim slojevima tla (Wu i sur., 2015), a slično je i s ostalim aktivnim tvarima. Nadalje, na mobilnost aktivne tvari dodatno utječe i vrsta pripravaka, odnosno pomoćne tvari koje se nalaze u pripravcima. Tako se zbog različitih dodataka EC pripravci brže ispiru iz tla nego SC pripravci (Khan i Brown, 2016). Zbog toga odabir aktivne tvari s najslabijim djelovanjem na neciljne organizme u kombinaciji s prikladnim pomoćnim tvarima predstavlja najbolje rješenje za stabilnost ekoloških sustava tla. Osim toga prilikom primjene komercijalnih pripravaka ključno je poštivanje preporučene doze i propisanih vremenske razmake prije ponovne primjene. Na taj način omogućuje se potpuna ili djelomična razgradnja aktivne tvari koja se već nalazi u tlu, ali i oporavak organizama nakon energetski zahtjevnog procesa obrane od oksidativnog stresa.

4. ZAKLJUČCI

- Prikupljeni rezultati predstavljaju prvi sveobuhvatni prikaz djelovanja strobilurinskih fungicida na enhitreje vrste *E. albidus* i *E. crypticus*.
- MXR mehanizam prisutan je kod enhitreja te je na njegovu aktivnost moguće utjecati modelnim inhibitorima (ciklosporin A, ivermektin i verapamil) i inducerima (deksametazon i rifampicin)
- Novoustanovljena metoda primjenjiva je u standardiziranim ekotoksikološkim testovima koji se provode u tlu te je uočena potreba za daljnjim istraživanjem MXR mehanizma enhitreja, ali i za korištenje promjene aktivnosti MXR mehanizma kao komparativnog biomarkera zagađenja.
- Izlaganje komercijalnom pripravku temeljenom na aktivnoj tvari propikonazol dovelo je do inhibicije MXR mehanizm vrsta *E. albidus* i *E. crypticus* dokazujući da ksenobiotici prisutni u okolišu mogu djelovati na aktivnost MXR mehanizma enhitreja.
- Komercijalni pripravak temeljen na aktivnoj tvari trifloksistrobin pokazao je snažniji utjecaj na preživljavanje i reprodukciju vrste *E. crypticus* u usporedbi s komercijalnim pripravcima temeljenima na aktivnoj tvari piraklostrobin i azoksistrobin.
- Primjena produljenog testa izlijeganja omogućila je razlikovanje odgode izlijeganja i njegova potpuna izostanka naglašavajući potrebu za istraživanjem djelovanja strobilurinskih fungicida na nižim razinama organizacije (biokemijski i stanični biomarkeri).
- Azoksistrobin u obliku čiste aktivne tvari i komercijalnog pripravka može uzrokovati promjene u aktivnosti MXR mehanizma, te izaziva oksidativni stres i promjene u količinama dostupne energije. Međutim,
- Veća dostupnost azoksistrobina u obliku komercijalnog pripravka u usporedbi s čistom aktivnom tvari prouzročila je inhibiciju aktivnosti MXR mehanizma enhitreja vrste *E. albidus* te indukciju oksidativnog stresa i promjenu količine dostupne energije. Negativan utjecaj pripravaka zabilježen je na okolišno relevantnim koncentracijama, što predstavlja opasnost za populacije enhitreja u poljoprivrednim tlima.
- Najveći utjecaj na reprodukciju vrste *E. albidus* pokazao je pripravak temeljen na aktivnoj tvari piraklostrobin, nakon koje slijede pripravci temeljeni na aktivnim tvarima trifloksistrobinu i krezoksim-metilu. Važnu ulogu u smanjenju štetnih utjecaja igraju MXR mehanizam i detoksikacijski sustav čija aktivnost može utjecati na ublažavanje štetnih posljedica na višim razinama organizacije.
-

- Svi testirani fungicidi djeluju na mitohondrijski respiratorni lanac smanjujući količinu dostupne energije u organizmu, te uzrokuju povećanje koncentracije ROS-a i posljedično pojavu oksidativnog stresa. Iako neki komercijalni pripravci nisu negativno utjecali na krajnje točke poput preživljavanja i reprodukcije, povećana aktivnost enzima koji sudjeluju u detoksikacijskom procesu i obrani organizma od oksidativnog stresa upućuje na izuzetnu važnost ovog mehanizma prilikom izloženosti strobilurinskim fungicidima.
- MXR mehanizam i detoksikacijski sustav dobro su razvijeni kod odraslih jedinki te igraju važnu ulogu u prevenciji štetnih posljedicama na višim razinama organizacije. Međutim, embrionalni razvoj se pokazao kao najosjetljivija točka životnog ciklusa enhitreja. Naime, dok je inhibicija reprodukcije opažena pri višim koncentracijama, niže koncentracije uzrokovale su negativno djelovanje na embrionalni razvoj koje je rezultiralo odgodom izlijeganja ili njegovim potpunim izostankom.
- Kako bi se spriječile dugoročne posljedice na populacije enhitreja i čitave ekološke sustave tla potrebno je odabrati komercijalne pripravke koji će pri okolišno relevantnim koncentracijama imati minimalno djelovanje na enhitreje, te ih primjenjivati u propisanim razmacima kako bi se omogućio oporavak organizama prije ponovne primjene.

5. LITERATURA

Abreu IA, Cabelli DE (2010) Superoxide dismutases-a review of the metal-associated mechanistic variations. Biochimica et Biophysica Acta 1804: 263-274.

Ács A, Imre K, Kiss G, Csaba J, Győri J, Vehovszky Á, Farkas A (2015) Evaluation of multixenobiotic resistance in dreissenid mussels as a screening tool for toxicity in freshwater sediments. Archives of Environmental Contamination and Toxicology 68(4):707-717.

Aebi H (1974) Catalase. In Methods of enzymatic analysis (pp. 673-684). Academic press.

Amorim MJB, Gomes SIL, Soares AMVM, Scott-Fordsmand JJ (2012) Energy basal levels and allocation among lipids, proteins, and carbohydrate in *Enchytraeus albidus*: Changes related to exposure to Cu-salt and Cu-nanoparticles. Water Air and Soil Pollution 223: 477–482.

Amorim MJB, Römbke J, Schallnass HJ, Soares AMVM (2005) Effect of soil properties and aging on the toxicity of copper for *Enchytraeus albidus*, *Enchytraeus luxuriosus*, and *Folsomia candida*. Environmental Toxicology and Chemistry 24: 1875–1885.

Amorim MJB, Scott-Forsmand JJ (2021) Plastic pollution - A case study with *Enchytraeus crypticus* – From micro - to nanoplastics. Environmental Pollution 271: 116363. https://doi.org/10.1016/j.envpol.2020.116363

Amorim MJB, Sousa JP, Nogueira AJ, Soares AMVM (2002) Bioaccumulation and elimination of 14C-lindane by *Enchytraeus albidus* in artificial (OECD) and a natural soil. Chemosphere 49: 323–329

Anselmo HMR, Van Den Berg JHJ, Rietjens IMCM, Murk ATJ (2012) Inhibition of cellular efflux pumps involved in multi xenobiotic resistance (MXR) in echinoid larvae as a possible mode of action for increased ecotoxicological risk of mixtures. Ecotoxicology 21, 2276e2287. https://doi.org/10.1007/s10646-012-0984-2

Balba H (2007) Review of strobilurin fungicide chemicals. Journal of Environmental Science and Health, Part B 42: 441e451.

Bardgett RD (2005) The Biology of Soil. Oxford University Press Inc., New York

Bart S, Amossé J, Lowe CN, Mougin C, Péry AR, Pelosi C (2018) *Aporrectodea caliginosa*, a relevant earthworm species for a posteriori pesticide risk assessment: current knowledge and recommendations for culture and experimental design. Environmental Science and Pollution Research, 25(34): 33867-33881.

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B (2002) The strobilurin fungicides. Pest Management Science 58: 649e662.

Bicho R, Santos F, Scott-Fordsmand J, Amorim M (2017a) Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in *Enchytraeus crypticus*. Environmental Pollution 224: 117–124.

Bicho R, Santos F, Scott-Fordsmand JJ, Amorim M (2017b) Multigenerational effects of copper nanomaterials (CuONMs) are different of those of CuCl2: exposure in the soil invertebrate *Enchytraeus crypticus*. Scientific Reports 7: 1–7.

Bicho RC, Faustino AMR, Rêma A, Scott-Fordsmand JJ, Amorim MJB (2021) Confirmatory assays for transient changes of omics in soil invertebrates – copper materials in a multigenerational exposure. Journal of Hazard Materials 402: 123500.

Bicho RC, Ribeiro T, Rodrigues NP, Scott-Fordsmand JJ, Amorim MJBB (2016) Effects of Ag nanomaterials (NM300K) and Ag salt (AgNO3) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*. Journal of Hazard Materials 318: 608–614.

Bicho RC, Santos FCF, Gonçalves MFM, Soares AMVM, Amorim MJB (2015) Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test—an optional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology 24 (5): 1053–1063.

Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O (2012) Oxidative stress and antioxidant defense. World Allergy Organization Journal 5 (1): 9-19.

Bošnjak I, Bielen A, Babić S, Šver L, Popović NT, Strunjak-Perović I, Čož-Rakovac R, Klobučar RS (2014) First evidence of the P-glycoprotein gene expression and multixenobiotic resistance modulation in earthworm. Arhiva Higijene Rada 65: 67e75. https://doi.org/10.2478/10004-1254-65-2014-2421.

Boxall AB, Hardy A, Beulke S, Boucard T, Burgin L, Falloon PD, Haygarth PM, Hutchinson T, Kovats RS, Leonardi G, Levy LS, Nichols G, Parsons SA, Potts L, Stone D, Topp E, Turley DB, Walsh K, Wellington EM, Williams RJ (2009) Impacts of climate change on indirect human exposure to pathogens and chemicals from agriculture. Environmental Health Perspectives 117(4): 508−514.

Briones MJI (2014) Soil fauna and soil functions: a jigsaw puzzle. Frontiers in Environmental Science 2. https://doi.org/10.3389/fenvs.2014.00007

Cao F, Martyniuk CJ, Wu PZ, Zhao F, Pang S, Wang CJ, Qiu LH (2019) Long-term exposure to environmental concentrations of azoxystrobin delays sexual development and alters reproduction in zebrafish (*Danio rerio*). Environmental Science & Technology 53: 1672e1679.

Cao F, Wu P, Huang L, Li H, Qian L, Pang S, Qiu L (2018) Short-term developmental effects and potential mechanisms of azoxystrobin in larval and adult zebrafish (*Danio rerio*). Aquatic Toxicology 198: 129e140.

Cao F, Zhu L, Li H, Yu S, Wang C, Qiu L (2016) Reproductive toxicity of azoxystrobin to adult zebrafish (*Danio rerio*). Environmental Pollution 219: 1109e1121.

Castro-Ferreira MP, Roelofs D, van Gestel CAM, Verweij RA, Soares AMVM, Amorim MJB (2012) *Enchytraeus crypticus* as model species in soil ecotoxicology. Chemosphere 87: 1222–1227.

Chelinho S, Domene X, Campana P, Andrés P, Römbke J, Sousa JP (2014) Toxicity of phenmedipham and carbendazim to *Enchytraeus crypticus* and *Eisenia andrei* (Oligochaeta) in Mediterranean soils. Journal of soils and sediments 14(3): 584-599.

Christensen B, Dózsa-Farkas K (2006) Invasion of terrestrial enchytraeids into two postglacial tundras: North-eastern Greenland and the Arctic Archipelago of Canada (Enchytraeidae, Oligochaeta). Polar Biol. 29, 454–466. 444

Chu X, Korzekwa K, Elsby R, Fenner K, Galetin A, Lai Y (2013) Intracellular drug concentrations and transporters: measurement modelling and implications for the liver. Nature 94: 126e141.

Clark A, Shamaan N, Sinclair M, Deuterman W (1986) Insecticide metabolism by multitple glutathione S-transferase in two strains of the house fly, *Musca domestica* (L). Pesticide Biochemistry Physiology 25: 169-175.

Cooper EM, Rushing R, Hoffman K, Phillips AL, Hammel SC, Zylka MJ, Stapleton HM (2020) Strobilurin fungicides in house dust: is wallboard a source? Journal of exposure science & environmental epidemiology 30(2): 247–252, PMID: 31636368.

Cui F, Chai T, Liu X, Wang C (2017) Toxicity of three strobilurins (kresoxim-methyl, pyraclostrobin, and trifloxystrobin) on *Daphnia magna*. Environmental Toxicology and Chemistry 36: 182e189.

Cunha V, Burkhardt-Medicke K, Wellner P, Santos MM, Moradas-Ferreira P, Luckenbach T, Ferreira M (2017) Effects of pharmaceuticals and personal care products (PPCPs) on multixenobiotic resistance (MXR) related efflux transporter activity in zebrafish (*Danio rerio*) embryos. Ecotoxicology and Environmental Safety 136: 14e23.

Cush R (2006) Back to basics: A review of pesticide formulation types. Golf Course Manag, 74(1): 143-145.

da Rocha AG, Pitombo LM, Bresolin JD, da Silva WTL, Espindola ELG, de Menezes Oliveira VB (2020) Single and combined toxicity of the pesticides abamectin and difenoconazole on soil microbial activity and *Enchytraeus crypticus* population. SN Applied Sciences 2(8): 1-10.

De Coen W, Janssen CR (1997) The use of biomarkers in *Daphnia magna* toxicity testing IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. Journal of Aquatic Ecosystem Stress and Recovery 6: 43–55.

de Coen WM, Janssen CR (2003) The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant - stressed *Daphnia magna* and corresponding population characteristics. Environmental Toxicology and Chemistry 22:1632–41.

Depledge MH, Fossi MC (1994) The role of biomarkers in environmental assessment (2). Invertebrates. Ecotoxicology 3:161–72.

Didden W (1991) Population ecology and functioning of Enchytraeidae in some arable farming systems. Laboratory of Soil Science and Geology. Wageningen University, pp. 57–61.

Didden W, Römbke J (2001) Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotoxicology and Environmental Safety 50: 25e43.

Druart C, Scheifler R, Millet M, De Vaufleury A (2012) Landsnail eggs bioassays: A new tool to assess embryotoxicity of contaminants in the solid, liquid or gaseous phase of soil. Applied soil ecology, 53:56-64.

Duryee MJ, Klassen LW, Schaffert CS, Tuma DJ, Hunter CD, Garvin RP, Anderson DR, Thiele GM (2010) Malondialdehyde-acetaldehyde adduct is the dominant epitope after MDA modification of proteins in atherosclerosis. Free Radical Biology and Medicine 49 (10): 1480–1486.

Epel D, Luckenbach T, Stevenson CN, Macmanus-Spencer LA, Hamdoun A, Smital T (2008) Efflux transporters: newly appreciated roles in protection against pollutants. Environmental Science & Technology 42: 3914e3920.

Eurostat Pesticide sales (2022) http://appsso.eurostat.ec.europa.eu/

Falfushynska H, Sokolov EP, Haider F, Oppermann C, Kragl U, Ruth W, Stock M, Glufke S, Winkel EJ, Sokolova IM (2019) Effects of a common pharmaceutical, atorvastatin, on energy metabolism and detoxification mechanisms of a marine bivalve *Mytilus edulis*. Aquatic Toxicology 208: 47e61.

Faria M, Navarro A, Luckenbach T, Pina B, Barata C (2011) Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants. Aquatic Toxicology 101: 78e87.

Feng Y, Huang Y, Zhan H, Bhatt P, Chen S (2020) An overview of strobilurin fungicide degradation: current status and future perspective. Frontiers in Microbiology 11:389.

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484(7393): 186−194.

Flury M (1996) Experimental evidence of transport of pesticides through field soils – a review. Journal of Environmental Quality 25: 24–45.

Forbes VE, Palmqvist A, Bach L (2006) The use and misuse of biomarkers in ecotoxicology. Environ Toxicol Chem 25:272–280.

Franzellitti S, Striano T, Pretolani F, Fabbri E (2017) Investigating appearance and regulation of the MXR phenotype in early embryo stages of the Mediterranean mussel (*Mytilus galloprovincialis*). Comparative Biochemistry and Physiology, part: C; Toxicology and Pharmacology 199: 1e10.

Gaupp-Berghausen M, Hofer M, Rewald B, Zaller JG (2015) Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. Scientific Reports 5:12886.

Gomes SI, Gonçalves MF, Bicho RC, Roca CP, Soares AM, Scott-Fordsmand JJ, Amorim MJ (2018) High-throughput gene expression in soil invertebrate embryos–Mechanisms of Cd toxicity in *Enchytraeus crypticus*. Chemosphere 212: 87-94.

Gomes SI, Novais SC, Gravato C, Guilhermino L, Scott-Fordsmand JJ, Soares AM, Amorim MJ (2012) Effect of Cu-nanoparticles versus one Cu-salt: analysis of stress biomarkers response in *Enchytraeus albidus* (Oligochaeta). Nanotoxicology 6(2): 134-143.

Gomes SI, Soares AM, Scott-Fordsmand JJ, Amorim MJ (2013) Mechanisms of response to silver nanoparticles on *Enchytraeus albidus* (Oligochaeta): survival, reproduction and gene expression profile. Journal of hazardous materials, 254, 336-344.

Gomes SIL, Ammendola A, Casini S, Amorim MJB (2021) Toxicity of fungicides to terrestrial non-target fauna – Formulated products versus active ingredients (azoxystrobin, cyproconazole,

prothioconazole, tebuconazole) – A case study with *Enchytraeus crypticus* (Oligochaeta). Science of the Total Environment 754: 142098.

Gomes SIL, Scott-Fordsmand JJ, Amorim MJB (2015b) Cellular energy allocation to assess the impact of nanomaterials on soil invertebrates (Enchytraeids): the effect of Cu and Ag. International Journal of Environmental Research and Public Health 12: 6858–6878.

Gomes SIL, Soares AMVM, Amorim MJB (2015a) Changes in cellular energy allocation in *Enchytraeus crypticus* exposed to copper and silver — linkage to effects at higher level (reproduction). Environmental Science and Pollution Research International 22: 14241–14247.

Gonçalves MFM, Bicho R, Rêma A, Soares AMVM, Faustino AMR, Amorim MJB (2015) Development of an embryotoxicity test for *Enchytraeus crypticus* – the effect of Cd. Chemosphere139: 386–392.

Gonçalves MFM, Gomes SIL, Scott-Fordsmand JJ, Amorim MJB (2017) Shorter lifetime of a soil invertebrate species when exposed to copper oxide nanoparticles in a full lifespan exposure test. Scientific Reports 7: 1–8.

Hackenberger BK, Velki M, Stepić S, Hackenberger DK (2012) First evidence for the presence of efflux pump in the earthworm *Eisenia andrei*. Ecotoxicology and Environmental Safety 75: 40e45.

Hakala K, Hannukkala AO, Huusela-Veistola E, Jalli M, Peltonen-Sainio P (2011) Pests and diseases in a changing climate: a major challenge for Finnish crop production. Agricultural and Food Science 20: 3−14.

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. 2nd ed. Oxford: Clarendon Press; pp. 543.

Han Y, Liu T, Wang J, Wang J, Zhang C, Zhu L (2016) Genotoxicity and oxidative stress induced by the fungicide azoxystrobin in zebrafish (*Danio rerio*) livers. Pesticide Biochemistry and Physiology 133: 13e19.

Han Y, Zhu L, Wang Jinhua, Wang Jun, Xie H, Zhang S (2014) Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin. Ecotoxicology and Environmental Safety 107: 214–219.

Hartman EA, Belden JB, Smith LM, McMurry ST (2014) Chronic effects of strobilurin fungicides on development, growth, and mortality of larval Great Plains toads (*Bufo cognatus*). Ecotoxicology 23: 396e403.

Hou K, Shi B, Liu Y, Lu C, Li D, Du Z, Li B, Zhu L (2022). Toxicity evaluation of pyraclostrobin exposure in farmland soils and co-exposure with nZnO to *Eisenia fetida*. Journal of Hazardous Materials, 433, 128794.

Howcroft CF, Amorim MJB, Gravato C, Guilhermino L, Soares AMVM (2009) Effects of natural and chemical stressors on *Enchytraeus albidus*: can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environment International 35: 318–324.

Howcroft CF, Gravato C, Amorim MJB, Novais SC, Soares AMVM, Guilhermino L (2011) Biochemical characterization of cholinesterases in *Enchytraeus albidus* and assessment of in vivo and in vitro effects of different soil properties, copper and phenmedipham. Ecotoxicology 20: 119–130.

Ighodaro OM, Akinloye OA (2018) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine 54: 287–293.

Iglesias J, Castillejo J, Ester A, Castro R, Lombardia MJ (2002) Susceptibility of the eggs of the field slug *Deroceras reticulatum* to contact with pesticides and substances of biological origin on artificial soil. Annals of Applied Biology 140:53–59.

Iglesias J, Castillejo J, Parama R, Mascato R, Lombardia MJ (2000) Susceptibility of the eggs of the pest slug *Deroceras reticulatum* to contact with metal salts. Journal of Molluscan Studie. 66:171–176.

Jarratt N, Thompson H (2009) A report to EFSA: comparison between the sensitivity of enchytraeids and Lumbricidae to chemicals, in particular plant protection products. The European Food Safety Authority 1–196.

Jensen KS, Holmstrup M (1997) Estimation of earthworm cocoon development time and its use in studies of in situ reproduction rates. Applied Soil Ecology 7(1):73–82.

Jiang JH, Lv L, Wu SG, An XH, Wang FD, Liu XJ, Zhao XP (2019b) Developmental toxicity of kresoxim-methyl during zebrafish (*Danio rerio*) larval development. Chemosphere 219: 517e525.

Jiang JH, Shi Y, Yu RX, Chen LP, Zhao XP (2018) Biological response of zebrafish after short-term exposure to azoxystrobin. Chemosphere 202: 56e64.

Jiang JH, Wu SG, Lv L, Liu XJ, Chen LZ, Zhao XP, Wang Q (2019a) Mitochondrial dysfunction, apoptosis and transcriptomic alterations induced by four strobilurins in zebrafish (*Danio rerio*) early life stages. Environmental Pollution 253: 722e730.

Joshi J, Sharma S, Guruprasad KN (2014) Foliar application of pyraclostrobin fungicide enhances the growth, rhizobial-nodule formation and nitrogenase activity in soybean (var. JS-335). Pesticide Biochemistry and Physiology 114: 61e66.

Junges CM, Peltzer PM, Lajmanovich RC, Attademo AM, Zenklusen MC, Basso A (2012) Toxicity of the fungicide trifloxystrobin on tadpoles and its effect on fish-tadpole interaction. Chemosphere 87:1348e1354.

Katsumiti A, Thorley AJ, Arostegui I, Reip P, Valsami-Jones E, Tetley TD, Cajaraville MP (2018) Cytotoxicity and cellular mechanisms of toxicity of CuONPs in mussel cells in vitro and comparative sensitivity with human cells. Toxicology in Vitro 48: 146e158.

Khan MA, Brown CD (2016) Influence of commercial formulation on leaching of four pesticides through soil. Science of the Total Environment 573: 1573-1579.

Kim C, Choe H, Park J, Kim G, Kim K, Jeon HJ, Moon JK, Kim MJ, Lee SE (2020) Molecular mechanisms of developmental toxicities of azoxystrobin and pyraclostrobin toward zebrafish (*Danio rerio*) embryos: visualization of abnormal development using two transgenic lines. Environmental Pollution 270: 116087.

Kim JH, Campbell BC, Mahoney N, Chan KL, Molyneux RJ, May GS (2007) Enhanced activity of strobilurin and fludioxonil by using berberine and phenolic compounds to target fungal antioxidative stress response. Letters in Applied Microbiology 45: 134e141.

Kobetičová K, Hofman J, Holoubek I (2009) Avoidance response of *Enchytraeus albidus* in relation to carbendazim ageing. Environmental Pollution 157(2): 704-706.

Kumar N, Willis A, Satbhai K, Ramalingam L, Schmitt C, Moustaid-Moussa N, Crago J (2020) Developmental toxicity in embryo-larval zebrafish (*Danio rerio*) exposed to strobilurin fungicides (azoxystrobin and pyraclostrobin). Chemosphere 241: 124980.

Kuperman RG, Amorim MJB, Römbke J, Lanno R, Checkai RT, Dodard SG, Sunahara GI, Scheffczyk A (2006) Adaptation of the enchytraeid toxicity test for use with natural soil types. European Journal of Soil Biology 42: S234–S243.

Kurelec B (1992) The multixenobiotic resistance mechanism in aquatic organisms. Critical Reviews in Toxicology 22: 23e43. https://doi.org/10.3109/10408449209145320.

Kurelec B (1997) A new type of hazardous chemical: the chemosensitizers of multixenobiotic resistance. Environmental Health Perspectives 105: 855e860.

Leitão S, Cerejeira MJ, Van den Brink PJ, Sousa JP (2014) Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. Applied Soil Ecology 76: 124–131.

Leslie EM, Deeley RG, Cole SPC (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicology and Applied Pharmacology 204 (3): 216e237.

Li H, Cao FJ, Zhao F, Yang Y, Teng MM, Wang CJ, Qiu LH (2018) Develop-mental toxicity, oxidative stress and immunotoxicity induced by three strobilurins (pyraclostrobin, trifloxystrobin and picoxystrobin) in zebrafish embryos. Chemosphere 207: 781e790.

Li H, Jing T, Li T, Huang X, Gao Y, Zhu J, Lin J, Zhang P, Li B, Mu W (2021) Ecotoxicological effects of pyraclostrobin on tilapia (*Oreochromis niloticus*) via various exposure routes. Environmental Pollution 285:117188.

Li H, Yu S, Cao F, Wang C, Zheng M, Li X, Qiu L (2018) Developmental toxicity and potential mechanisms of pyraoxystrobin to zebrafish (*Danio rerio*). Ecotoxicology and Environmental Safety 151: 1e9.

Li H, Zhao F, Cao FJ, Teng MM, Yang Y, Qiu LH (2019) Mitochondrial dysfunction-based cardiotoxicity and neurotoxicity induced by pyraclostrobin in zebrafish larvae. Environmental Pollution 251: 203e211.

Li JZ, Wu X, Hu JY (2006) Determination of fungicide kresoxim-methyl residues in cucumber and soil by capillary gas chromatography with nitrogen-phosphorus detection. Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes 41(4): 427-36.

Licht A, Schneider E (2011) ATP binding cassette systems: structures, mechanisms, and functions. Central European Journal of Biology 6: 785e801.

Liu L, Jiang C, Wu ZQ, Gong YX, Wang GX (2013) Toxic effects of three strobilurins (trifloxystrobin, azoxystrobin and kresoxim-methyl) on mRNA expression and antioxidant enzymes in grass carp (*Ctenopharyngodon idella*) juveniles. Ecotoxicology and Environmental Safety 98:297e302.

Liu T, Liu Y, Fang K, Zhang X, Wang X (2020) Transcriptome, bioaccumulation and toxicity analyses of earthworms (*Eisenia fetida*) affected by trifloxystrobin and trifloxystrobin acid. Environmental Pollution 265:115100.

Lock K, Janssen CR (2001) Tolerance changes of the potworm *Enchytraeus albidus* after long-term exposure to cadmium. Science of the total environment 280(1-3): 79-84.

Lock K, Janssen CR (2002a) Ecotoxicity of nickel to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. Chemosphere, 46(2): 197-200.

Lock K, Janssen CR (2002b) Multi-generation toxicity of zinc, cadmium, copper and lead to the potworm *Enchytraeus albidus*. Environmental Pollution 117: 89–92.

Lock K, Janssen CR (2003) Influence of aging on metal availability in soils. Reviews of Environmental Contamination and Toxicology 178: 1–21.

Lopes BCH, Zeppelini D, Pontes DS, Amado EM (2021) First characterization of multixenobiotic activity in Collembola: An approach on cadmium-induced response. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 239: 108850.

Lu B (2018) Azoxystrobin and pyraclostrobin dominate the market of Strobilurin fungicides. Shangdong Pesticide News 4: 24e30.

Luz AL, Kassotis CD, Stapleton HM, Meyer JN (2018) The high-production volume fungicide pyraclostrobin induces triglyceride accumulation associated with mitochondrial dysfunction, and promotes adipocyte differentiation independent of PPARγ activation, in 3T3-L1 cells. Toxicology 393: 150e159.

Ma J, Cheng C, Du Z, Li B, Wang Jinhua, Wang, Jun, Wang Z, Zhu L (2019) Toxicological effects of pyraclostrobin on the antioxidant defense system and DNA damage in earthworms (*Eisenia fetida*). Ecological Indicators 101:111–116.

Mao LG, Jia W, Zhang L, Zhang YN, Zhu LZ, Sial MU, Jiang HY (2020) Embryonic development and oxidative stress effects in the larvae and adult fish livers of zebrafish (*Danio rerio*) exposed to the strobilurin fungicides, kresoxim-methyl and pyraclostrobin. Science of the Total Environment 729: 139031.

Maria VL, Ribeiro MJ, Guilherme S, Soares AM, Scott-Fordsmand JJ, Amorim MJ (2018) Silver (nano) materials cause genotoxicity in *Enchytraeus crypticus*, as determined by the comet assay. Environmental toxicology and chemistry 37(1): 184-191.

Marques C, Pereira R, Gonçalves F (2009) Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. Journal of Soils and Sediments 9: 137–147.

Matković K (2016) Analiza primjene fungicida i insekticida u Osječko-baranjskoj županiji. Završni specijalistički rad. Sveučilište Josipa Jurja Strossmayera u Osijeku, Fakultet agrobiotehničkih znanosti Osijek https://urn.nsk.hr/urn:nbn:hr:151:965063

Mazur CS, Marchitti SA, Zastre J (2015) P-glycoprotein inhibition by the agricultural pesticide propiconazole and its hydroxylated metabolites: implications for pesticide-drug interactions. Toxicology Letters 232:37e45.

McDougall P (2015) AgriService: Products Section, 2014 Market, p. 220

Međunarodna organizacija za standardizaciju (ISO - International Organization for Standardization ISO) (2008) Avoidance Test for Testing the Quality of Soils and Effects of Chemicals on Behaviour — Part 1: Test with Earthworms (*Eisenia fetida* and *Eisenia andrei*). Dostupno na: https://www.iso.org/obp/ui/#iso: std:38402:en.

Međunarodna organizacija za standardizaciju (ISO - International Organization for Standardization ISO) (2014) Soil Quality - Effects of Contaminants on Enchytraeidae (*Enchytraeus* sp.) - Determination of Effects on Reproduction. Geneva.

Mendes LA, Amorim MJB, Scott-Fordsmand JJ (2018) Interactions of soil species exposed to CuO NMs are different from Cu salt: a multispecies test. Environmental Science & Technology 52: 4413–4421.

Mendes LA, Amorim MJB, Scott-Fordsmand JJ (2019) Assessing the toxicity of safer by design CuO surface-modifications using terrestrial multispecies assays. Science of the Total Environment 678: 457–465.

Mendonça MCP, de Jesus MB, van Gestel CA (2020). Protective effect of N-acetylcysteine on the toxicity of silver nanoparticles: Bioavailability and toxicokinetics in *Enchytraeus crypticus*. Science of the Total Environment, 715, 136797.

Menezes-Oliveira VB, Scott-Fordsmand JJ, Soares AMVM, Amorim MJB (2013) Effects of temperature and copper pollution on soil community—extreme temperature events can lead to community extinction: effect of temperature and copper pollution on soil community. Environmental Toxicology and Chemistry 32: 2678–2685.

Menezes-Oliveira VBB, Scott-Fordsmand JJ, Soares AMVM, Amorim MJB (2014) Development of ecosystems to climate change and the interaction with pollution—unpredictable changes in community structures. Applied Soil Ecology 75: 24–32.

Menta C. (2012). Soil fauna diversity-function, soil degradation, biological indices, soil restoration. Biodiversity conservation and utilization in a diverse world 59-94.

Mesnage R, Antoniou MN (2018) Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Public Health Front. 5, 361.

Mesnage R, Defarge N, Spiroux de Vendômois J, Séralini G-E (2014) Major pesticides are more toxic to human cells than their declared active principles. BioMed Research International 1–8.

Ministarstvo poljoprivrede (2022) - https://fis.mps.hr/trazilicaszb/ pristupljeno svibanj 2022.

Moolman LJHJ, Van Vuren JHJ, Wepener V (2007) Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. Ecotoxicology and Environmental Safety, 68(3):443-450.

Nagy K, Duca RC, Lovas S, Creta M, Scheepers PT, Godderis L, Ádám B (2020) Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. Environmental Research 181:108926.

Nnadi NE, Carter DA (2021) Climate change and the emergence of fungal pathogens. PLoS Pathogens 17(4):e1009503.

Novais SC, Amorim MJ (2013) Changes in cellular energy allocation in *Enchytraeus albidus* when exposed to dimethoate, atrazine, and carbendazim. Environmental toxicology and chemistry 32(12): 2800-2807.

Novais SC, Gomes NC, Soares AM, Amorim MJ (2014) Antioxidant and neurotoxicity markers in the model organism *Enchytraeus albidus* (Oligochaeta): mechanisms of response to atrazine, dimethoate and carbendazim. Ecotoxicology 23(7): 1220-1233.

Novais SC, Gomes SI, Gravato C, Guilhermino L, De Coen W, Soares AM, Amorim MJ (2011) Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environmental Pollution 159(7): 1836-1843.

Novais SC, Soares AM, Amorim MJ (2010) *Enchytraeus albidus* (Oligochaeta) exposed to several toxicants: effects on survival, reproduction and avoidance behaviour. Interdisciplinary Studies on Environmental Chemistry—Biological Responses to Contaminants 237-242.

Novais SC, Soares AMVM, de Coen W, Amorim MJB (2013) Exposure of *Enchytraeus albidus* to Cd and Zn—Changes in cellular energy allocation (CEA) and linkage to transcriptional, enzymatic and reproductive effects. Chemosphere 90: 1305–1309.

Organizacija za ekonomsku suradnju i razvoj (Organisation for Economic Cooperation and Development - OECD) (1984) Test broj 207: Acute Toxicity Tests. Guideline for Testing Chemicals. OECD Publishing, Pariz, Francuska

Organizacija za ekonomsku suradnju i razvoj (Organisation for Economic Cooperation and Development - OECD) (2010) Test broj 317: Bioaccumulation in Terrestrial Oligochaetes, OECD Guidelines for the Testing of Chemicals, Section 3. OECD Publishing, Pariz, Francuska

Organizacija za ekonomsku suradnju i razvoj (Organisation for Economic Cooperation and Development - OECD) (2016) Test broj 220: Enchytraeid reproduction test. OECD Guidelines for Testing of Chemicals. OECD Publishing, Pariz, Francuska.

Palmeira CM (1999) Herbicide-induced mitochondrial and cellular toxicity: A review of paraquat, dinoseb and 2,4-D effects. Toxic Substances Mechanisms, 18: 187 – 204.

Patrício Silva AL, Holsmtrup M, Amorim MJB (2013) Soil salinity increases survival of freezing in the enchytraeid *Enchytraeus albidus*. The Journal of Experimental Biology 216: 2732–2740.

Pereira R (2009) Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: Commercial formulation versus active ingredient. Ecotoxicology 18: 455–463.

Puurtinen HM, Martikainen EAT (1997) Effect of soil moisture on pesticide toxicity to an enchytraeid worm, Enchytraeus sp. Archives of environmental contamination and toxicology 33(1): 34-41.

R Studio Team (2022) RStudio: Integrated Development for R. RStudio, Inc., Boston, MA http://www.rstudio.com.

RCore Team (2022) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.

Ribeiro MJ, Maria VL, Scott-Fordsmand JJ, Amorim MJ (2015) Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those of AgNO3: effects in the soil invertebrate *Enchytraeus crypticus*. International journal of environmental research and public health, 12(8), 9589-9602.

Ribeiro MJ, Scott-Fordsmand JJ, Amorim MJB (2019) Multigenerational exposure to cobalt (CoCl2) and WCCo nanoparticles in *Enchytraeus crypticus*. Nanotoxicology 13: 751–760.

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLOs ONE 598 (10), e0146021.

Rodrigues ET, Lopes I, Pardal MA (2013) Occurrence, fate and effects of azoxystrobin in aquatic ecosystems: a review. Environment International 53:18–28.

Römbke J (1988) Die Enchytraeen eines Moderbuchenwaldes. Ihre Rollebeim Streuabbau und ihre Reaktion auf Umweltbelastungen. Diss.,Frankfurt an Main.

Römbke J (2003) Ecotoxicological laboratory tests with enchytraeids: a review. Pedobiologia 47, 607–616.

Römbke J, Knacker T (1989) Aquatic toxicity test for enchytraeids. Hydrobiologia 180: 235-242.

Römbke J, Moser T (1999) Organisation and performance of an international ring test for the validation of the enchytraeid reproduction test. Vols. I and II.; BA-πexte4/99.

Römbke J, Moser T (2002) Validating the enchytraeid reproduction test: organisation and results of an international ring test. Chemosphere 46 (7): 1117-40.

Roßberg D (2009) NEPTUN - Weinbau. Berichte aus der BBA, 151.

Santos FCF, Gomes SIL, Scott-Fordsmand JJ, Amorim MJB (2017) Hazard assessment of nickel nanoparticles in soil—the use of a full life cycle test with *Enchytraeus crypticus*. Environmental Toxicology and Chemistry 36: 2934–2941.

Sauerborn Klobučar R, Žaja R, Franjević D, Brozović A, Smital T (2010) Presence of ecotoxicologically relevant Pgp and MRP transcripts and proteins in Cyprinid fish. Arhiv za higijenu rada i toksikologiju 61, 175-182

Sauter H, Ammerman E, Roehl F (1996) Strobilurins—From natural products to a new class of fungicides. PP 50-81 u: Crop Protection Agents from Nature: Natural Products and Analogues. Urednik: LG Copping, The Royal Society of Chemistry Information Services, Essex, UK.

Sauter H, Ammermann E, Benoit R, Brand S, Gold RE, Grammenos W, Koehle H, Lorenz G, Muller B, Rohl F, Schirmer U, Speakman JB, Wenderoth B, Wingert H (1995) Mitochondrial respiration as a target for antifungals: Lessons learned from research on strobilurins. PP 173-191 u: Antifungal Agents—Discovery and Mode of Action. Urednici: GK Dixon, LG Copping, DW Hollomon, BIOS, Oxford.

Schmelz RM, Collado R (1999) Enchytraeus luxuriosus sp. nov., a new terrestrial oligochaete species (Enchytraeidae, Clitellata, Annelida). Carolinea 93–100.

Schmelz RM, Collado R (2010) A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta).

Schnug L, Ergon T, Jakob L, Scott-Fordsmand JJ, Joner EJ, Leinaas HP (2015) Responses of earthworms to repeated exposure to three biocides applied singly and as a mixture in an agricultural field. Science of the Total Environment 505: 223–235.

Schnug L, Jakob L, Hartnik T (2013) The toxicity of a ternary biocide mixture to two consecutive earthworm (*Eisenia fetida*) generations. Environmental Toxicology and Chemistry 32: 937–947.

Schnug L, Jensen J, Scott-Fordsmand JJ, Leinaas HP (2014a) Toxicity of three biocides to springtails and earthworms in a soil multi-species (SMS) test system. Soil Biology and Biochemistry 74: 115–126.

Schnug L, Leinaas HP, Jensen J (2014b) Synergistic sub-lethal effects of a biocide mixture on the springtail Folsomia fimetaria. Environmental Pollution 186: 158–164.

Shi Z, Tang Z, Wang C (2017) A brief review and evaluation of earthworm biomarkers in soil pollution assessment. Environmental Science and Pollution Research 24(15):13284-13294.

Shoaib MA, Mahmoud MF, Loutfy N, Tawfic MA, Barta M (2010) Effect of botanical insecticide Nimbecidine® on food consumption and egg hatchability of the terrestrial snail Monacha obstructa. Journal of Pest Science 83:27–32.

Silva V, Mol HGJ, Zomer P, Tienstra M, Ritsema CJ, Geissen V (2019) Pesticide residues in European agricultural soils – A hidden reality unfolded. Science of the Total Environment 653: 1532-1545.

Singh S, Eapen S, D'Souza SF (2006) Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in an aquatic plant, Bacopa monnieri L. Chemosphere 62: 233–246.

Smolders R, De Boeck G, Blust R (2003) Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). Environmental Toxicology and Chemistry: An International Journal, 22(4):890-899.

Sokolova IM, Frederich M, Bagwe R, Lannig G, Sukhotin AA (2012) Energy homeostasis as an integrative tool for assessing the limits of environmental stress tolerance in aquatic invertebrates. Environmental Research 79: 1–15.

Solé, M. (2020) Biomarkers in earthworms. Interaction and Fate of Pharmaceuticals in Soil-Crop Systems 311-337.

Štefan L, Tepšić T, Zavidić T, Urukalo M, Tota D, Domitrović R (2007) Lipidna peroksidacija uzroci i posljedice. Medicina 43: 84-93.

Stegeman JJ, Brouwer M, Di Giulio RT, Förlin L, Fowler BA, Sanders BM, Van Veld PA (1992) Molecular responses to environmental contamination: enzyme and protein systems as

indicators of chemical exposure and effect. In: Huggett RJ, Kimerle RA, Mehrle PM, Bergman HL, editors. Biomarkers, biochemical, physiological, and histological markers of anthropogenic stress. USA: Lewis Publishers; pp. 235–335.

Stenersen J (1984) Detoxification of xenobiotics in earthworms. Comparative Biochemistry and Physiology C: Toxicology and Pharmacology 78: 249–252.

Strange RN, Scott PR (2005) Plant disease: a threat to global food security. Annual Review of Phytopathology 43: 83−116.

Štraus B, Barišić K, Čepelak I, Čvorišćec D, Dodig S, Đurić K, Fumić K, Petlevski R, Petrik J, Plavšić F, Plavšić V, Rogić D, Rumora L, Trbojević-Čepe M, Žuntar I, Wolf A (2005) Štrausova Medicinska biokemija. 3. izdanje. Zagreb, Medicinska naklada, 655 pp.

Suter GW (1990) Use of biomarkers in ecological risk assessment. In: McCarthy JF, Shugart LR, editors. Biomarkers of environmental contamination. Boca Raton, FL, USA: Lewis Publishers; pp. 419–28.

Swift MJ, Andren O, Brussaard L, Briones M, Couteaux MM, Ekschmitt K, Kjoller A, Loiseau P, Smith P (1998) Global change, soil biodiversity, and nitrogen cycling in terrestrial ecosystems: three case studies. Global Change Biology 4, 729e743.

Tarazona JV, Ramos-Peralonso MJ (2014) Ecotoxicology, Encyclopedia of Toxicology (Third Edition), PP: 276-280, urednik: Wexler P, Academic Press ISBN 9780123864550. https://doi.org/10.1016/B978-0-12-386454-3.00494-2.

Tominack RL (2000) Herbicide Formulations. Clinical Toxicology 38(2):129–135. doi:10.1081/clt-100100927

Topić Popović N, Strunjak-Perović I, Klobučar RS, Barišić J, Babić S, Jadan M, Kepec S, Kazazić SP, Matijatko V, Ljubić B, Car I, Repec S, Stipanićev D, Klobučar GIV, Čož-Rakovac

R (2015) Impact of treated wastewater on organismic biosensors at various levels of biological organization. Science of the Total Environment 538: 23e37.

Topoliantz S, Ponge JF, Viaux P (2000) Earthworm and enchytraeid activity under different arable farming systems, as exemplified by biogenic structures. Plant Soil 225: 39–51.

Topuz E, van Gestel CA (2017) The effect of soil properties on the toxicity and bioaccumulation of Ag nanoparticles and Ag ions in *Enchytraeus crypticus*. Ecotoxicology and environmental safety, 144, 330-337.

Tripathi G, Kachhwaha N, Dabi I (2010) Comparative studies on carbofuran-induced changes in some cytoplasmic and mitochondrial enzymes and proteins of epigeic, anecic and endogeic earthworms. Pesticide Biochemistry and Physiology 96: 30–35.

Twentyman PR (1992) Cyclosporins as drug resistance modifiers. Biochemical Pharmacology 43(1):109e117.

U.S. Environmental Protection Agency (2020) Pesticide Product Label, Azoxystrobin 2SC.https://www3.epa.gov/pesticides/chem_search/ppls/094730-00006-20200806.pdf [pristupljeno 1. lipnja 2022].

U.S. Environmental Protection Agency (2022) Azoxystrobin; tolerances forresidues. Code of Federal Regulations, https://www.ecfr.gov/current/title-40/chapter-I/subchapter-E/part-180/subpart-C/section-180.507 [pristupljeno 1. lipnja 2022].

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazir M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology 39: 44-84.

van Gestel CA (2012) Soil ecotoxicology: state of the art and future directions. ZooKeys, 176: 275.

van Gestel CAM, Borgman E, Verweij RA, Diez Ortiz M (2011) The influence of soil properties on the toxicity of molybdenum to three species of soil invertebrates. Ecotoxicology and Environmental Safety 74: 1–9.

Velki M, Hackenberger BK (2012) Species-specific differences in biomarker responses in two ecologically different earthworms exposed to the insecticide dimethoate. Comparative Biochemistry and Physiology part: C; Toxicology and Pharmacology 156: 104e112.

Velki M, Hackenberger BK (2013b) Inhibition and recovery of molecular biomarkers of earthworm *Eisenia andrei* after exposure to organophosphate dimethoate. Soil Biology and Biochemistry 57: 100e108.

Velki M, Hackenberger BK, (2013a) Biomarker responses in earthworm *Eisenia andrei* exposed to pirimiphos-methyl and deltamethrin using different toxicity tests. Chemosphere 90: 1216e1226.

Velki M, Stepić, S, Hackenberger BK (2013) Effects of formalin on some biomarker activities of earthworms pre-exposed to temephos. Chemosphere 90: 2690e2696.

Velki M, Weltmeyer A, Seiler TB, Hollert H (2018) Acute toxicities and effects on multixenobiotic resistance activity of eight pesticides to the earthworm *Eisenia andrei*. Environmental Science and Pollution Research 26(5):4821-4832.

Voua OP, Wepener V, Meboeta MS (2014) Single and mixture toxicity of gold nanoparticles and gold (III) to *Enchytraeus buchholzi* (Oligochaeta). Applied Soil Ecology 84: 231–234.

Wallwork JA (1970) Ecology of soil animals. McGraw-Hill, London

Wang X, Li X, Wang Y, Qin Y, Yan B, Martyniuk CJ (2021) A comprehensive review of strobilurin fungicide toxicity in aquatic species: Emphasis on mode of action from the zebrafish model. Environmental Pollution 275: 116671.

Wang YH, Dai DJ, Yu YJ, Yang GL, Shen WF, Wang Q, Weng HB, Zhao XP (2018) Evaluation of joint effects of cyprodinil and kresoxim-methyl on zebrafish, *Danio rerio*. Journal of Hazardous Materials 352: 80e91.

Wang YH, Wu SG, Chen LP, Wu CX, Yu RX, Wang Q, Zhao XP (2012) Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*. Chemosphere 88: 484–491.

Westheide W, Bethke-Beilfuβ D, Hagens M, Brockmeyer V (1989) Enchytraeiden als Testorganismen - Voraussetzungen för ein terrestrisches Testverfahren und Testergebnisse. Verh Ges Ökol 17: 793- 798.

Westheide W, Graefe U (1992) Two new terrestrial Enchytraeus species (Oligochaeta, Annelida). Journal of Natural History 26: 479–488.

Winston GW, Di Giulio RT (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. Aquatic Toxicology 19:137–61.

Wu P, Wu WZ, Han ZH, Yang H (2016) Desorption and mobilization of three strobilurin fungicides in three types of soil. Environmental monitoring and assessment 188(6): 1-11.

Wu R, Zhou T, Wang Jun, Wang Jinhua, Du Z, Li B, Juhasz A, Zhu L (2021) Oxidative stress and DNA damage induced by trifloxystrobin on earthworms (*Eisenia fetida*) in two soils. Science of the Total Environment 797: 149004.

Xu Y, Li B, Hou K, Du Z, Allen SC, Zhu Lusheng, Li W, Zhu Lei, Wang Jinhua, Wang Jun (2021) Ecotoxicity evaluation of azoxystrobin on *Eisenia fetida* in different soils. Environmental Research 194: 110705.

Yang J, Chen X, Yin D, Yin H, Zhang Y, Liu M, Zhao W, Zhang L, Liu Y, Yang X, Zhong Z (2021) Applicability of *Enchytraeus bulbosus* as a model species in ecotoxicology and risk assessment. Ecotoxicology and Environmental Safety 224: 112660.

Ypema HL, Gold RE (1999) Kresoxim-methyl: modification of a naturally occurring compound to produce a new fungicide. Plant Disease 83(1): 4-19.

Zhang C, Wang J, Zhang S, Zhu L, Du Z, Wang J (2017) Acute and subchronic toxicity of pyraclostrobin in zebrafish (*Danio rerio*). Chemosphere 188: 510e516.

Zhang C, Zhang J, Zhu L, Du Z, Wang J, Wang J, Li B, Yang Y (2020c) Fluoxastrobin-induced effects on acute toxicity, development toxicity, oxidative stress, and DNA damage in *Danio rerio* embryos. Sci. Total Environ. 715, 137069.

Zhang C, Zhou T, Xu Y, Du Z, Li B, Wang Jinhua, Wang Jun, Zhu L (2020a) Ecotoxicology of strobilurin fungicides. Science of the Total Environment 742: 140611.

Zhang C, Zhou TT, Du ZK, Juhasz A, Zhu LS, Wang J, Wang JH, Li B (2020b) Applying fungicide on earthworms: biochemical effects of *Eisenia fetida* exposed to fluoxastrobin in three natural soils. Environmental Pollution 258: 113666.

Zhang C, Zhu LS, Wang J, Wang JH, Du ZK, Li B, Zhou TT, Cheng C, Wang ZB (2018b) Evaluating subchronic toxicity of fluoxastrobin using earthworms (*Eisenia fetida*). Science of the Total Environment 642: 567–573.

Zhang Q, Yu Y, Jin M, Deng Y, Zheng B, Lu T, Qian H (2022) Oral azoxystrobin driving the dynamic change in resistome by disturbing the stability of the gut microbiota of *Enchytraeus crypticus*. Journal of Hazardous Materials 423: 127252.

Zhang Q, Zhu D, Ding J, Zheng F, Zhou S, Lu T, Zhu Y-G, Qian H (2019) The fungicide azoxystrobin perturbs the gut microbiota community and enriches antibiotic resistance genes in *Enchytraeus crypticus*. Environment international 131: 104965.

Zhou H, Xiang N, Xie J, Diao X (2019) Ecotoxicology: The History and Present Direction, Encyclopaedia of Ecology (Second Edition), Elsevier, PP 415-423, Urednik: Fath B. ISBN 9780444641304.

Zhu B, Liu GL, Liu L, Ling F, Wang GX (2015) Assessment of trifloxystrobin uptake kinetics, developmental toxicity and mRNA expression in rare minnow embryos. Chemosphere 120: 447e455.

Zubrod JP, Bundschuh M, Arts G, Brühl CA, Imfeld G, Knäbel A, Payraudeau S, Rasmussen JJ, Rohr J, Scharmüller A, Smalling K, Stehle S, Schulz R, Schäfer RB (2019) Fungicides: An Overlooked Pesticide Class?. Environmental Science & Technology 53: 3347−3365.

6. ŽIVOTOPIS

Marija Kovačević rođena je 12. svibnja 1994. godine u Požegi. Nakon završetka osnovne škole u Pleternici pohađala je prirodoslovno-matematički program Gimnazije Požega. Preddiplomski i diplomski studij biologije pohađala je na Odjelu za biologiju Sveučilišta Josipa Jurja Strossmayera u Osijeku, te je za izvrsne uspjehe tijekom studiranja primila Pročelnikova nagrada za najboljeg studenta I. godine diplomskog sveučilišnog studija biologije; smjer: znanstveni za akademsku 2016./2017. godinu i Rektorovu nagradu za akademsku 2017./2018. godinu. Osim toga 2018. godine je nagrađena Godišnjom nagrada grada Pleternice za obrazovanje. Tijekom studija sudjelovala je u izvođenju nastave kao demonstrator na vježbama i terenskoj nastavi iz kolegija "Stablašice" i "Kralježnjaci" preddiplomskog sveučilišnog studija. Nadalje, sudjelovala je u raznim projektima inventarizacije flore i faune, te u floističkim istraživanjima koja su osim završnog i diplomskog rada rezultirala prvim kongresnim priopćenjima.

Nakon završetka diplomskog studija zaposlila se na Zavodu za kvantitativnu biologiju, Odjelu za biologiju Sveučilišta Josipa Jurja Strossmayera u Osijeku u okviru HRZZ projekta „Različiti učinci okolišno relevantnih mješavina metal temeljenih nanočestica i pesticida na faunu tla: Nove smjernice za procjenu rizika (DEFENSoil)" (IP-2014-09-4459) pod mentorstvom prof.dr.sc. Branimira K. Hackenberger. Tijekom posljediplomskog studija stručno se usavršavala na Zavodu za botaniku i zoologiju, Sveučilišta Masaryk u Brnu (Češka), te na Zavodu za ekoznanosti, Sveučilišta Aarhus u Silkeborgu (Danska). Sudjelovala je u izvođenju nastave iz kolegija Animalna fiziologija 1, Molekularna ekotoksikologija, Toksikologija, Ekologija tla, te provođenju terenske nastave.

Do sada je kao koautor objavila 8 znanstvenih radova u časopisima citiranima u CC (Curent Contents) bazi podataka, te 19 kongresnih priopćenja koja su bila prezentirana na domaćim i međunarodnim kongresima.

Popis objavljenih publikacija:

1. Merdić E, Kujavec M, **Kovačević M**, Žulj M, Graovac N, Vignjević G, Turić N (2018) *Culex torrentium* (*Martini*), a new species in Croatian mosquito fauna. Natura Croatica: periodicum Musei historiae naturalis Croatici. 2:323-329.

2. Kovačević M, Hackenberger DK, Lončarić Ž, Hackenberger BK (2021) Measurement of multixenobiotic resistance activity in enchytraeids as a tool in soil ecotoxicology. Chemosphere. 279:130549.

3. Kovačević M, Hackenberger DK, Hackenberger BK (2021) Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifoxystrobin) on survival, reproduction and hatching success of *Enchytraeus crypticus*. Science of the Total Environment. 790:148143.

4. Trigui S, Hackenberger DK, **Kovačević M**, Stjepanović N, Palijan G, Kallel A, Hackenberger BK (2021) Effects of olive mill waste (OMW) contaminated soil on biochemical biomarkers and reproduction of *Dendrobaena veneta*. Environmental Science and Pollution Research. 29(17), 24956-24967.

5. Kovačević M, Stjepanović N, Trigui S, Hackenberger DK, Lončarić Ž, Glavaš OJ, Kallel A, Hackenberger BK (2022) Assessment of adverse effects of olive mill waste water and olive mill waste contaminated soil on springtail *Folsomia candida*. Chemosphere, 300:134651.

6. Trigui S, Hackenberger DK, Stjepanović N, Lončarić Ž, **Kovačević M**, Hackenberger BK, Kallel A (2022) Mitigation of OMW toxicity toward *Enchytraeus albidus* with application of additives. Environmental Science and Pollution Research https://doi.org/10.1007/s11356-022-21668-y

7. Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Toxicity of fungicide azoxystrobin to *Enchytraeus albidus*: Differences between the active ingredient and formulated product. Pesticide Biochemistry and Physiology, 187: 105198.

8. **Kovačević M**, Amorim MJB, Hackenberger BK, Scott-Fordsmand JJ (2022) Avoidance Behaviour of Six Collembolan Species Shows Species-Specific Sensitivity—Impact of Ag NM300K. Nanomaterials, 12(19):3276. https://doi.org/10.3390/nano12193276

9. **Kovačević M**, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Comprehensive study of the effects of strobilurin-based fungicide formulations on Enchytraeus albidus. Ecotoxicology, 31:1554–1564 https://doi.org/10.1007/s10646-022-02609-4

Kongresna priopćenja:

1. Špoljarić Maronić D, Žuna Pfeiffer T, **Kovačević M**, Zahirović V, Stević F, Mihaljević M (2016) Orchid diversity in a special habitat mosaic on the southern slopes of Požeška Gora hill

(Croatia). Međunarodni simpozij “Advances in research on the flora and vegetation of the Carpato Pannonian region”, Budimpešta. (postersko priopćenje)

2. Mandir T, **Kovačević M**, Šimić M, Žuna Pfeiffer T, Špoljarić Maronić D, Stević F (2017) Raznolikost flore stepolike travnjačke površine u Bistrincima, 6. Simpozij s međunarodnim sudjelovanjem "Kopački rit jučer, danas, sutra, 2017.", Tikveš. (postersko priopćenje)

3. Kovačević M, Plavac T, Hackenberger BK (2018) TBARS in horse hair as an indicator of oil industry pollution, SETAC (Society of Environmental Toxicology and Chemistry) Europe 28th annual meeting, Rim. (postersko priopćenje)

4. Komljenović J, Kristić T, Mandir T, Đurić A, **Kovačević M**, Bjedov, D., Mikuška, A (2018) Breeding of rooks (*Corvus frugilegus* L. 1785) In the eastern Slavonia, 1st International Students' GREEN Conference, Osijek. (postersko priopćenje)

5. Mandir T, **Kovačević M**, Šimić M, Grgošević M, Žuna Pfeiffer T, Špoljarić Maronić D (2018) Flora of steppe like grasland a rea in Bistrinci (East Croatia), 1st International Students' GREEN Conference, Osijek. (postersko priopćenje)

6. Žuna Pfeiffer T, Špoljarić Maronić D, **Kovačević M**, Šag M, Domjan L, Stević F, Lončarić Z (2018) Comparative study of plant morphology and leaf anatomy of near threatened terrestrial species, *Orchis morio* L., 3rd International Conference on Plant Biology (22nd SPPS Meeting), Beograd. (postersko priopćenje)

7. Kovačević M, Hackenberger DK, Lončarić Ž, Hackenberger BK (2019) Aktivnost efluks pumpe enhitreida, 2nd Young Scientists Days Conference, Osijek. (predavanje)

8. Mandir T, **Kovačević M**, Šimić M, Grgošević M, Žuna Pfeiffer T, Špoljarić Maronić D (2019) Raznolikost flore subpanonskog travnjaka u Bistrincima, 2nd Young Scientists Days Conference, Osijek. (predavanje)

9. Kovačević M, Hackenberger DK, Lončarić Ž, Hackenberger BK (2019) Efflux pump activity in Enchytraeidae, SETAC (Society of Environmental Toxicology and Chemistry) Europe 29th Annual Meeting, Helsinki. (predavanje)

10. Galinović T, Hackenberger DK, **Kovačević M**, Zelić L, Lončarić Ž (2019) The effect of silver nanoparticle size on the reproduction of *Enchytraeus albidus*, Scientific symposium Soil ecotoxicology Theory and Application, Osijek. (predavanje)

11. Kovačević M, Hackenberger DK (2019) Effect of fungicide azoxystrobin on hatching delay in potworm *Enchytraeus crypticus*, Scientific symposium Soil ecotoxicology Theor y and Application, Osijek. (predavanje)

12. Kovačević M, Hackenberger DK, Hackenberger BK (2021) Učinci strobilurinskih fungicida na preživljavanje, reprodukciju i izlijeganje vrste *Enchytraeus crypticus*, 5. simpozij studenata doktorskih studija PMF-a, Zagreb, Hrvatska. (predavanje)

13. Hackenberger DK, Hackenberger BK, Jelić M, **Kovačević M** (2021) Earthworm fauna of Croatian part of the Pannonian island mountains, XVIII International Colloquium of Soil Zoology (ICSZ 2021) XV International Colloquium on Apterygota (ICA 2021), Bolzano, Italija. (postersko priopćenje)

14. Kovačević M, Hackenberger BK, Hackenberger DK (2021) Effects of strobilurins (azoxystrobin, pyraclostrobin, and trifloxystrobin) on reproduction and hatching delay in *Enchytraeus crypticus*, CROTOX 2021, Rabac, Hrvatska. (predavanje)

15. Kovačević M, Stjepanović N, Hackenberger DK, Hackenberger BK (2021) Procjena toksičnosti aktivne tvari azoksistrobin i komercijalnog pripravka (Quadris®) na neciljni organizam tla *Enchytraeus albidus*. 3rd Young Scientists Days - Conference, Osijek, Hrvatska. (predavanje)

16. Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Effects of three strobilurin fungicides on survival, reproduction and biochemical parameters in *Enchytraeus albidus*. SETAC (Society of Environmental Toxicology and Chemistry) 11th Young Environmental Scientists Meeting. (predavanje)

17. Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Sveobuhvatna procjena utjecaja tri strobilurinska fungicida na vrstu *Enchytraeus albidus*. 6. simpozij studenata doktorskih studija PMF-a, Zagreb, Hrvatska. (predavanje)

18. Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Biomarker responses of *Enchytraeus albidus* to fungicide azoxystrobin: differences between active ingredient and commercial product, SETAC (Society of Environmental Toxicology and Chemistry) Europe 32nd Annual Meeting, Kopenhagen, Danska. (predavanje)

19. Kovačević M, Stjepanović N, Hackenberger DK, Lončarić Ž, Hackenberger BK (2022) Assessment of adverse effects of olive mill waste water and olive mill waste contaminated soil

on springtail *Folsomia candida*, SETAC (Society of Environmental Toxicology and Chemistry) Europe 32nd Annual Meeting, Kopenhagen, Danska. (predavanje)

Projekti:

Različiti učinci okolišno relevantnih mješavina metal temeljenih nanočestica i pesticida na faunu tla: Nove smjernice za procjenu rizika (DEFENSoil) (2014-09-4459). Voditelj projekta: prof.dr.sc. Branimir K. Hackenberger, Odjel za biologiju; Sveučilište Josipa Jurja Strossmayera, Osijek. Hrvatska zaklada za znanost (HRZZ).