Sveučilište u Zagrebu
Fakultet kemijskog
inženjerstva i tehnologije
Zavod za industrijsku ekologiju

Kolegij:
PRIMJENA EKOTOKSIKOLOGIJE
3. predavanje

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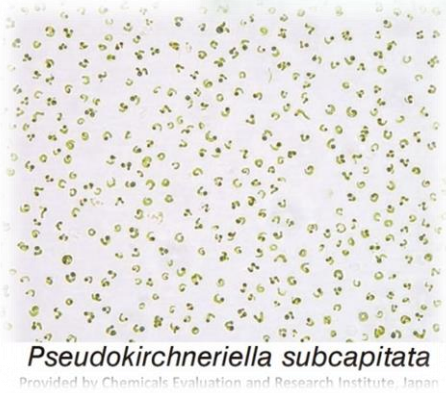
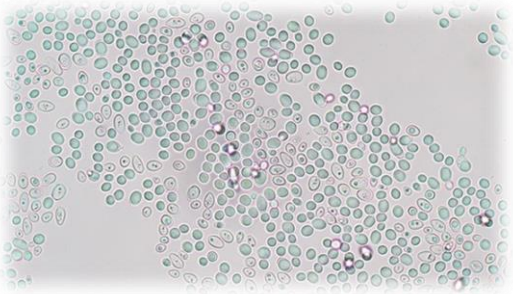
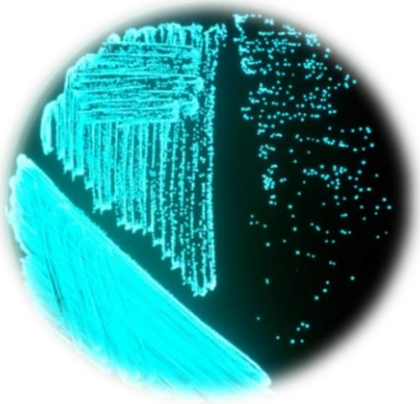
SADRŽAJ

Testovi ekotoksičnosti:

1. *Vibrio fischeri*
2. *Pseudomonas putida*
3. *Saccharomyces cerevisiae*
4. *Daphnia magna* (račić)
5. Zebrafish *(Danio rerio)*
6. Zelena alga (*Pseudokirchneriella subcapitata*)
7. Glodavci
8. Nematode







TESTOVI TOKSIČNOSTI

- *In vivo* i *in vitro* tehnike
- **Prednosti** *in vitro* tehnike – ekonomski isplative i kraćeg trajanja
- **Nedostatci:** iz njih je teško zaključiti o učinku na organizam i zdravlje čovjeka
- In vivo testovi – bolje predviđaju biološke odgovore organizama

- Karakteristike MODELNOG ORGANIZMA:

  (1) jednostavno građeni organizam uz istovremenu prisutnost osnovnih staničnih procesa koje imaju i složeniji organizmi

  (2) dostupnost jedinki za istraživanje i mogućnost jednostavne manipulacije organizama

  (3) jednostavno i jeftino održavanje u laboratoriju

  (4) podložnost genetičkoj manipulaciji

  (5) relativno mali i stabilni genom

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

Carstvo: Animalia

Koljeno: Chordata

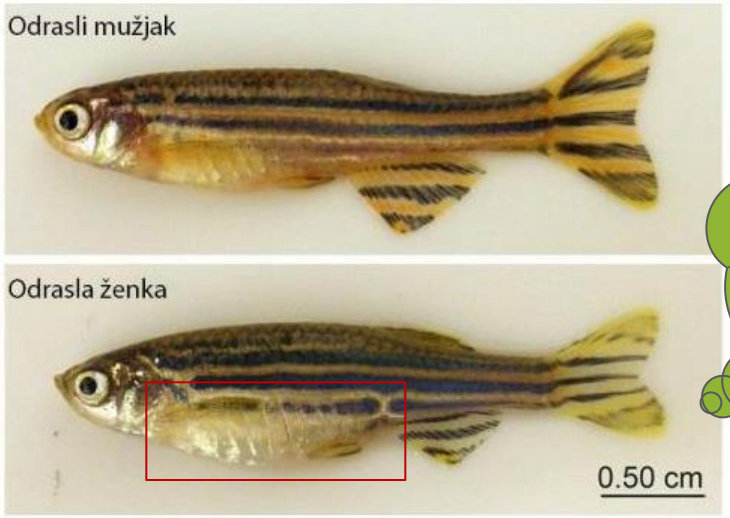
Razred: Actinopterygii

Red: Cypriniformes

Porodica: Cyprinidae

Rod: *Danio*

Vrsta: *Danio rerio* (Hamilton, 1822)



Natečeni trbuh kod ženki - jedna ženka rađa između 50 i 200 jaja na dan

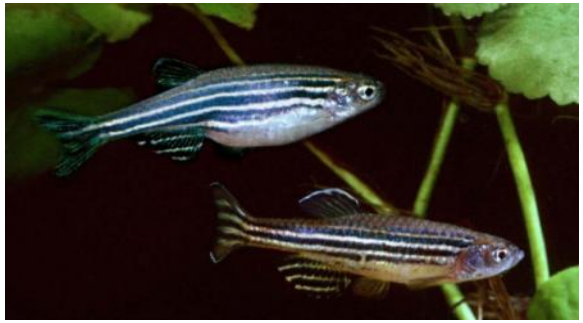
Slika 1. Usporedba odraslog mužjaka i ženke. Tijelo mužjaka ima žutu nijansu i vretenastiji oblik, naspram ženke koja je krupnija te zaobljena s bijelom nijansom (Preuzeto iz Holtzman i sur., 2016).

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

- Ciljevi testova toksičnosti koji se provode na RIBAMA su procjene:
  - opasnosti za okoliš od pojedinačnih tvari,
  - klasifikacija opasnosti tvari,
  - procjena opasnosti otpadnih voda
- Potrebno je procijeniti AKUTNU I KRONIČNU TOKSIČNOSTI za ribe te odrediti tvari koje se potencijalno mogu AKUMULIRATI U TKIVIMA RIBE
- RIBE – vodeni kralježnjaci
- Često se koriste za MONITORING KAKVOĆE VODE
- Testovi se mogu provoditi za ispitivanje:
  - AKUTNE TOKSIČNOSTI,
  - TOKSIČNOSTI U RANOM ŽIVOTNOM STADIJU,
  - KRATKOROČNI TESTOVI TOKSIČNOSTI NA RIBLJIM EMBRIJIMA I
  - TOKSIČNOST PRILIKOM ODRASTANJA ODNOSNO U STADIJU LARVE

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

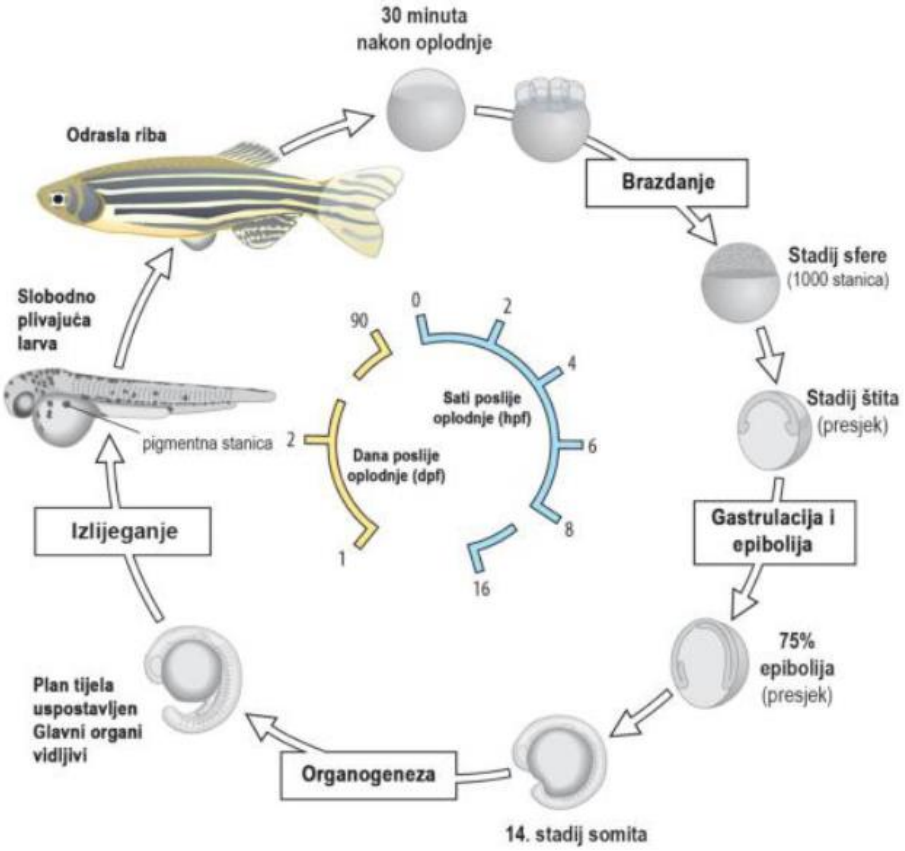
- *Danio rerio* ili zebrica – slatkovodna, bentopelagička tropska riba
- Ime dobila po indijskoj riječi *dhani* – od "rižinih polja", nalazi se uz rubove rižinih polja Indije
- P. Matte – 1905. unosi zebricu u Europu kao akvarijsku ribu
- Tijelo dugo oko 5 cm
- Tijekom odrasle faze spolni dimofrizam – odlikuje se kroz različitu veličinu i oblik tijela te pigmentaciju
- Spolnu zrelost dosežu ovisno o tipu ishrane – 5 mj
- Prosječni životni vijek – 5 god.
- Par zebrica može po mrijestu dati od 100 – 200 embrija



TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

- Zebrice - male bentopelagičke ciprinidne ribe sa srednjom dužinom odraslih jedinki između 3 i 5 cm
- Zebrice brzo rastu na 26 °C i završavaju svoj životni ciklus u roku od tri mjeseca
- Vrsta je dostupna, jeftina, lako održiva i, u odgovarajućim uvjetima, daje velik broj prozirnih jaja
- GLAVNI MODEL U NEUROBIOLOGIJI I TOKSIKOLOGIJI, KAO I OPĆENITOJ MOLEKULARNOJ I RAZVOJNOJ BIOLOGIJI
- Treba izbjegavati korištenje divljih zebrica
- MRIJEST SE AKTIVIRA SVJETLOŠĆU
- Ženske zebrice koje se koriste za mrijest trebaju imati biti između 4 i 15 mjeseci starosti
- VEĆINA ORGANA RAZVIJA SE UNUTAR JEDNOG DANA, A EMBRIJI SE IZLEGU NAKON TRI DO ČETIRI DANA

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

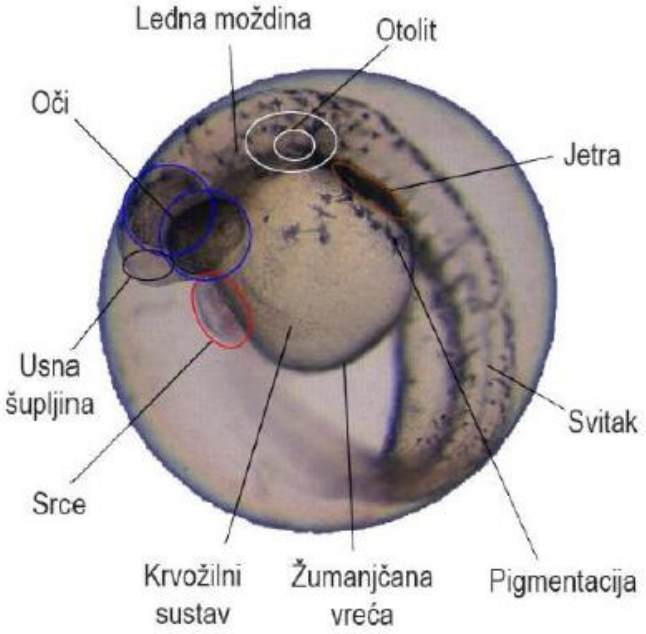


Slika 2. Razvojni ciklus zebrice. Prilagođeno prema URL 1.

Vrijeme [h]	Stadij	Karakteristike
0	Oplodnja	Zigota
0	Period zigote	Citoplazma se akumulira na životinjskom polu, jednoćelijski stadij
0,75	Razdoblje cijepanja	1. (vertikalna srednja) podjela: dvostanični
1		2. (vertikalna) podjela: četverostanični stadij
1,25		3. (okomita i paralelna s prvom ravninom) podjela: 8-stanični stadij
1,5		4. (okomita i paralelna s drugom) podjela: 16-stanični stadij
2	Razdoblje blastule	Početak stadija blastule
3		Kasno cijepanje; sadrži otprilike 256 blastomera
4		Ravno sučelje između blastoderme i žumanjka
5,25	Radoblje grastule	50 % epiboličkih pokreta; blastoderma se smanjuje i sučelje između periblasta i blastoderme postaju zakrivljeni
8		75 % epiboličkog pokreta
10		Epiblički pokret završava, blastopore su gotovo zatvorene
10,5	Razdoblje segmentacija	Najprije brazda somita
12		Somiti su razvijeni, nediferencirana mesodermalna komponenta rano tijelo, rep segmentiran ili metameričan
20		Mišićno trzanje; rep dobro ispružen
22		Razvoj unutarnjeg uha (otolita)
24	Razdoblje faringule	Spontani pokreti, rep odvaja se od jajeta; rana pigmentacija
30		Smanjeno spontano kretanje; mrežnica pigmentirana, stanična degeneracija kraja repa; cirkulacija u aortnom luku vidljiva
36		Pigmentacija repa; jaka cirkulacija; jedan par aortalnih lukova; otkucaji srca
72-96	Period izmrzavanja	Otkucaji srca redoviti; nastavak žumanjka koji se počinje sužavati; leđne i ventralne pigmentirane trake susreću se na repu; otkrivene segmentarne krvne žile: zadebljani sakulus s dvije vidljive komore; razvoj prednjeg crijeva

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

- Zebrice – brza izmjena generacija sa velikim brojem potomaka, kratkim *ex utero* embrionalnim razvojem od svega 72 h
- Tijekom cijelog embrionalnog razvoja – tijela prozirna – pogodni za promatranje učinaka različitih spojeva
- Jeftino održavanje
- Pogodan model u toksikološkim istraživanjima



Slika 3. Embrij zebrice star 48 hpf sa razvijenim glavnim organima i krvožilnim sustavom (Prilagođeno prema Lillicrap, 2010).

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

- Sličnosti između zebrica i ostalih kralježnjaka:

  (1) sličnost u staničnoj strukturi

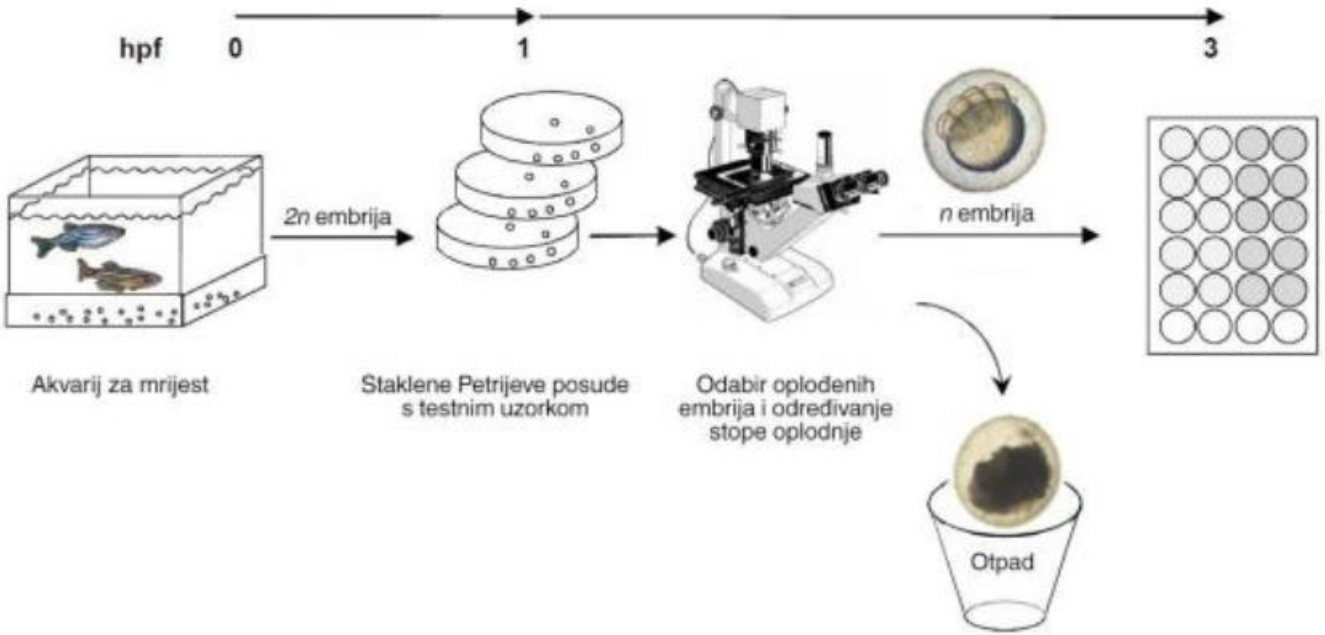
  (2) sličnost u signalnim putevima

  (3) sličnost u anatomiji i fiziologiji
- Ljudi i zebrice dijele 70 % GENOMA, unutar koje je 84 % GENA povezano s istim genetskim bolestima u čovjeka i zebrica
- Velika sposobnost regeneracije tkiva – srca, bubrega, mozga, leđne moždine
- Standardni modalni organizam – BIOMEDICINSKA ISTRAŽIVANJA
- Modalni organizam u EKOTOKSIKOLOGIJI i BIOMONITORINGU STANJA VODENIH EKOSUSTAVA

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

- Žarište istraživanja – s odraslih jedinki na najranije razvojne stadije – EMBRIJI
- Utvrđena izvrsna korelacija akutnog izlaganja embrija sa kroničnim izlaganjem odraslih jedinki
- TEST EMBRIOTOKSIČNOSTI NA ZEBRICAMA (eng. *Zebrafish embryotxicity test)* (ZET, OECD (236:2013))
- Najčešće korišteni test
- Princip – praćenje letalnih i subletalnih učinaka testiranog spoja/uzorka u najranijim razvojnim stadijima do 96 sati nakon oplodnje
- Letalni učinak – prestanak rada srca, koagulacija embrija
- Subletalni učinci – nepotpuno formiranje pigmentacije
- Embriji zebrica u potpunosti zadovoljavaju 3R načela (*Replacement, Reduction, Refinement*)

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)



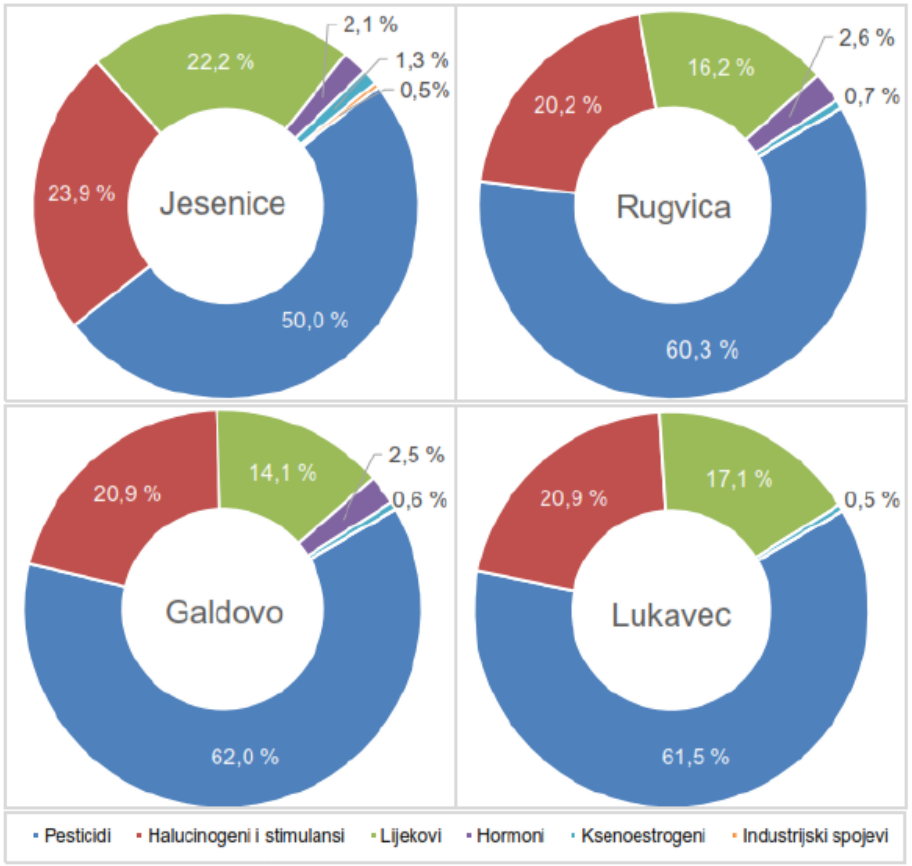
Slika 5. Shematski prikaz provođenja testa embriotoksičnosti na zebricama (Prilagođeno prema Lammer i sur., 2009)

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

PRIMJER: Određivanje toksičnosti sedimenta rijeke Save sa Zebrafish

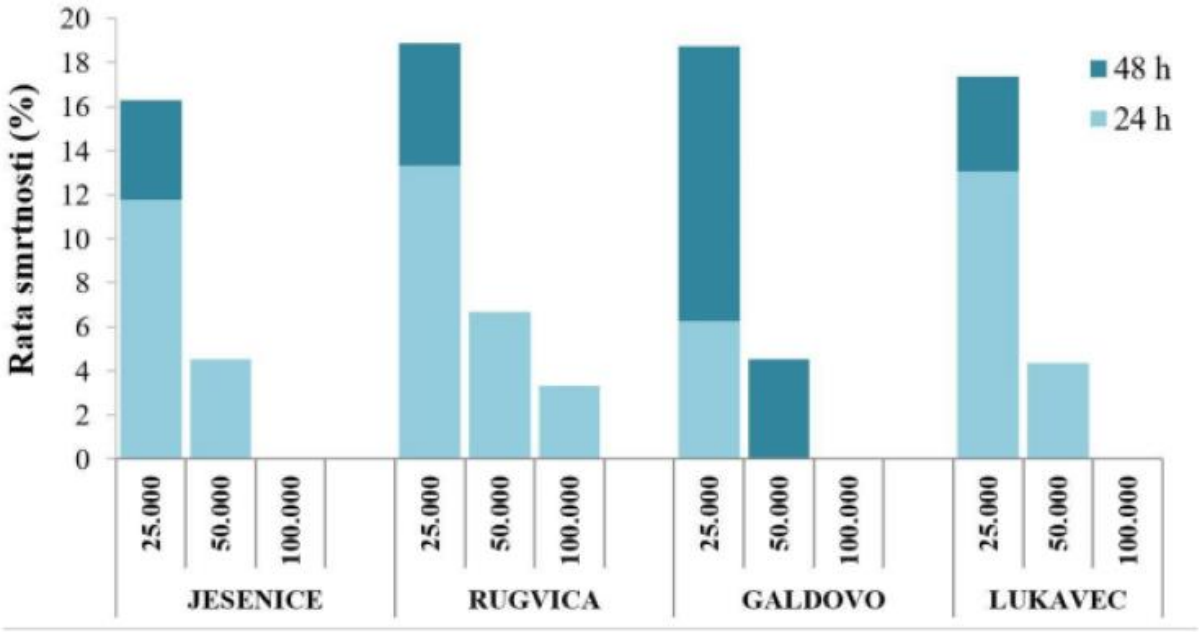
Slika 4. Prikaz lokacija uzorkovanja (ArcGIS 10.1 program).

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)



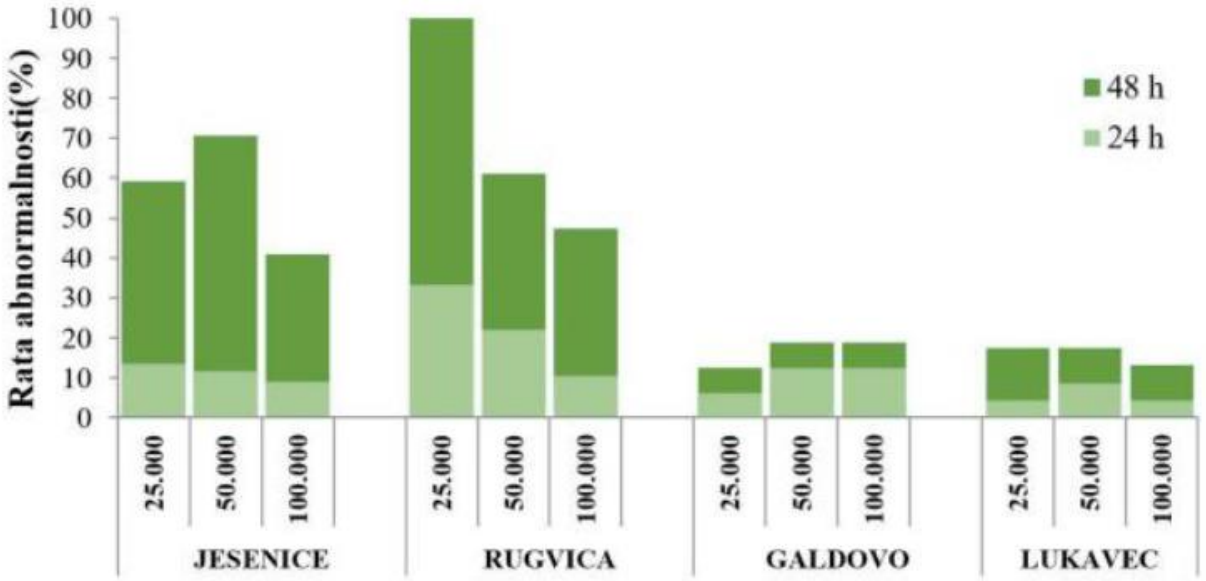
Slika 6. Prikaz brojnosti kemijskih skupina pronađenih u ekstraktima sedimenta na analiziranim postajama u rijeci Savi.

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)



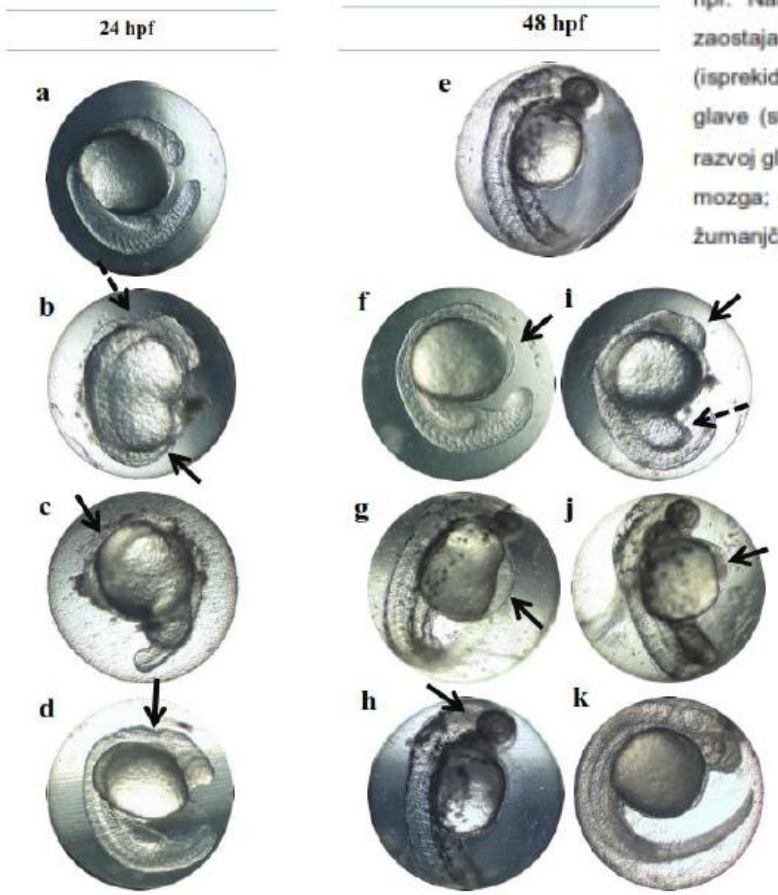
Slika 7. Rata smrtnosti embrija *Danio rerio* nakon 24 i 48-satnog izlaganja ekstraktima sedimenta.

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)



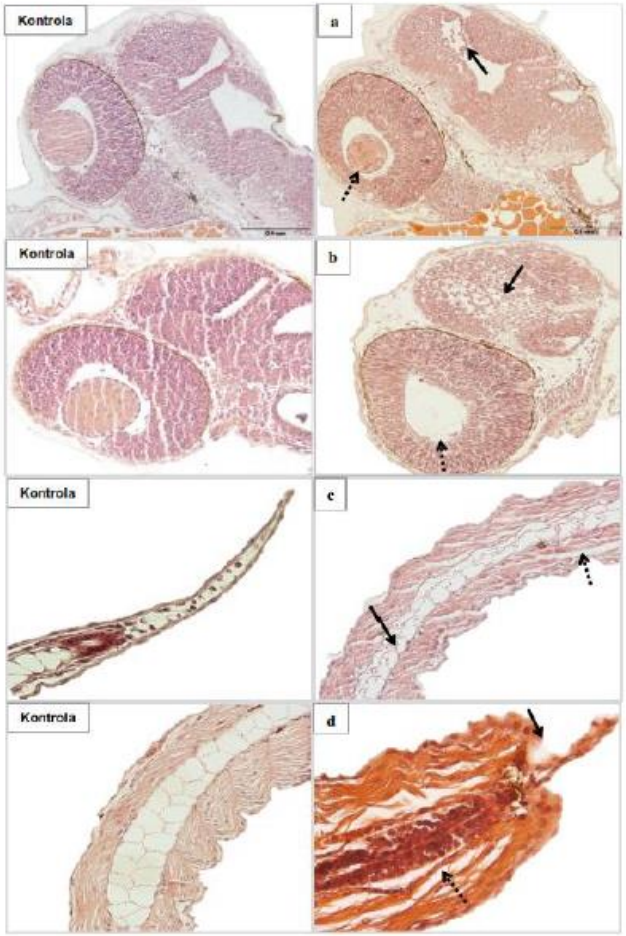
Slika 8. Rata abnormalnosti embrija *Danio rerio* nakon 24 i 48 h izlaganja ekstraktima sedimenta.

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)



Slika 9. Razvojne abnormalnosti embrija *D. rerio* 24 i 48 hpf tijekom izlaganja različitim koncentracijama sedimenata: a) normalno razvijeni embrij 24 hpf, e) normalno razvijeni embrij 48 hpf. Nakon 24 h od izlaganja embrija uzorcima, primiječene su slijedeće abnormalnosti: b) zaostajanje u razvoju, ne odvajanje repa od žumanjčane vrećice (strelica), deformacije glave (isprekidana strelica); c) deformacije cijelog embrija, nerazvijena glava (strelica), d) deformacije glave (strelica). Nakon 48 h od izlaganja embrija uzorcima, primiječeno je slijedeće: f) nepotpuni razvoj glave; g) edem u području žumanjčane vrećice, h) manje nakupljanje krvi u području srednjeg mozga; i) zaostajanje u razvoju, slabo razvijeno oko (strelica); j) nakupljanje krvi u području žumanjčane vrećice; k) izostanak pigmentacije tijela i oka.

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)



Slika 10. Histopatološke promjene zabilježene u poprečnom presjeku embrija *Danio rerio* 48 h nakon izlaganja ekstraktima sedimenta. Kontrola: normalno razvijena glava, očna leća, trup i rep embrija; a) nekroza mozga (strelica), slabo razvijena očna čašica (isprekidana strelica); b) edem mozga i apoptoza (strelica), poremećaja u razvoju leće (isprekidana strelica); c) oštećenje svitka: dezintegracija septi (isprekidana strelica), nepravilan raspored mišićnih vlakana (strelica); d) nekroza vršnog dijela repa (strelica), oštećenje mišića (isprekidana strelica).

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

Tablica 3. Zabilježene razvojne abnormalnosti embrija zebrica 24 i 48 hpf u odnosu na različita razrjeđenja ekstrakata sedimenta.

		Analizirane postaje i razrjeđenja ekstrakata sedimenta											
		Jesenice			Rugvica			Lukavec			Galdovo		
	Toksikološke krajnje točke	25.000	50.000	100.000	25.000	50.000	100.000	25.000	50.000	100.000	25.000	50.000	100.000
24 hpf	Koagulacija embrija	†	†		†	†	†	†	†		†	†	
	Nerazdvajanje repa		†				†						
	Nestvaranje somita				†								
	Slabo razvijeno oko	•		•	•		•	•	•		•	•	
	Zaostajanje u razvoju		•	•	•		•		•				
	Neformirani rep				•								
	Slabo razvijena glava				•		•						
	Deformacije glave	•		•					•		•		
48 hpf	Koagulacija embrija				†						†	†	
	Snižen broj otkucaja srca	†			†			†					
	Nakupljanje krvi u žumanjčanoj vrećici	•	•	•	•	•		•	•	•			
	Nakupljanje krvi u mozgu									•	•		•
	Perikardijalni edem	•			•		•	•	•		•	•	
	Edem u području žumanjčane vrećice	•	•		•		•						
	Nepotpun razvitak ili izostanak pigmentacije tijela i oka				•	•	•	•					
	Zaostajanje u razvoju			•		•			•			•	

† = Letalni učinak (ISO 2007:15088)
• = Subletalni učinak (Hollert, 2003; Beekhuijzen i sur., 2015)

Tablica 4. Zabilježene histopatološke promjene na embrijima zebrica 48 hpf u ovisnosti o različitim razrjeđenjima ekstrakata sedimenta.

		Analizirane postaje i razrjeđenja ekstrakata sedimenta											
		Jesenice			Rugvica			Lukavec			Galdovo		
	Histopatološke promjene	25.000	50.000	100.000	25.000	50.000	100.000	25.000	50.000	100.000	25.000	50.000	100.000
48 hpf	Oštećenje svitka	+	++	+	-	-	+++	+	+	+	+	++	+++
	Promjene trupnih mišića	+	++	+	+	-	++	+	+	+	-	-	+++
	Nekroza mišića	+	++	++	+	+++	+	+	-	-	-	-	+
	Nekroza mozga	+	+	+	+	+++	+++	-	+	+	-	+	+
	Deformacija očne leće	+	++	++	+	+++	+++	-	+	++	++	++	++
	Nekroza repa	-	-	-	+++	+++	+++	+	++	+	-	-	-
	Nakupljanje krvi u žumanjčanoj vrećici	-	-	++	+	-	-	-	++	+++	+++	+++	+++
	Nakupljanje krvi u području mozga	-	-	-	+	-	-	-	+	++	+	+	++
	Nakupljanje krvi u području trupa	-	-	+	+	-	-	+	++	+++	++	++	++
	Edem žumanjčane vrećice	-	-	-	-	-	-	-	+	++	+	+	+
	Edem mozga	-	-	+	+	-	+	-	+	++	+	+	++
	Poremećaj u razvoju oka	+	++	++	++	+++	+++	+	+	++	-	-	++
	Poremećaj u razvoju mozga	+	++	++	++	+++	+++	+	+	++	-	-	+

Blagi učinak (+)
Osrednji učinak (++)
Izraziti učinak (+++)

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

PRIMJER: Bioaccumulation of polystyrene nanoplastics and their effect on the toxicity of Au ions in zebrafish embryos

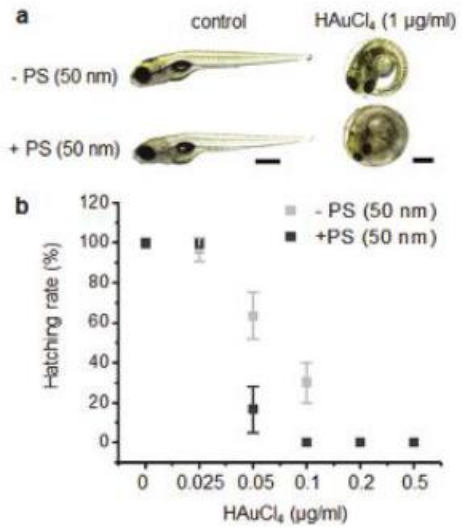


Fig. 4 Hatching rate of ZFEs. (a) Optical image of ZFE at 120 hpf after exposure to none as control and $HAuCl_4$ without or with of $PS_{50\ nm}$. (b) Hatching rate of ZFE exposed to various concentration of $HAuCl_4$ without or with $PS_{50\ nm}$. Samples were exposed to ZFE at 24 hpf until 120 hpf. Scale bar: 500 μm.

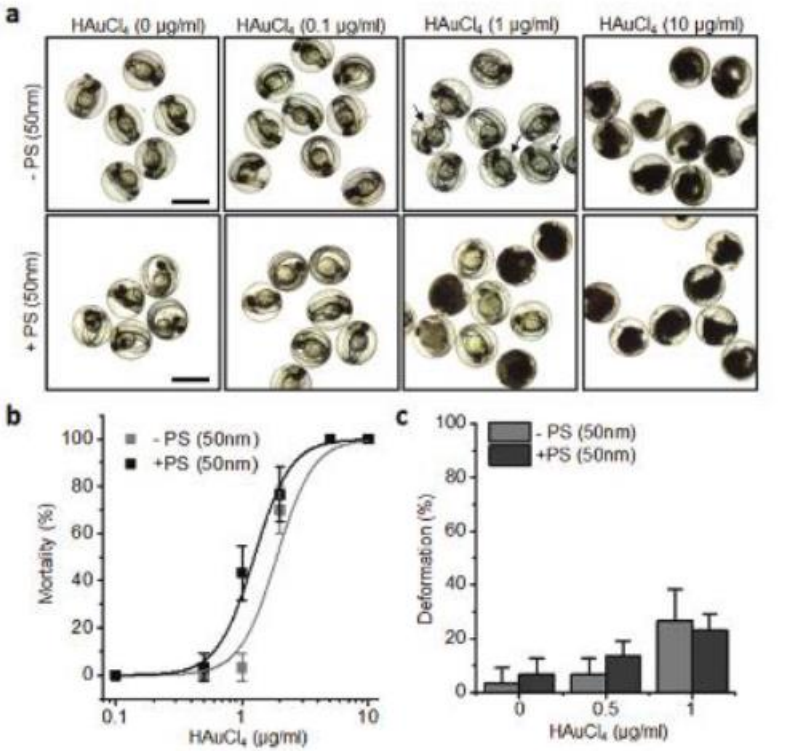


Fig. 3 Toxicity of Au ion and PS nanoplastics. (a) Optical images of ZFE exposed to various concentration of $HAuCl_4$ at 0, 0.1, 1, 10 μg ml^{-1} without or with $PS_{50\ nm}$ (100 μg ml^{-1}) for 24 h starting at 24 hpf. (b) Mortality rate and (d) deformation rate of ZFEs exposed to $HAuCl_4$ without or with PS nanoplastics. The asterisk (*) indicates a significant difference between the treatment group and control ($p < 0.05$).

TEST TOKSIČNOSTI SA ZEBRICAMA (*Danio rerio*)

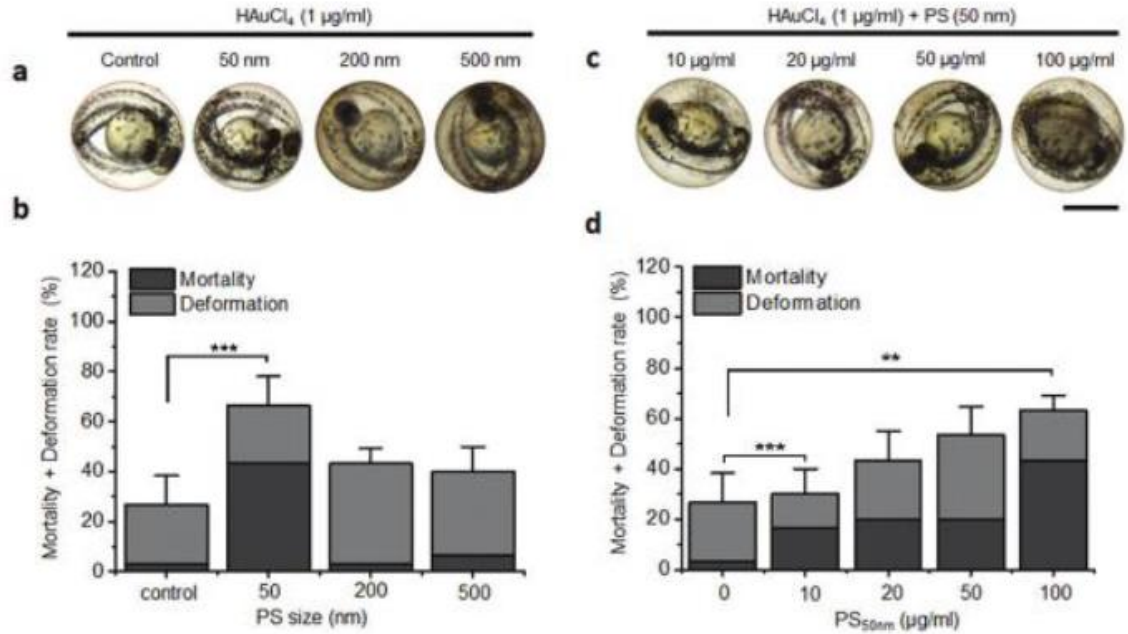
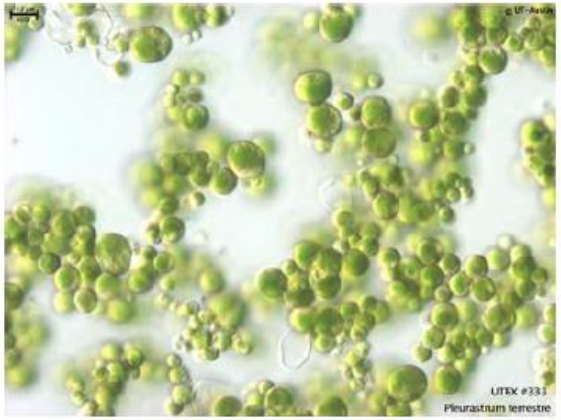


Fig. 5 Size and dose-dependent toxicity of PS nanoplastics. (a) Optical image of ZFE at 48 hpf after exposure to different sized PS nanoplastics (0, 50, 200, 500 nm) in presence of $HAuCl_4$ (1 μg ml^{-1}) for 24 h. (b) Mortality and deformation rate of ZFEs exposed to each sample. (c) Optical image of ZFE at 48 hpf after exposure to different concentration of $PS_{50\ nm}$ (0, 10, 20, 50, 100 μg ml^{-1}) in presence of $HAuCl_4$ (1 μg ml^{-1}). (d) Mortality and deformation rate of ZFEs exposed to each sample. Deformation features was counted as heart edema, yolk edema, and eye development. The asterisks indicate significant differences between treatment groups and control in term of mortality (** for $p < 0.01$, and *** for $p < 0.005$). All experiment were performed triplicate with $n = 10$ (total 30 eggs) for each. Scale bar: 500 μm.

TEST TOKSIČNOSTI S MIKROALGAMA

- Alge – fotoautotrofi
- Eukariotski organizmi
- Mikroskopska veličina i makroskopska veličina - do duljine od 60 m
- Jednostanične i višestanične
- Jednostanične – zavijene, slične štapiću ili kuglaste
- Spolno i nespolno razmnožavanje
- Zelene alge – često svrstavaju u biljke jer u staničnim stjenkama sadržavaju celulozu, imaju klorifil *a* i *b*, te kao biljke pohranjuju škrob



TEST TOKSIČNOSTI S MIKROALGAMA

- Slatkovodne planktonske alge – koriste se u laboratorijskim testovima toksičnosti, češće od drugih vrsta vodenih biljaka
- Testovi s algama vrlo osjetljivi, relativno brzi i jeftini
- Vrste koje se najčešće koriste u istraživanjima:

Pseudokirchneriella subcapitata

Chlorella vulgaris

Scenedesmus quadricauda

Scenedesmus subspicatus

Alge razreda – Chrysophyceae

Pojedine cijanobakterije

TEST TOKSIČNOSTI S MIKROALGAMA

- *Pseudokirchneriella subcapitata* – pripada skupini zelenih alga (Chlorophyta)
- Jednostanična zelena alga koja je sastavni dio fitoplanktona kopnenih voda
- Stanice imaju oblik polumjeseca, nemaju bičeva i često dolaze u kolonijama od 4 do 16 stanica
- Svaka stanica ima po jedan dugi kloroplast i pirenoid, a kao pričuvni polisaharid stvara škrob
- Razmnožava se nespolno – autosporama

- Princip rada:

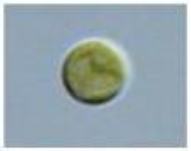
  – nekoliko generacija alga uzgaja se na hranjivoj podlozi pogodnoj za intenzivan rast

  - u eksponencijalnoj fazi rasta dodaju se različite koncentracije onečišćujuće tvari i tijekom 72 sata prati se inhibicija alga u odnosu na kontrolni pokus

  - rast alga prati se svaka 24 sata brojanjem stanica u Thominoj komorici

TEST TOKSIČNOSTI S MIKROALGAMA

Table 1.2: Appearance, characteristics and distributions of the algal test species

	P. subcapitata	*D. subspicatus*	*C. vulgaris*	*A. flos-aquae*	*S. leopoliensis*	*N. pelliculosa*	*P. tricornutum.*
Strain	CCAP 278/4	CCAP 258/137	CCAP 211/11b	CCAP 1403/13A	CCAP 1405/1	CCAP 1050/9	CCAP 1052/1b
Test meidum and pH	Kuhl, 6.8	Kuhl, 6.8	Kuhl, 6.8	JM, pH 7.8	JM, pH 7.8	ESAW + f/2, 8.2	ESAW + f/2, 8.2
Picture[a]							
Appearance[b]	Curved, twisted single cells	Oval, mostly single cells	Spherical, single	Chains of oval cells	Rods	Rods	Fusiform, triradiate, and ova (paper)
Size (LXW) µm[b]	8-14 X 2-3	7-15 X 3-12	3 (diameter)[d]	4.5 X 3	6 X 1	7.1 X 3.7	n.a
Cell volume	40-60	60-80	n.a.	30-40	2.5	40-50	n.a

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TEST TOKSIČNOSTI S MIKROALGAMA

Table 2.1 Summary of ecotoxicity data of pharmaceuticals to algae

Pharmaceutical class	Mode of action for human	Example of pharmaceuticals in this class	EC_{50} range (mg L^{-1})		
			Chlorophytes	Cyanobacteria	Others (e.g. diatom)
Analgesic	Inhibit both isoforms of cyclooxygenase, COX-1, COX-2, and COX-3 enzymes involved in prostaglandin (PG) synthesis	Fentanyl Paracetamol	0.98-134		
Androgenic	Activate the androgen receptor; activate certain estrogen receptors by conversing to estradiol	Testosterone	0.5		
Anesthetic	Block the sodium-channel and decrease chances of depolarization and consequent action potentials	Prilocaine Ropivacaine,	0.045-154		
Antiarrhythmic	Inhibit voltage gated sodium (Na+) channels	Lidocaine Dronedarone	0.045-780	0.25	
	Na, K-activated myocardial adenosine triphosphatase	Amiodarone			
Antiasthmatic	Antagonize leukotriene D_4 (LTD_4) at the cysteinyl leukotriene receptor	Montelukast	100		
Antibiotic	Inhibit ptidyl transferase; inhibit amino acids	(Macrolide) Clarithromycin Erythromycin, Tylosin,	0.002-1.38	0.034	
	Inhibit cell-wall synthesis enzyme	(β-lactam) Amoxicillin, Cefradine	1.77-630	0.0022-1.38	
	N.A.	Chloramphenicol Florfenicol	0.1-1283		1.3-38[b]

TEST TOKSIČNOSTI S MIKROALGAMA

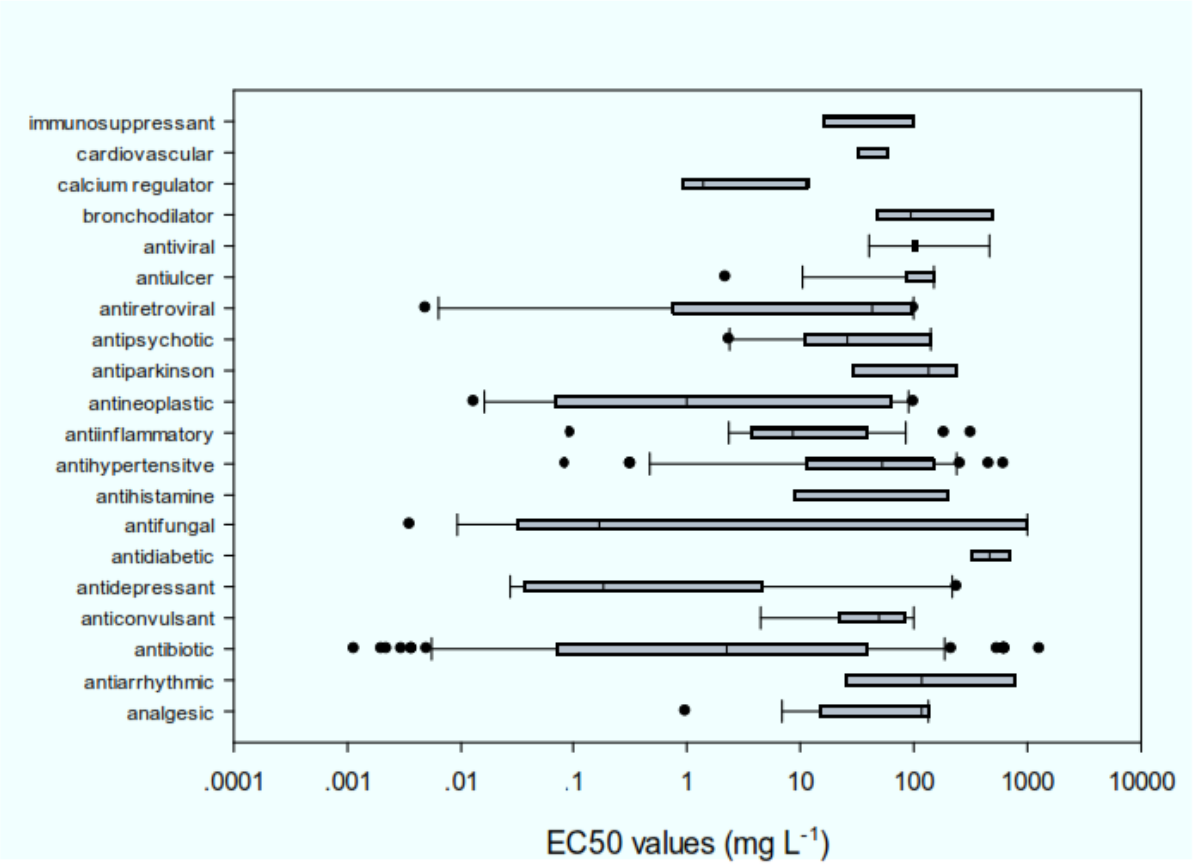


Figure 2.2 Toxicity value comparisons for selected therapeutic classes of pharmaceuticals

TEST TOKSIČNOSTI S MIKROALGAMA

Table 2.2 Toxicity of veterinary pharmaceuticals and environmental risk assessment to algae

Species	Pharmaceuticals	Test duration	EC_{50} (mg L^{-1})	Reference	PEC (mg L^{-1})	PEC:PNEC ratio*
Chlorella pyrenoidosa	Florfenicol	72h	215	(Lai et al., 2009)[1]	0.046	0.021
Chlorella vulgaris	Oxytetracycline	48h	6.4	(Pro et al., 2003)[3]	0.00021	0.0033
Desmodesmus subspicatus	Paracetamol	72h	134	(FASS, 2012)[3]	0.09	0.067
Microcystis aeruginosa	Amoxicillin	7d	0.008	(Liu et al., 2012)[3]	0.0099	122.98
	Amoxicillin	7d	0.0037	(FASS, 2012)[3]	0.0099	266.9
Pseudokirchneriella subcapitata	Tetracycline	72h	0.09	(Halling-Sorensen, 2000)[2]	0.00017	0.19
	Tiamulin	72h	0.003		0.0033	108.25
	Tylosin	72h	0.034		0.0035	10.42
	Oxytetracycline	72h	0.6	(van der Grinten et al., 2010)[2]	0.00021	0.035
	Trimethoprim	72h	9		0.49	5.46
	Tylosin	72h	0.0069		0.0035	39.81
	Erythromycin	72h	0.02	(Isidori et al., 2005b)[3]	0.0093	46.56
	Lincomycin	72h	0.07		0.044	62.46
	Oxytetracycline	72h	0.17		0.00021	0.12
	Amoxicillin	72h	630	(FASS, 2012)[3]	0.0099	0.0016
	Chlortetracycline	72h	3.1		0.00016	0.0053
	Fentanyl	72h	15.1		5.7E-06	3.77E-05
	Tetracycline	72h	2.2		1.7E-4	0.0076
	Tiamulin	72h	0.17		0.0032	1.97
	Tylosin	72h	1.36		0.0035	0.26
Scenedesmus obliguus	Enrofloxacin	72h	45.1	(Qin et al., 2012)[3]	2.29E-05	5.09E-05
Synechococcus leopoliensis	Amoxicillin	96d	0.0022	(FASS, 2012)[3]	0.0099	444.84
Tetraselmis chuii	Florfenicol	96h	6.06	(Goncalves et al., 2007)[3]	0.046	0.76
	Oxytetracycline	96h	11.18		0.00021	0.0019

*PNEC= EC_{50}/100

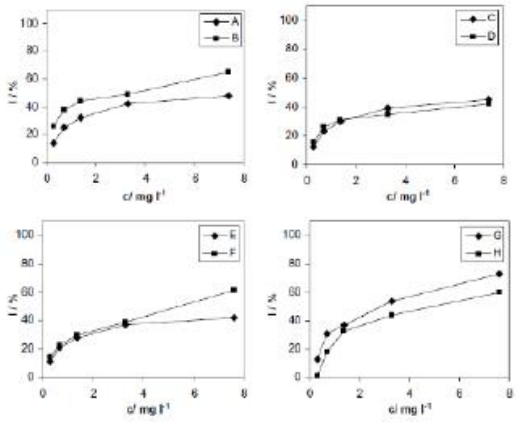
1 real concentration used; 2 nominal concentration used; 3 unknown

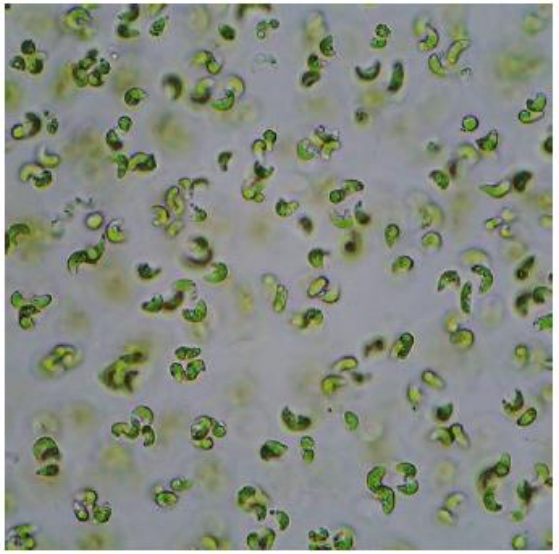
TEST TOKSIČNOSTI S MIKROALGAMA

PRIMJER: Utjecaj tenzida na toksičnost algi

- TENZIDI – površinske aktivne tvari
- Koriste se u: sredstvima za pranje u kućanstvu i industriji, u proizvodima za osobnu njegu, zaštitu od korozije

Primjer krivulje inhibicije rasta zelene alge *Pseudokirchneriella subcapitata* pod utjecajem pojedinih tenzida (A - natrij laurileterSulfat, B - natrij laurilmiristiletersulfat, C – amonij laurilsulfat, D – trietanolaminlaurilsulfat, E – dinatrij laurileterSulfosukcinat, F – kokosova masna kiselina kondenzat, G – decil poliglikozid, H – alkilamidopropilbetain)





Slika 4. Krivulja inhibicije rasta zelene alge *Pseudokirchneriella subcapitata* (Pavić, 2005)

TEST TOKSIČNOSTI S MIKROALGAMA

PRIMJER: Toksičnost pesticida na alge

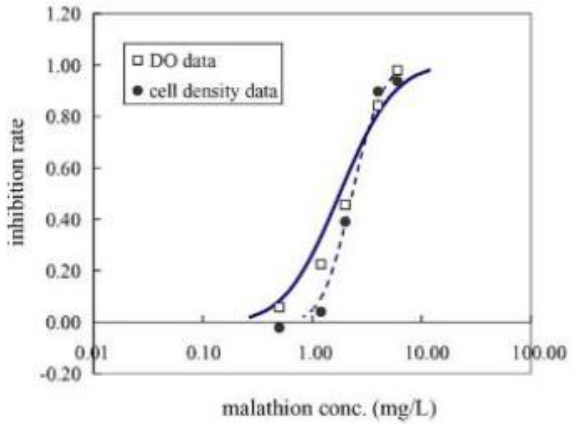


Fig. 1. The dose–response relationships of malathion on *Pseudokirchneriella subcapitata*.

Table 3
EC50 values and 95% confidence intervals of pesticides

Toxicants	EC50 (DO)	95% C.I.			EC50 (cell density)	95% C.I.		
Atrazine	0.0899	0.086	–	0.095	0.0784	0.075	–	0.082
MCPA	3.397	2.13	–	8.88	3.115	2.68	–	3.69
Dichlorvos	0.737	0.698	–	0.77	1.616	1.54	–	1.69
Parathion	1.162	1.10	–	1.22	0.927	0.88	–	0.97
Malathion	2.04	1.24	–	4.86	2.32	1.47	–	1.94
Fenthion	1.288	1.02	–	2.15	1.049	0.64	–	1.53
PCP	0.0035	3.03E-3	–	3.79E-3	0.0067	0.003	–	0.012

Unit: mg/L. C.I. = confidence interval.

TEST TOKSIČNOSTI S MIKROALGAMA (mikroplastika)

Vrsta alge	Vrsta plastičnih čestica	Rezultat istraživanja
Dunaliella tertiolecta	Peleti PS-a	Nema učinka na fotosintezu, ali smanjeni rast nakon izloženost koncentracijama 25 i 250 mg/L.
Thalassiosira pseudonana	Negativno nabijeni peleti PS-a	Nema učinka na fotosintezu nakon izloženost koncentracijama 25 i 250 mg/L.
Chlorella vulgaris	Negativno nabijene peleti PS-a	Nema učinka na fotosintezu nakon izloženost koncentracijama 25 i 250 mg/L.
Tetraselmis chuii	Sferične čestice PE-a	Nema učinka na rast nakon izlaganja koncentracijama 0,046–1,5 mg/L tijekom 96 h.
Chlorella sp.	Pozitivno nabijene nanočestice PS-a	Smanjena fotosinteza i povećano stvaranje reaktivnih oksidacijskih vrsta.
Scenedesmus spp.	Pozitivno nabijene nanočestice PS-a	Smanjena fotosinteza i povećano stvaranje reaktivnih oksidacijskih vrsta.
Skeletonema costatum	Mikročestice PVC-a	Inhibirani rast, smanjen klorofil i reducirana fotosinteza nakon izloženost koncentracijama 0–50 mg/L tijekom 96 h.
Scenedesmus obliquus	Nanočestice PS-a	Inhibirani rast i smanjen klorofil nakon izlaganja koncentracijama 44–1100 mg/L tijekom 72 sata.
Chlamydomonas reinhardtii	PE visoke gustoće i fragmenti PP-a	Nema utjecaja na rast.

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PRIMJER: Toxic effects of microplastic on marine microalgae *Skeletonema costatum*: Interactions between microplastic and algae

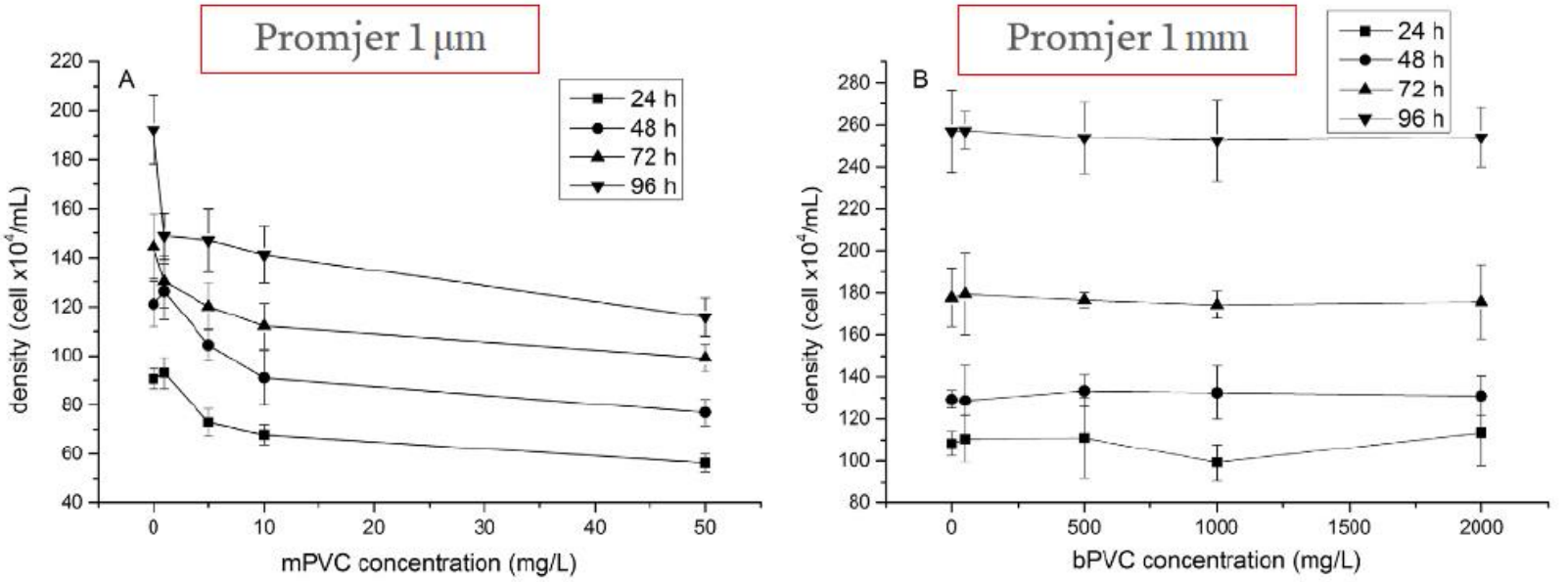


Fig. 3. Toxic effects of mPVC and bPVC of different concentrations on growth of microalgae with time. Square, circle, triangle and inverted triangle represented cell density under different mPVC concentrations at 24 h, 48 h, 72 h and 96 h, respectively. A: mPVC B: bPVC.

Utjecaj veličine na toksičnost algi

PRIMJER: Toxic effects of microplastic on marine microalgae *Skeletonema costatum*: Interactions between microplastic and algae

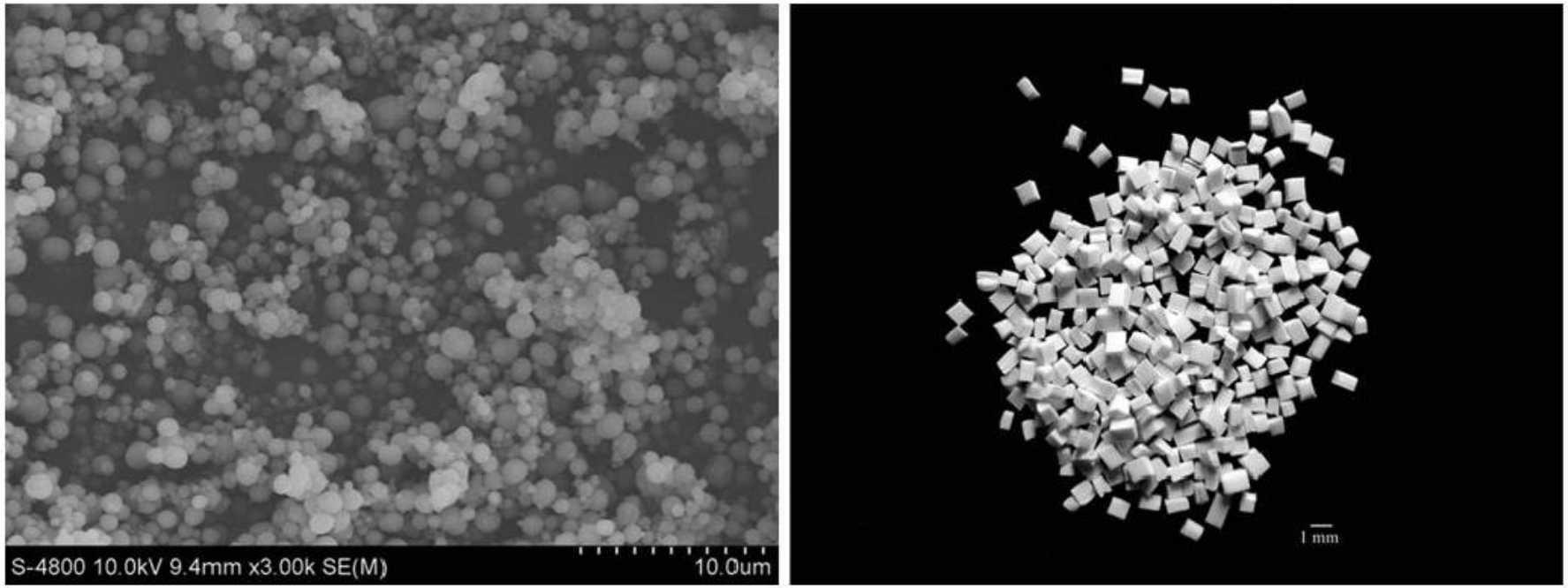


Fig. 1. The SEM image of mPVC and the photo of bPVC.

PRIMJER: Toxic effects of microplastic on marine microalgae *Skeletonema costatum*: Interactions between microplastic and algae

Učinkovitost fotosinteze

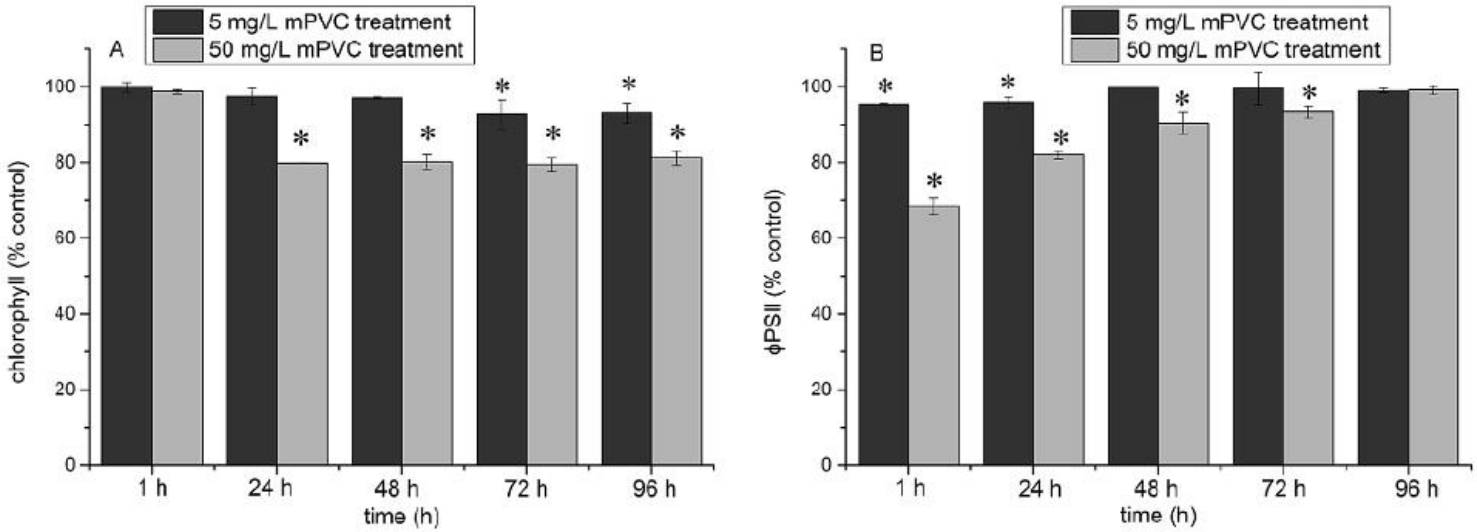


Fig. 4. Effects of mPVC of 5 and 50 mg/L concentrations on photosynthesis of microalgae with time. * represented significant difference ($p < 0.05$) with control (100%). A: chlorophyll B: ΦPSII.

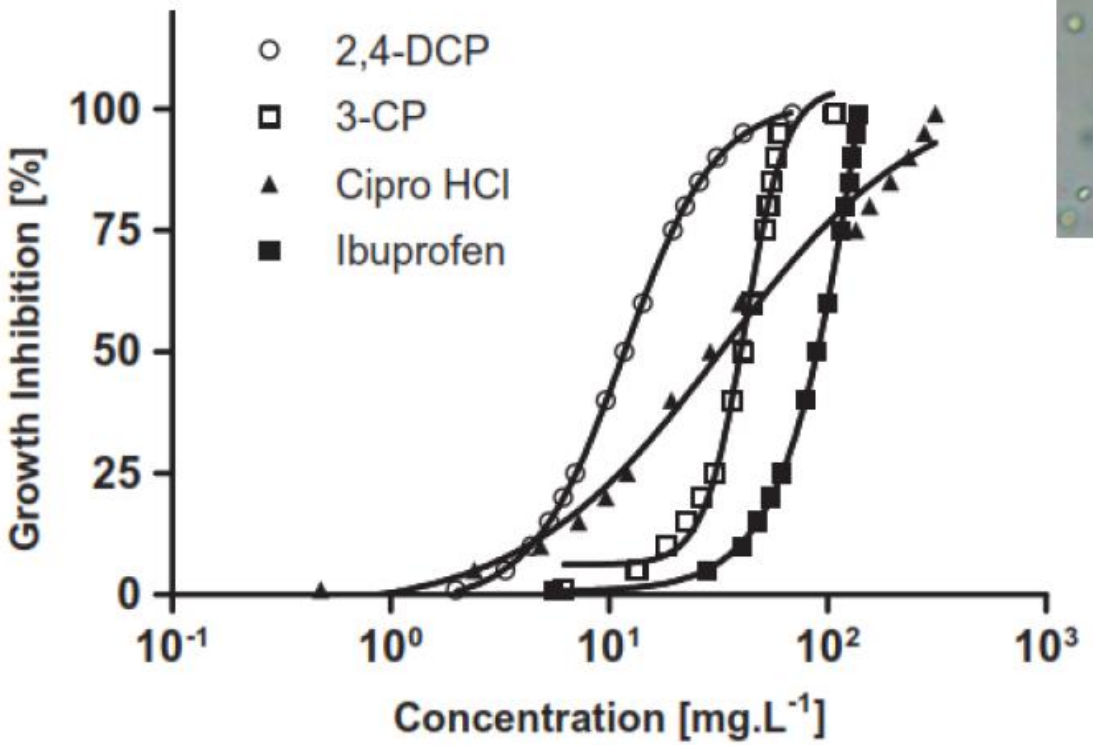
Povećanjem koncentracije PVC-a smanjuje se učinkovitost fotosinteze

Table 1
The ability of algal photosynthesis with time in algal growth inhibition tests.

mPVC concentration	Photosynthesis ability (%)				
	1 h	24 h	48 h	72 h	96 h
5 mg/L	95.3 ± 1.5[a]	93.4 ± 2.6	96.8 ± 0.8	92.2 ± 3.9	92.0 ± 4.2
50 mg/L	67.6 ± 2.4	65.4 ± 3.1	72.4 ± 1.4	74.2 ± 2.9	80.4 ± 2.4

[a] Results were expressed as average ± standard deviation.

PRIMJER: Single and mixture toxicity of pharmaceuticals and chlorophenols to freshwater algae *Chlorella vulgaris*



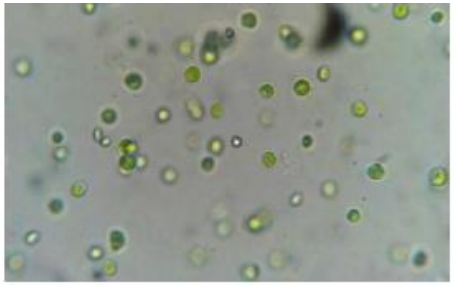


Fig. 1. Concentration-response curve for *C. vulgaris* exposed to individual compounds, 2,4-dichlorophenol, 3-chlorophenol, ciprofloxacin and ibuprofen based on specific growth rate and ICp calculations after exposure period of 96-h.

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PRIMJER: Single and mixture toxicity of pharmaceuticals and chlorophenols to freshwater algae *Chlorella vulgaris*

Table 2
50% and 20% inhibitory concentrations (IC_{50} and IC_{20}) calculated at the end of 48, 72 and 96 h based on different methods executed in ToxCalc software using specific growth rate (SGR) calculations, no-observed effect concentration (NOEC), lowest-observed effect concentration (LOEC) for *C. vulgaris*.

Compound	Method	48 h IC_{50} [mg L^{-1}]	48 h IC_{20} [mg L^{-1}]	72 h IC_{50} [mg L^{-1}]	72 h IC_{20} [mg L^{-1}]	96 h IC_{50} [mg L^{-1}]	96 h IC_{20} [mg L^{-1}]	NOEC [mg L^{-1}][a]	LOEC [mg L^{-1}][a]
2,4-dichlorophenol	ICp	11.04 (10.4–11.7)[b]	5.95 (4.9–6.8)	10.78 (10.2–11.5)	6.10 (4.9–6.7)	10.76 (10.1–11.6)	6.31 (6.0–6.7)	< 0.73	0.73
	Weibull	11.16(8.1–24.6)	5.30(0.9–7.5)	10.97(7.7–26.7)	4.80(0.7–7.0)	10.83(8.0–19.9)	5.49(1.0–7.6)		
	Probit	11.17(7.2–2623)	5.29(0.0–8.0)	10.83(7.2–97.5)	5.35(0.2–8.2)	10.73(7.2–94.3)	5.65(0.1–8.1)		
3-chlorophenol	ICp	40.52 (36.4–45.6)	23.02 (19.2–27.4)	39.98 (36.4–44.5)	24.67 (20.2–30.7)	40.92 (36.2–44.6)	26.54 (20.9–32.3)	15	30
	Weibull	38.99 (30.4–47.6)	24.42 (13.5–31.1)	38.10 (32.5–45.2)	26.46 (19.2–31.2)	39.03 (33.7–45.7)	27.78 (21.1–32.4)		
	Probit	37.21 (28.7–49.8)	25.23 (12.4–31.7)	36.24 (31.2–51.7)	27.63 (18.0–32.1)	36.90 (32.2–52.1)	28.72 (21.0–33.0)		
Ciprofloxacin HCl	ICp	34.62 (3.6–93.3)	9.83 (5.9–18.8)	27.89 (8.9–40.6)	9.23 (5.7–19.7)	29.09 (8.36–40.7)	9.67 (5.5–23.6)	< 20	20
	Weibull	40.66 (0.0–92.9)	5.80 (0.0–22.3)	30.34 (0.0–69.9)	3.97 (0.0–17.8)	31.63 (0.0–70.8)	4.46 (0.0–18.5)		
	Probit	38.02 ND	8.09 ND	29.45 ND	6.37 ND	30.65(0.0–75.3)	7.03(0.0–22.3)		
Ibuprofen25	ICp	82.25 (57.4–101.8)	48.20 (35.6–67.7)	86.43 (51.6–103.7)	51.05 (36.0–78.3)	89.65 (71.3–103.5)	54.84 (38.7–86.6)	35	70
	Weibull	77.39 (65.6–120.4)	52.94 (18.7–63.2)	80.72 (69.7–120.9)	56.57 (28.7–66.2)	82.34 (71.2–112.9)	57.80 (35.3–67.4)		
	Probit	75.70 ND	58.11 ND	77.19 ND	62.56 ND	77.69 ND	65.42 ND		

ND: not determined.
[a] NOEC and LOEC values were calculated using 72-h toxicity data.
[b] 95 % confidence intervals [mg L^{-1}].

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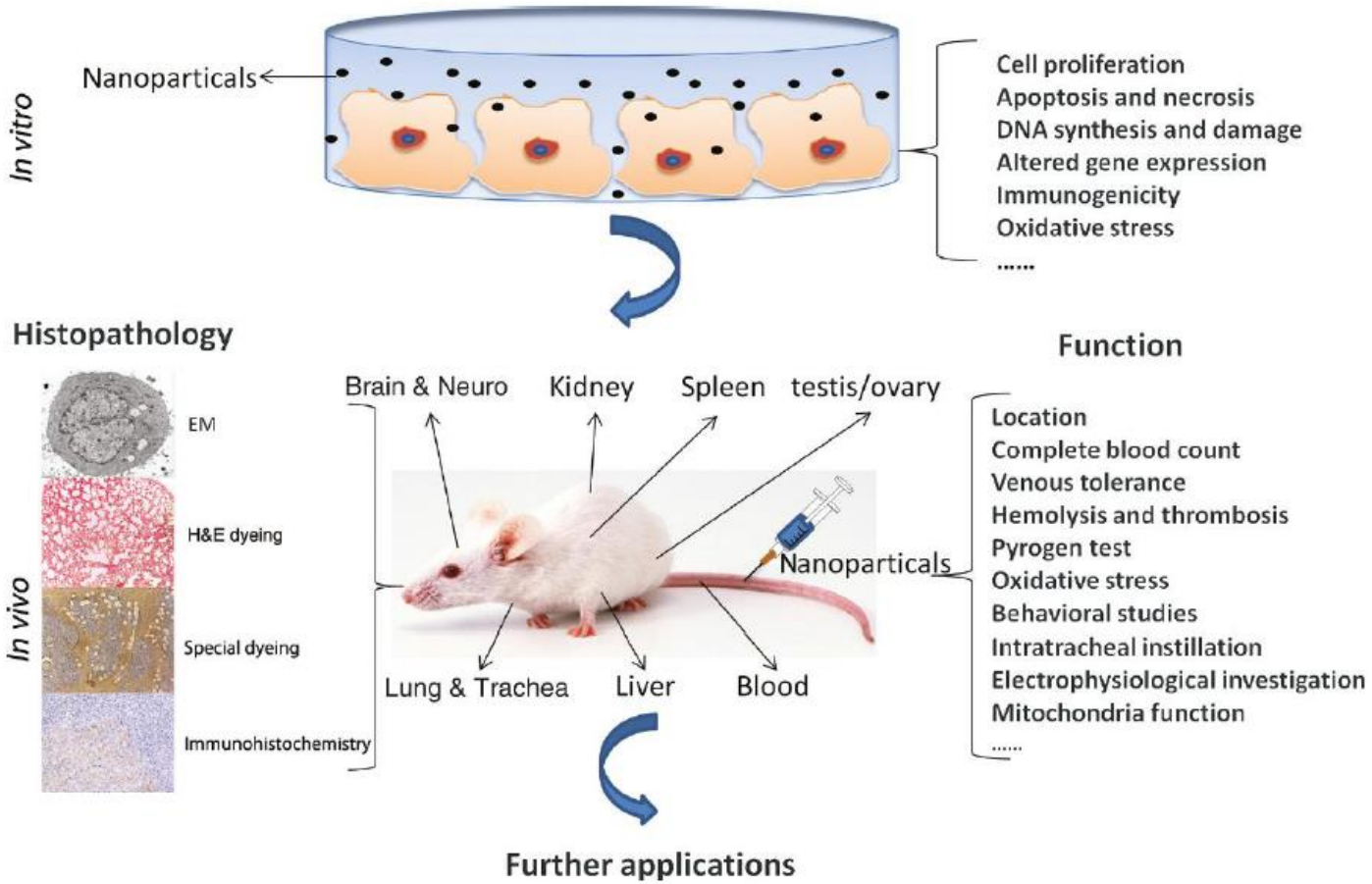
PRIMJER: Single and mixture toxicity of pharmaceuticals and chlorophenols to freshwater algae *Chlorella vulgaris*

Table 3
Model deviation ratios (MDRs) and Combination Index (CI) values at different growth inhibitory concentrations for binary mixtures for *C. vulgaris*.

Growth inhibition [%]	2,4-DCP + 3-CP IC_{50} = 28.2 [mg L^{-1}]			Ciprofloxacin HCl + Ibuprofen IC_{50} = 60.71 [mg L^{-1}]			2,4-DCP + ciprofloxacin HCl IC_{50} = 19.40 [mg L^{-1}]			3-CP + ciprofloxacin HCl IC_{50} = 38.88 [mg L^{-1}]			2,4-DCP + Ibuprofen IC_{50} = 48.18 [mg L^{-1}]			3-CP + Ibuprofen IC_{50} = 83.87 [mg L^{-1}]		
	MDR[a]		CI values[b]	MDR		CI values	MDR		CI values	MDR		CI values	MDR		CI values	MDR		CI values
	CA	IA		CA	IA		CA	IA		CA	IA		CA	IA		CA	IA	
1	0.17	0.09		**0.55**	0.14		**1.11**	0.17		**0.60**	0.11		2.06	**0.76**		0.32	**0.50**	
5	0.45	0.31	0.44	**0.55**	0.32	0.61	**1.31**	**0.63**	1.31	**0.68**	0.33	0.83	**1.71**	**1.27**	1.50	0.44	0.36	0.55
10	**0.81**	**0.56**	0.51	**0.59**	0.40	0.63	**1.67**	**1.08**	1.15	**0.75**	0.45	0.81	**1.36**	**1.00**	1.37	**0.89**	**0.63**	0.64
15	**1.10**	**0.75**	0.56	**0.63**	0.44	0.64	**1.66**	**1.20**	1.08	**1.51**	**1.00**	0.80	**1.25**	**0.87**	1.29	**1.13**	**0.76**	0.71
20	**1.07**	**0.73**	0.60	**0.67**	0.48	0.66	**1.47**	**1.11**	1.03	**1.35**	**0.95**	0.80	**1.17**	**0.81**	1.24	**1.17**	**0.79**	0.76
25	**1.05**	**0.73**	0.63	**0.70**	**0.51**	0.68	**1.35**	**1.04**	0.99	**1.26**	**0.91**	0.81	**1.11**	**0.76**	1.20	**1.21**	**0.82**	0.81
40	**1.08**	**0.74**	0.72	**0.91**	**0.67**	0.74	**1.15**	**0.90**	0.93	**1.18**	**0.85**	0.85	**1.01**	**0.70**	1.10	**1.28**	**0.85**	0.94
50	**1.09**	**0.75**	0.78	**1.02**	**0.81**	0.79	**0.97**	**0.83**	0.90	**1.11**	**0.87**	0.89	**0.96**	**0.67**	1.04	**1.28**	**0.85**	1.02
60	**1.10**	**0.77**	0.85	**1.05**	**0.85**	0.84	**1.08**	**0.96**	0.88	**1.17**	**0.94**	0.94	**0.97**	**0.69**	0.99	**1.29**	**0.84**	1.12
75	**1.08**	**0.77**	0.97	**0.84**	**0.85**	0.97	**0.88**	**1.09**	0.86	**0.96**	**0.96**	1.06	**1.00**	**0.73**	0.91	**1.29**	**0.83**	1.30
80	**1.06**	**0.76**	1.03	**0.84**	**0.83**	1.03	**0.90**	**1.14**	0.86	**1.04**	**1.00**	1.12	**1.00**	**0.73**	0.88	**1.29**	**0.82**	1.38
85	**1.03**	**0.73**	1.11	**0.84**	**0.80**	1.11	**0.87**	**1.14**	0.86	**1.17**	**1.08**	1.21	**0.98**	**0.71**	0.84	**1.30**	**0.80**	1.49
90	**0.99**	**0.70**	1.22	**1.01**	**0.89**	1.24	**0.82**	**1.08**	0.87	**1.29**	**1.10**	1.35	**0.95**	**0.68**	0.80	**1.30**	**0.77**	1.65
95	**0.92**	**0.63**	1.42	**1.29**	**0.95**	1.51	**0.71**	**0.91**	0.90	**1.40**	**0.99**	1.65	**0.90**	**0.62**	0.73	**1.30**	**0.73**	1.94

[a] The model deviation ratios (MDRs) are the ratios of observed experimental effect concentration divided by the effect concentration predicted by CA or IA. Bold text shows MDRs within a factor of two.
[b] CI values derived from CompuSyn (Chou and Martin, 2005). CI < 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively.

TESTOVI TOKSIČNOSTI



TESTOVI TOKSIČNOSTI – *In vivo*

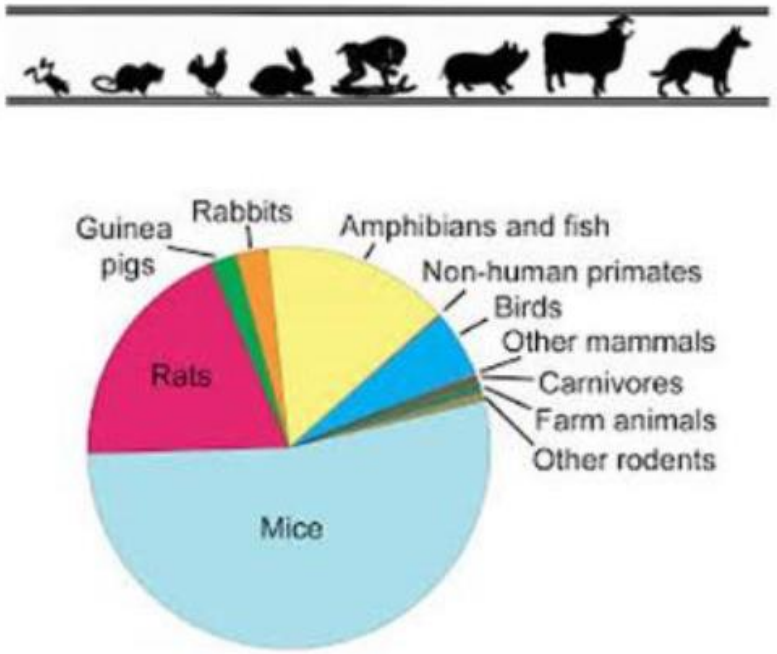


Figure 1 Percentage of animals used in testing.

TEST TOKSIČNOSTI S GLODAVCIMA

- Tri vrste glodavca se koriste za određivanje toksičnosti: ŠTAKOR, MIŠ, HRČAK
- *In vivo* test toksičnosti
- Mnoge vrste biomarkera - koriste za određivanje stanja ispitivanog glodavca nakon unošenja štetne tvari (masa tijela, hematologija)

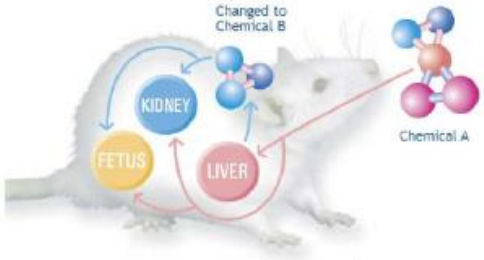


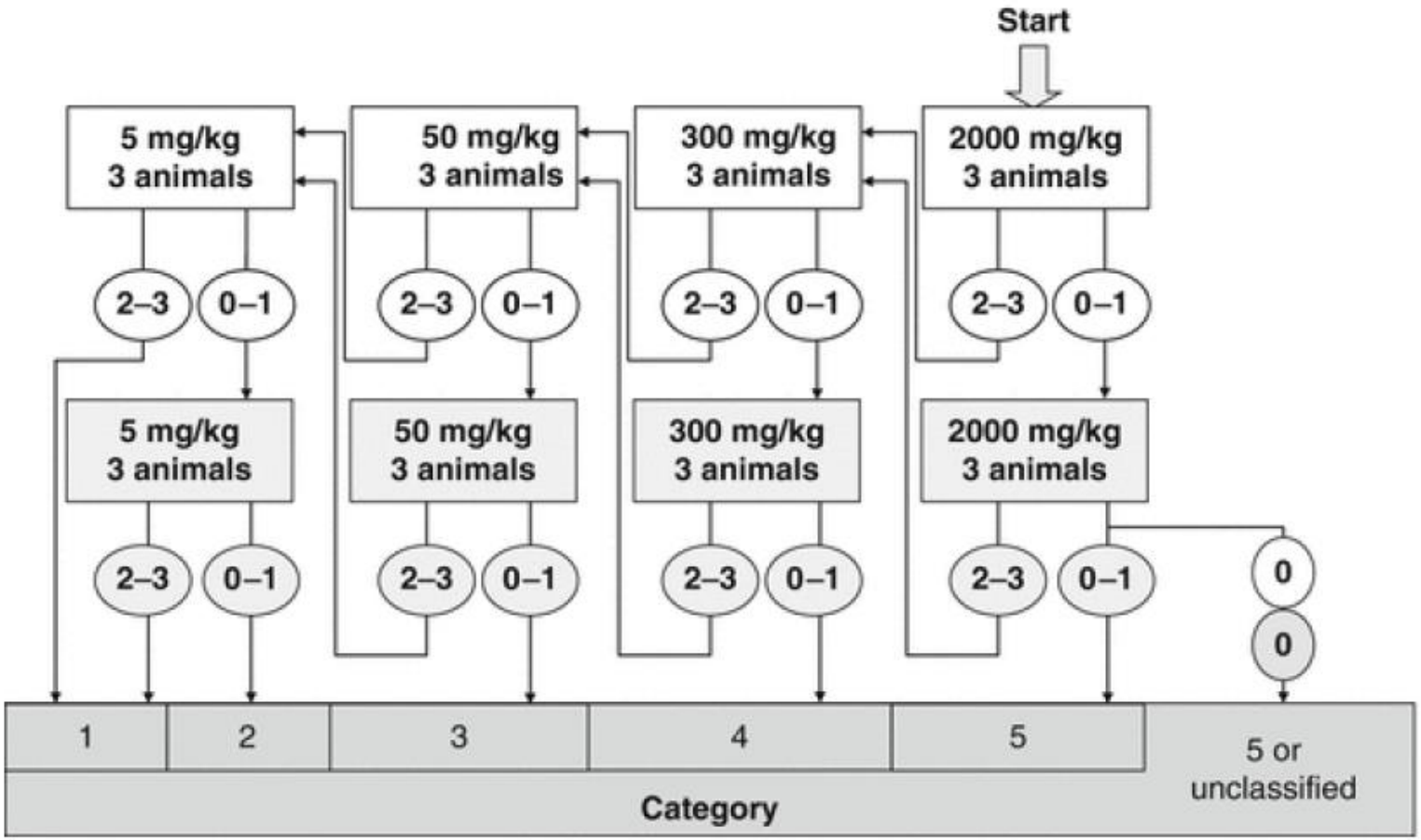
TABLE 2.1 Classic associations in toxicology

Liver Toxicity	Renal Toxicity
Increased plasma activity of liver marker enzymes: e.g. alanine and aspartate aminotransferases.	Increased water consumption and urine volume. Urine parameters may change, e.g. enzymes and cellular debris.
Decreased plasma total protein concentration.	Increased plasma concentrations of urea and creatinine. Proteinuria.
Increased coagulation times due to decreased synthesis of coagulation factors.	Severe renal toxicity may lead to decreased erythrocyte parameters due to effects on erythrocyte synthesis.
Increased liver weight due to enzyme induction or accumulation of lipid or glycogen.	Increased kidney weight.
Change in color or size at necropsy.	Change in color or size at necropsy.
Histological findings such as necrosis or centrilobular hypertrophy due to enzyme induction.	Histological change, e.g. basophilic tubules or necrosis, papillary necrosis, or glomerular changes.

TEST TOKSIČNOSTI S GLODAVCIMA

Akutna toksičnost	
Vrsta životinje	Štakor se preporučuje za inhalacijsku i dermalnu toksičnost, za dermalne testove preferiraju se zečevi kao i za testove iritacija oka (Draizeov test, primjenjuje se od 1944); osim glodavaca vrlo rijetko se koriste ostale životinje
Dob	Mlade životinje, najčešće oko 3-6 mjeseci
Broj životinja	5-10 za srođene sojeve, 10 ili više za nesrođene sojeve
Doza	Minimalno tri doze, niska (najčešće NOEL), srednja i visoka koja se aplicira jednokratno ako je aplikacija intraperitonealna, oralna, subkutana, intradermalna, intramuskularna; Za inhalacijsku aplikaciju izloženost minimum 4 sata a za dermalnu izloženost vrijeme izlaganja je do 24 sata (do analize)
Vrijeme trajanja pokusa i vrijeme analize	Do 24 sata od aplicirane doze, iznimno do 48 sati ako se istražuje eliminacija neke kemikalije

TEST TOKSIČNOSTI S GLODAVCIMA



TEST TOKSIČNOSTI S GLODAVCIMA

Subkronična toksičnost	
Vrsta životinje	Glodavci i to najčešće štakori a rjeđe miševi; zečevi uglavnom za dermalnu toksičnost;
Dob	Mlade životinje, najčešće oko 3-6 mjeseci
Broj životinja	Minimum 10 po spolu za nesrođene sojeva glodavaca; 5 za srođene sojeve, 4 za druge veće životinjske vrste (pas svinja) iznimno 1 životinja ako je pokusna životinja primat
Doza	Minimalno tri doze, niska (najčešće NOEL), srednja i visoka, preporučuje se svakodnevna aplikacija pojedinoj dozi osim za bioakumulativne kemikalije koje zahtijevaju posebne protokole
Vrijeme trajanja pokusa i vrijeme analize	28 dana ili 90 dana, ovisno o vrsti životinje

TEST TOKSIČNOSTI S GLODAVCIMA

Kronična toksičnost	
Vrsta životinje	Glodavci i to najčešće štakori, miševi i psi, pokusi se moraju provoditi na minimalno dvije vrste kako bi se kompenzirale razlike u fiziologiji
Dob	Mlade životinje, najčešće oko 3-6 mjeseci
Broj životinja	Minimum 20 po spolu za nesrođene sojeva glodavaca; 5 za srođene sojeve, 4 za druge veće životinjske vrste (pas, svinja) iznimno 1 životinja ako je pokusna životinja primat
Doza	Minimalno tri doze, niska (najčešće NOEL), srednja i visoka, preporučuje se svakodnevna aplikacija pojedinoj dozi osim za bioakumulativne kemikalije koje zahtijevaju posebne protokole
Vrijeme trajanja pokusa i vrijeme analize	12-24 mjeseci, ovisno o vrsti životinje, za aditive u hrani minimalno 24 mjeseca do tri godine

TEST TOKSIČNOSTI S GLODAVCIMA

- **Promjene** koje se prate:
  - smrtnost ili preživljavanje
  - kliničko praćenje (težina tijela, veličina tumora, apetit, promjene u ponašanju)
  - klinički parametri (biokemija krvi, hematologija, poremećaji u zgrušavanju, analiza urina i dr.)
  - patološki parametri (patohistološke analize)
  - procjena toksičnosti na staničnoj razini (genotoksičnost, citotoksičnost)
  - u studijama kancerogeneze (pojava tumora)

TEST TOKSIČNOSTI S GLODAVCIMA

Parameter	Units	Females		Males	
Hematology					
Age	Weeks	8	16	8	16
White blood cell count (WBC)	10^3 cells/µL	3.71	3.52	2.41	2.80
Red blood cell count (RBC)	10^6 cells/µL	10.51	10.35	10.79	10.68
Hemoglobin	g/dL	16.6	16.8	17.2	17.0
Hematocrit	%	48.3	46.8	48.5	48.0
Mean cell volume (MCV)	fL	45.9	45.2	45.0	45.0
Mean cell hemoglobin (MCH)	pg	15.8	16.2	15.9	15.9
Mean cell hemoglobin concentration (MCHC)	g/dL	34.4	35.9	35.4	35.4
Platelet count	10^3 cells/µL	956	927	1041	1005
Mean platelet volume (MPV)	fL	6.5	5.1	6.9	5.5
Percent reiticulocytes	%	3.0	3.0	2.9	3.1
Reticulocyte hemoglobin	pg	16.6	16.5	16.4	16.2
Reticulocyte count	10^3 cells/L	319.9	307.3	313.3	327.2
Percent neutrophils	%	15.5	22.2	22.2	24.3
Percent Lymphocytes	%	80.7	72.9	71.9	70.4
Percent Monocytes	%	0.8	1.2	1.3	1.3
Percent Eosinophils	%	2.3	2.7	4.0	3.0
Percent Basophils	%	0.2	0.5	0.4	0.6
Neutrophil count	10^3 cells/µL	0.58	0.77	0.53	0.67
Lymphocyte count	10^3 cells/µL	3.00	2.58	1.74	1.98
Monocyte count	10^3 cells/µL	0.03	0.04	0.03	0.04
Eosinophil count	10^3 cells/µL	0.08	0.09	0.09	0.08
Basophil count	10^3 cells/µL	0.01	0.02	0.01	0.02

Biochemistry					
Albumin	g/dL	3.7	2.8	3.4	2.7
Total protein	g/dL	5.8	5.6	5.8	5.9
Blood urea nitrogen	mg/dL	21	21	25	24
Calcium	mg/dL	10.2	9.8	9.9	9.8
Phosphorous	mg/dL	9.5	7.1	9.7	7.9
Cholesterol	mg/dL	76	69	102	113
HDL cholesterol	mg/dL	74.1	67.9	100.6	112.5
Triglycerides	mg/dL	123	175	182	128
Free fatty acids	mEq/L	2.75	2.59	2.61	2.73
Glucose	mg/dL	94	115	140	97
Alanine tranferase	IU/L	73	58	91	82
Creatine kinase	IU/L	1111	609	545	443
Thyroxine/T4	µg/dL	5.8	5.3	6.0	5.5

Parameter	Units	Females		Males	
Organ Weights					
Age	Weeks	8	16	8	16
Brain	g	0.386	0.385	0.377	0.379
	% of body weight	1.99	1.57	1.58	1.31
Heart	g	0.109	0.123	0.127	0.144
	% of body weight	0.56	0.50	0.53	0.50
Liver	g	1.144	1.412	1.366	1.408
	% of body weight	5.88	5.74	5.72	4.87
Left kidney	g	0.128	0.154	0.194	0.248
	% of body weight	0.66	0.63	0.81	0.86
Right kidney	g	0.133	0.162	0.192	0.252
	% of body weight	0.69	0.66	0.81	0.87
Spleen	g	0.091	0.106	0.078	0.084
	% of body weight	0.47	0.43	0.33	0.29

Body Composition by DEXA Analysis					
DEXA body weight	g; Total Tissue	19.99	23.51	23.62	29.38
Bone mineral density	g/cm^2	0.050	0.056	0.047	0.055
Bone mineral content	G	0.393	0.471	0.367	0.461
Bone area	cm^2	7.90	8.35	7.59	8.34
Lean tissue	g	15.8	18.4	18.2	23.2
Fat tissue	g	4.1	5.1	5.4	6.1
Percent fat tissue	%	20.7	21.5	22.8	20.8

Flow Cytometry - Spleen					
Lymphoid cells					
B cells					
B cells (B220+)	%	43.58	52.87	48.35	55.24
T cells					
CD4 T cells (CD3+, CD4+)	%	19.48	15.23	20.78	17.17
CD8 T cells (CD3+, CD8+)	%	10.75	7.88	10.37	8.15
NK cells (CD3-, NKG2D+)	%	3.50	2.78	2.25	2.71
NKT cells (CD3+, NKG2D+)	%	1.26	0.79	0.90	0.60
Myeloid cells					
Granulocytes (MAC1+, GR1+)	%	1.14	0.99	1.11	0.72
Monocytes (MAC1+, GR1-)	%	4.32	1.98	3.96	2.02

TEST TOKSIČNOSTI S GLODAVCIMA

Štakori – fiziološka i anatomska sličnost sa drugim vrstama

Razlika - dišu samo kroz nos, placenta je poroznija, crijevna mikroflora drugačija (razlika u metabolizmu)

The 3Rs - definitions

Replacement: methods which avoid or replace the use of animals in an area where animals would otherwise have been used.

Reduction: methods which minimise animal use and enable researchers to obtain comparable levels of information from fewer animals or to obtain more information from the same number of animals, thereby reducing future use of animals.

Refinement: improvements to husbandry and procedures which minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare.

TEST TOKSIČNOSTI S GLODAVCIMA

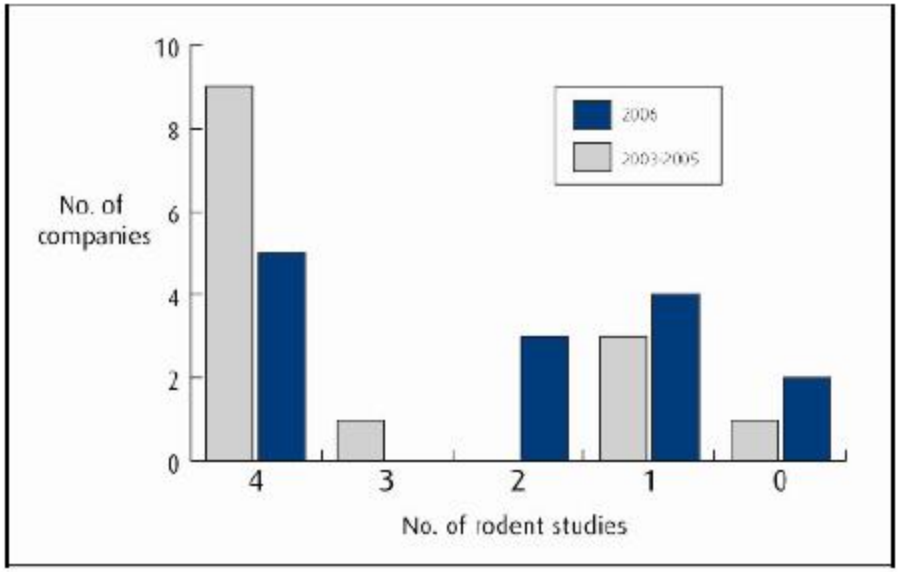


Figure 2: Number of rodent studies conducted per investigative compound in 2003-2005 compared to 2006

The maximum number of studies conducted by companies was four, which accounted for acute toxicity tests in two species using two different routes of administration. On the basis of the working group sharing best practice, many companies moved away from carrying out the full four studies, leading to a reduction in the number of studies conducted per compound.

TEST TOKSIČNOSTI S GLODAVCIMA

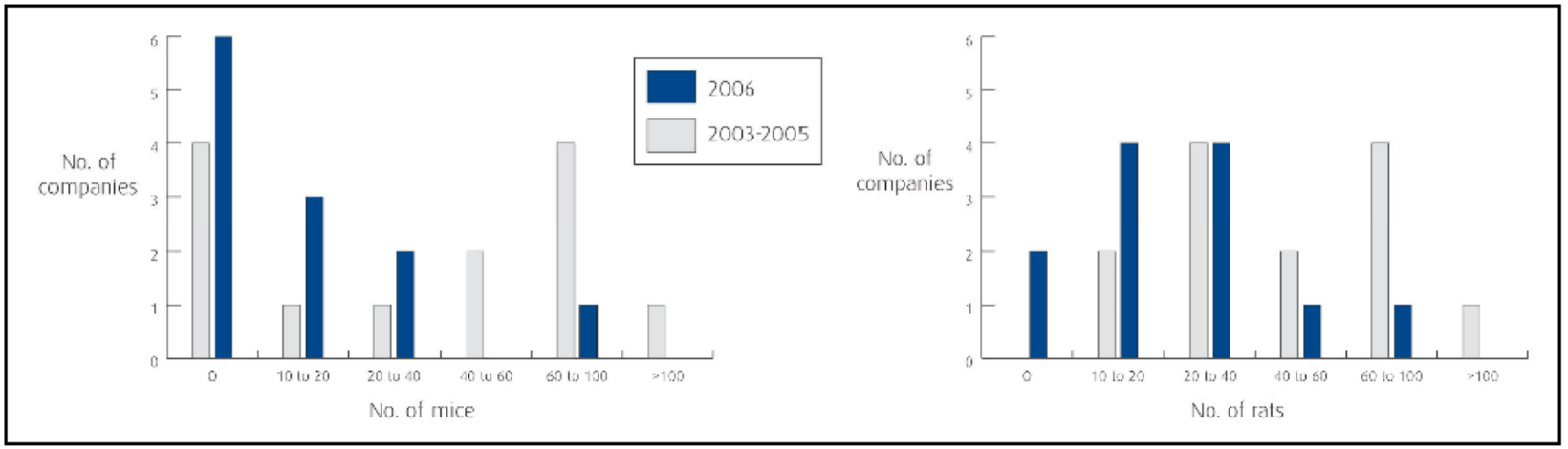
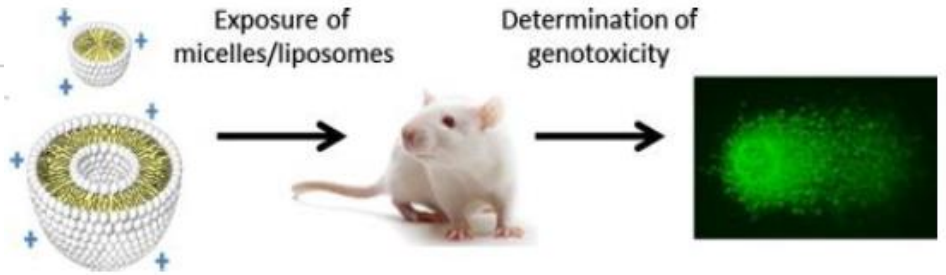


Figure 3: Number of mice used per compound in each company
The sharing of data and experience has led to a reduction in the number of mice used per conventional acute toxicity study. In 2003-2005 seven companies were using from 40 to over 100 mice per study. In 2006, since the working group shared best practice, most companies use less than 40 mice and more companies use no mice at all.

Figure 4: Number of rats used per compound in each company
The sharing of data and experience has led to a reduction in the number of rats used per conventional acute toxicity study. In 2003-2005 seven companies were using from 40 to over 100 rats per study. In 2006, since the working group shared best practice, most companies use less than 40 rats and two companies use no rats at all.

TEST TOKSIČNOSTI S GLODAVCIMA - MIŠ



- Testovi toksičnosti štetne tvari ispituju se na najmanje dvije vrste – glodavcima i na organizmu koji ne pripada skupini glodavaca
- Bitan faktor kod određivanja toksičnosti– **način primjene** (obavezno ispitati onaj način koji će se primjenjivati kod ljudi)
- **Prednosti** miša kao testnog organizma:
  - mali, relativno jeftini, lagani za rukovanje,
  - kratko vrijeme trudnoće,
  - kratak prirodni životni vijek
- U medicini –koriste se stotinama godina
- Najčešće se koriste za određivanje akutne toksičnosti
- Štakori se češće primjenjuju za određivanje – kronične toksičnosti

TEST TOKSIČNOSTI S GLODAVCIMA - MIŠ

FIZIOLOŠKE VRIJEDNOSTI MIŠA – prije izlaganja štetnoj tvari

TABLE 2.17 Normal physiological values general and reproductive

General	
Life span	
average	1–3 years
maximum reported	4 years
Adult weight	
male	20–40 g
female	18–40 g
Surface area	0.03–0.06 cm^2
Chromosome number (diploid)	40
Food consumption	4–5 g/day
Water consumption	5–8 mL/day *ad libitum*
Body temperature	36.5°C
oxygen consumption	1.69 mL/g/hr
Reproductive	
Age, sexual maturity	
male	50 days (20–35 g)
female	18–40 g
Breeding season	continuous, cyclic
Estrus cycle	4–5 days
Gestation period	
average	19 days
range	17–21 days
Litter size	
average	12
range	1–23
Birth weight	1.5 g
Age begin dry food	10 days
Age at weaning	16–21 days (10–12 g)

Source: Data derived from Jacoby and Fox (1984).

TABLE 2.18 Normal physiological values cardiovascular and respiratory

Cardiovascular	
Heart rate	
average	600/min
range	320–800/min
Blood pressure	
Systolic	113–160 mm Hg
Diastolic	102–110 mm Hg
Blood volume	
plasma	45 mL/kg
whole	78 mL/kg
Hematocrit	41.5%
RBC life span	20–30 days
RBC diameter	6.6 microns
Plasma pH	7.2–7.4
Respiratory	
Rate	
average	163/min
range	320–800/min
Tidal volume	
average	0.18 mL
range	0.09–0.38 mL
Minute volume	
average	24 mL/min
range	11–36 mL/min

Source: Data derived from Jacoby and Fox (1984), and from the Animal Diet Reference Guide, Purina Mills, Inc. (Ralston Purina Company, 1987).

TEST TOKSIČNOSTI S GLODAVCIMA - MIŠ

TABLE 2.22 Typical acute toxicity study design for mice

Number of mice/sex/dose group	3–5
Number of dose groups	1–3
Number of control groups	None
Dosing frequency	Single dose
Dosing days	1 day
Survival checks	Not done (part of Clin. Obs.)
Clinical observations	4 or more on day of treatment, then 1–2 daily
Physical examinations	Not done
Body weights	Prior to dosing
Feed consumption	Not done
Number of reversal mice	None
Duration of reversal period	Not applicable
Blood collection	Not done
Hematology parameters	Not done
Clinical chemistry parameters	Not done
Urine collection	Not done
Necropsy	Gross (increasingly, but rarely useful)
Tissue collection	Rarely (specific cause only)

TABLE 2.23 Typical short-term toxicity study design for mice

Number of mice/sex/dose group	5–40
Number of dose groups	3–4
Number of control groups	1
Dosing frequency	Once, daily
Dosing days	Daily for 7–90 days
Survival checks	1–2 daily
Clinical observations	Daily
Physical examinations	Weekly
Body weights	Weekly
Feed consumption	Weekly
Number of reversal mice	None
Duration of reversal period	None
Blood collection	Terminal, all animals
Hematology parameters	None
Clinical chemistry parameters	Limited
Urine collection	Not done
Necropsy	Gross, all animals
Tissue collection	Limited list, all animals

TEST TOKSIČNOSTI S GLODAVCIMA - MIŠ

TABLE 2.24 Typical chronic toxicity study design for mice

Number of mice/sex/dose group	20–50
Number of dose groups	3
Number of control groups	1
Dosing frequency	Once, daily
Dosing days	Daily 26–52 weeks
Survival checks	Daily
Clinical observations	Not done
Physical examinations	Weekly
Body weights	Weekly
Feed consumption	Weekly
Number of reversal mice	25–35% of main groups
Duration of reversal period	2–4 weeks
Blood collection	Terminal, all animals
Hematology parameters	Dif. smear, RBC, WBC
Clinical chemistry parameters	Limited list
Urine collection	Not done
Necropsy	Gross, all animals
Tissue collection	Comprehensive list, all animals

TABLE 2.25 Typical carcinogenicity study design for mice

Number of control groups	2
Dosing frequency	Once, daily
Dosing days	Daily for 18–24 months
Survival checks	Daily
Clinical observations	Not done
Physical examinations	Monthly during first year, 2 × /month thereafter
Body weights	Weekly during first 2 months, 2 × /month thereafter
Feed consumption	Weekly during first 2 months, 2 × /month thereafter
Blood collection	Optional periodic, terminal
Hematology parameters	Periodic peripheral smears, terminal smears, RBC, WBC
Clinical chemistry parameters	Not done
Necropsy	Gross, all animals (including those found dead during study)

TEST TOKSIČNOSTI S GLODAVCIMA - HRČAK

- Najčešće se koriste za ispitivanje **kancerogenosti** neke štetne tvari (mala prirodna mogućnost nastanka tumora)
- **Prednosti:**
  - Brza reprodukcija
  - Jedinstvene anatomske i fiziološke značajke
  - Brzi fiziološki razvoj
  - Kratak životni vijek
  - Mala učestalost spontanih bolesti
  - Model za ispitivanje dijabetesa

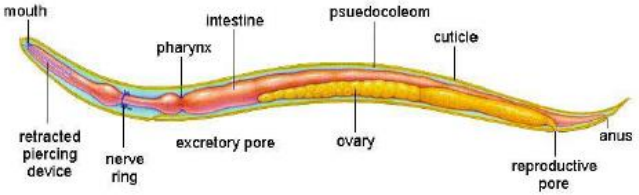
TABLE 2.31 Common and species names of hamsters and chromosome number

Common Name	Species Name	Chromosome Number
Syrian (Golden)	*Mesocricetus auratus*	44
Chinese (Striped, Black)	*Cricetus griseus* or *barabensis*	22
European (Common, Black, Field)	*Cricetus cricetus*	22
South African	*Mystromys albicaudatus*	32
Rumanian (Newtoni's)	*Mesocricetus newtoni*	38
Turkish (Kurdanti)	*Mesocricetus brandti*	42/44
Armenian (Gray, Migratory)	*Cricetulus migratorius*	22
Djungarian (Striped hairy-footed)	*Phodopus sungorus*	28

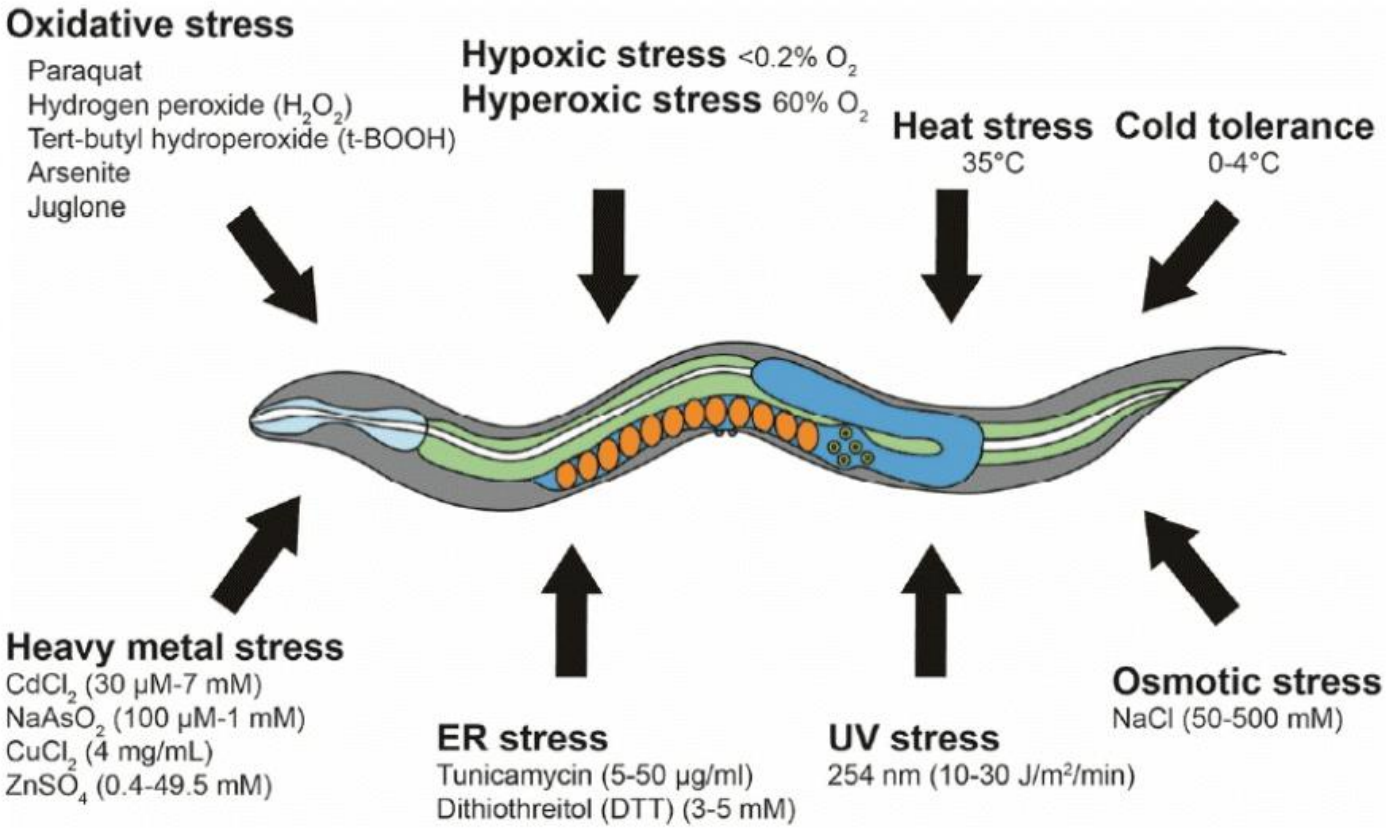
TEST TOKSIČNOSTI S NEMATODAMA

NEMATODE

- skupina organizma koja naseljava brojne ekosustave
- najdominantnija skupina živih organizama u tlu
- grč. riječ *nema* – konac, nit i *eidos* slično
- oblik tijela okrugao, končast, vretenast i sl.
- sudjeluju u brojnim životnim procesima
- koriste se u ekotoksikološkim ispitivanjima i monitoringu onečišćenja
- tijelo – mliječno-bijele do žućkaste boje, ne posjeduju pigment
- tijelo nematoda "cijev u cijevi" (kožni pokrov građen od nekoliko sloja)
- Veličina nematoda varira – nevidljive ljudskom oku (μm) do 8 m
- Neke vrste nemetoda su nametnici ljudi i životinja

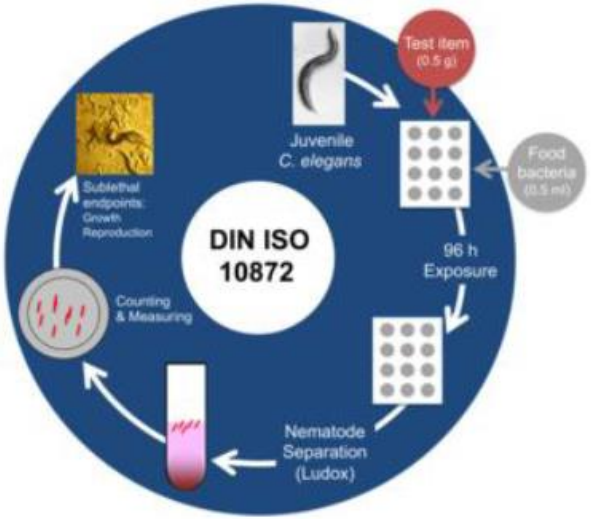


TEST TOKSIČNOSTI S NEMATODAMA

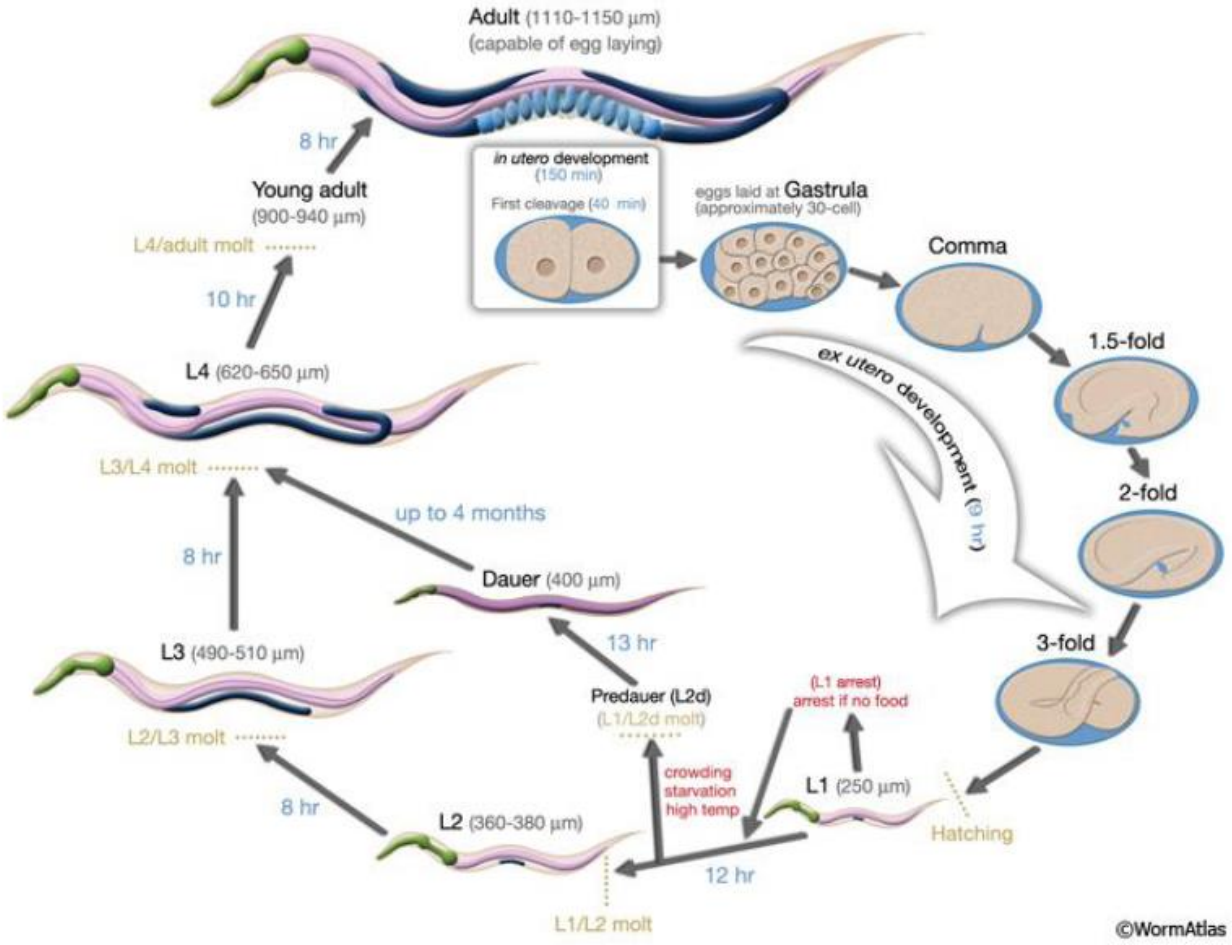


TEST TOKSIČNOSTI S NEMATODAMA

- Nematoda koja se često koristi u znanstvenim istraživanjima – *Chenorhabditis elegans*
- Nematodama za rast i razmnožavanje vrlo bitna voda, ali i poroznost, aeriranost, sadržaj organske tvari
- Nematode – ukazuju na indeks zrelosti tla
- *Chenorhabditis elegans* - primjenjuje se za različita neurološka ispitivanja
- DIN ISO 10872: Nematode Toxicity Test - Water quality - Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)
- Prednosti:
  - Primjenjuju se za ispitivanje ekotoksičnosti sedimenta/tla i vode
  - Traje 96 h
  - Kronična toksičnost (cijeli generacijski ciklus obuhvaća)
  - Potrebna je mala količina nematoda (0,5 g)



TEST TOKSIČNOSTI S NEMATODAMA



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TEST TOKSIČNOSTI S NEMATODAMA

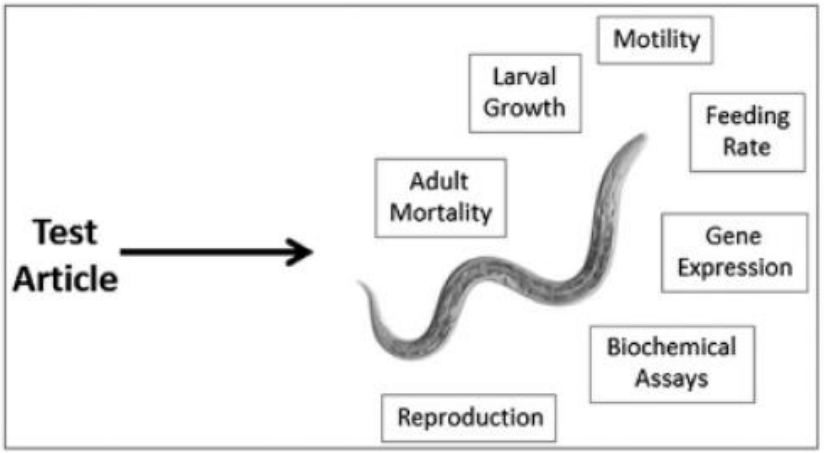


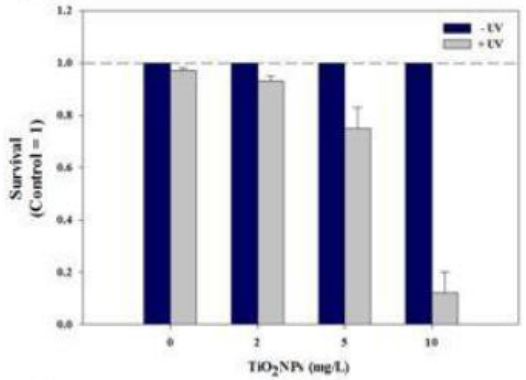
Figure 1. Toxicity testing in *C. elegans* can provide a bridge between *in vitro* and mammalian *in vivo* testing.

Table 1. *C. elegans* culture standardization factors for toxicology and GCeCP

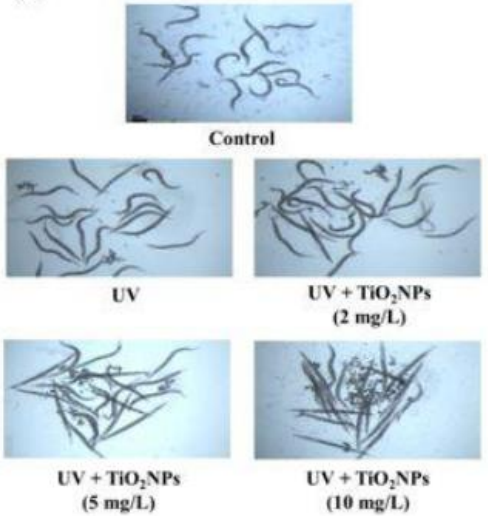
Factor	Details
Temperature	Small temperature differences have a large effect on *C. elegans* growth rate, motility, lifecycle, lifespan, and gene expression. The method and duration of vessel contact with human hands and metal surfaces can significantly affect many endpoints. Handling culture vessels by edges that are not in contact with medium, and a layer of Styrofoam on work surfaces will reduce heat exchange.
Humidity	Low humidity will alter test article and nutrient concentration, the smaller the volume the larger the effect. Unless equipment is enclosed and carefully climate controlled, it is unlikely that the small volumes used in HTS will work well with *C. elegans* assays. For larger test volumes, incubators can be humidified with an open vessel of water that is cleaned and refilled regularly.
pH	Extreme pH is required to alter adult *C. elegans* viability, but other endpoints are more sensitive to pH. The appropriate pH range should be determined for each assay, and the pH of test articles in assay medium must be assessed and reported.
Worm Density	*C. elegans* gene expression and life cycle respond to nutrient availability and secreted hormones. Given a 3-day generation time and ~300 progeny per worm, cultures can easily outstrip nutrients if they are not consistently monitored. Conversely, *C. elegans* do not grow well if maintained too sparsely. Note that the worms will not necessarily die in these conditions, instead they will adapt epigenetically (Hall *et al.*, 2010), potentially resulting in altered toxicity test results for several generations.
Cohort Synchronization	A cohort of 1st larval stage (L1) *C. elegans* can be isolated by hypochlorite treatment of gravid hermaphrodites (an egg prep) followed by hatching of the released eggs in non-nutrient buffer. In the absence of nutrients, these L1's halt development just after hatching. At 20 °C, about 12 hours are required for all the eggs to hatch. After more than 18 hours in buffer, gene expression is altered resulting in delayed and unsynchronized development, and increased stress resistance (Jobson *et al.*, 2015; Nass and Hamza, 2007). Some genetics protocols state that hatched L1's can be maintained in non-nutrient buffer and used for a week or more, but this will result in variable toxicity outcomes.
Dauers	The *C. elegans* dauer larva is a stress resistant, long-lived alternate to the 3rd larval stage (L3). Dauers must revert to the normal lifecycle in order to grow and reproduce. *C. elegans* dauers secrete dauer pheromone, which promotes conversion to the dauer state in other larvae and induces increased stress resistance in exposed adults. This will both reduce apparent growth rates as measured by worm length (dauers can remain at the L3 length for months or even years), and increase viability in the presence of many toxins. Dauers are thinner and darker than L3s of similar length, and lack the clearly defined gonadal region and visible intestinal lumen identifiable in developing *C. elegans*. Liquid cultures are unlikely to produce dauers if they are consistently maintained with adequate nutrient supply and are started from agarose cultures that were well fed for at least 3 generations. Daily media exchange along with a few sequential egg preps as soon as each generation becomes gravid can sometimes free a culture of dauers.
Genetic Drift	Genetics labs often maintain commonly used *C. elegans* strains at room temperature as dauers. If *C. elegans* cultures are consistently well fed for optimal toxicity studies, use of frozen stocks must be scheduled in order to prevent genetic drift.
Males	Non-disjunction of the X chromosome results in XO males. This happens rarely in nature, and is induced by toxins and stress. *C. elegans* males can be identified by their flared tail and single gonad arm. Males are smaller than the XX hermaphrodites, which will result in apparent reduced growth if automated methods are used and technicians are not trained to recognize males. Mating with males more than doubles the progeny per hermaphrodite relative to selfing, so males in a culture will increase reproductive output. Removing males from a culture requires isolating developing hermaphrodites away from the males.
Solid vs. Liquid Medium	When the test article is mixed into molten agar, or spread in solution onto solidified agar, and then the *E. coli* feeder organism is grown in a lawn on top of the dosed agar, the true exposure will depend on many factors such as humidity, compound solubility, compound-agar interaction, and feeder organism uptake and metabolism. Dosing in liquid medium provides a measureable exposure, but limits the test to water-soluble compounds.
E. coli vs. Axenic Medium	Axenic medium avoids the complicating factor of the metabolic response of the feeder organism, which is especially important for test articles with antibiotic activity. Killed *E. coli* are sometimes used (usually heat or UV), but the method and exposure time must be carefully controlled to avoid the bacteria producing toxins which will alter assay results.

TEST TOKSIČNOSTI S NEMATODAMA (toksičnost nanočestica)

(A)



(B)



(C)

	24h LC (mg/L)	Interval of Confidence (95%)
LC 10	4.696	2.238<LC 10<6.000
LC 50	**7.880**	6.250<LC 50<10.04
LC 90	13.22	10.29<LC 90<28.52

(D)