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Nikotinski acetilholinski receptor kot farmakološka tarča pri pljučnem raku

Nicotinic acetylcholine receptor as a pharmacological target in lung cancer

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Izvleček: Rak pljuč je zelo razširjena oblika raka z nizko stopnjo preživetja. Kajenje tobaka predstavlja glavni dejavnik tveganja za razvoj raka pljuč, saj v tobačnem dimu najdemo veliko rakotvornih snovi. Nikotin, ki sicer ni opredeljen kot rakotvoren, je glavna komponenta tobaka odgovorna za zasvojenost, poleg tega pa raziskave nakazujejo, da neodvisno od ostalih komponent tobaka v telesu sproži različne učinke, ki vplivajo na razvoj in napredovanje raka. Kot agonist nikotinskih acetilholinskih receptorjev (nAChR) nikotin spodbuja proliferacijo celic, preprečuje njihovo apoptozo, ter igra pomembno vlogo pri pospeševanju angiogeneze in zasevanju rakavih celic. Antagonisti nAChR, ki bi zavirali proliferacijo rakavih celic ter spodbujali njihovo apoptozo, predstavljajo velik terapevtski potencial. Trenutno je znanih le malo antagonistov nAChR, pri katerih je bila protirakava učinkovitost že raziskana, poleg tega pa po večini tudi niso dovolj selektivni ligandi za podvrste nAChR, ki se prekomerno izražajo v celicah pljučnega raka, zato je pričakovati škodljive stranske učinke. V izogib slednjim, se išče načine za ciljano dostavo antagonista nAChR do rakavih celic. Precej obetajo nanodostavni sistemi, ki omogočajo prednostni vnos aktivne učinkovine v celice raka. V našem članku predstavljamo najnovejše dosežke razvoja zdravil za zdravljenje pljučnega raka na osnovi antagonstov nAChR, dostavljenih na mesto delovanja s pomočjo nanodelcev.

Ključne besede: agonist, antagonist, apoptoza, nAChR, nanodostavni sistem, nikotin, pljučni rak

Abstract: Lung cancer is a widespread form of cancer with a low survival rate. Tobacco smoking is a major risk factor for the development of lung cancer, as tobacco smoke contains many carcinogens. Nicotine, which is not classified as a carcinogen, is the main component of tobacco, responsible for addiction and recent research suggests that nicotine, independent of other tobacco components, may contribute to the development and progression of cancer. Nicotine, as an agonist of nicotinic acetylcholine receptors (nAChRs), promotes cell proliferation, prevents apoptosis, and has an important role in promoting angiogenesis and metastasis of cancer cells. The realisation that nAChRs are involved in the development and progression of lung cancer has raised the idea of using nAChR antagonists that would counteract the adverse effects of nicotine. Currently, there are only a few nAChR antagonists for which anticancer efficacy has been investigated. Many of the known antagonists do not act selectively on nAChR subtypes that are overexpressed in lung cancer cells. Nonselective nAChR antagonists can cause adverse side effects by acting on nAChR subtypes expressed on non-cancerous cells. In order to avoid such side effects, it is necessary to ensure that a given antagonist acts predominantly on cancer cells. This can be achieved by using nanodelivery systems that are preferentially uptaken by cancer cells. In this article, we present the latest achievements in the development of drugs for the treatment of lung cancer based on nAChR antagonists delivered to the site of action by nanoparticles.

Keywords: agonist, antagonist, apoptosis, lung cancer, nAChR, nanodelivery system, nicotine

Uvod

Rak pljuč spada med najpogosteje diagnosticirane oblike raka. Odgovoren je za več kot 18 % vseh z rakom povezanih smrti (Bray in sod. 2018). Za posledicami pljučnega raka na svetu letno zboli 2 milijona, umre pa 1.8 milijona ljudi (Thandra in sod. 2021). Tudi v Sloveniji imamo visoko stopnjo obolevnosti za rakom pljuč; letno zboli več kot 1400 ljudi, incidenca obolenj pa v zadnjih letih narašča (Onkološki inštitut Ljubljana 2022). Razlikujemo več histoloških oblik pljučnega raka, ki se v osnovi delijo na drobnocelični karcinom pljuč (angl. small-cell lung carcinoma oz. SCLC), ki predstavlja približno 20 % vseh rakov pljuč, in nedrobnocelični karcinom pljuč (angl. non-small-cell lung carcinoma oz. NSCLC), ki predstavlja 70-80 % vseh rakov pljuč. NSCLC lahko razdelimo na ploščatocelični karcinom (25-30 % vseh rakov pljuč), pljučni karcinom velikih celic (10-15 % vseh rakov pljuč) in adenokarcinom pljuč (približno 40 % vseh rakov pljuč), slednji je od vseh oblik pljučnega raka njegova najpogostejša oblika (Shiraishi in sod. 2012, Wang in Hu 2018).

Epidemiološke študije kažejo, da je kajenje tobaka glavni etiološki dejavnik za razvoj velike večine oblik raka pljuč (Schuller 2019), saj je odgovorno za kar 90 % vseh primerov (Thandra in sod. 2021). Tobačni dim vsebuje več kot 7000 različnih kemikalij, od katerih jih je več kot 70 s strani Mednarodne agencije za raziskave raka (International Agency for Research on Cancer – IARC) uvrščenih med rakotvorne snovi (El-Bayoumy in Stoner 2022). V tobačnem dimu najdemo številne mutagene in rakotvorne kemikalije, kot so aldehidi, policiklični aromatski ogljikovodiki in nitrozamini (derivati nikotina) (Mao in sod. 2016). Konzumacija nikotina spet narašča zaradi vse večje popularnosti uporabe elektronskih cigaret (e-cigaret), ki jih promovirajo kot ″varnejšo″ alternativo klasičnim cigaretam. Pomanjkanje znanja o biomedicinskih učinkih polnilnih tekočin za e-cigarete predstavlja veliko skrb za zdravje, še posebej, ker proces segrevanja teh tekočin vodi do njihove razgradnje in nastanka novih, potencialno strupenih spojin (Marques in sod. 2021). Čeprav nikotina pogosto ne uvrščamo med rakotvorne snovi, ampak kot glavno sestavino tobaka, odgovorno za zasvojenost, pa je znano, da s svojim agonističnim delovanjem na nikotinske acetilholinske receptorje (nAChR), spodbuja proliferacijo rakavih celic, preprečuje njihovo apoptozo in pospešuje angiogenezo, s tem pa zasevanje rakavih celic (Friedman in sod. 2019). Poleg tega so nekatere raziskave pokazale, da nikotin lahko deluje genotoksično in tudi na tak način spodbuja nastanek tumorjev (Grando 2014, Sanner in Grimsrud 2015).

Čedalje boljše poznavanje biologije pljučnega raka je pripeljalo do razvoja novejših metod zdravljenja raka, kot je imunoterapija raka, pri kateri spodbudimo imunski sistem pacienta, da prepozna in uniči celice raka preko naravnih mehanizmov (Calles in sod. 2019, Doroshow in sod. 2019, Riley in sod. 2019). Kljub velikemu napredku diagnostike, genske tehnologije, operacijskih postopkov ter bioloških in kombiniranih terapij, je stopnja preživetja pri raku pljuč za obdobje petih let le okoli 10-20 % (Mao in sod. 2016). Velik problem pri zdravljenju raka pljuč predstavlja neselektivno delovanje zdravil tudi na zdrave celice in pojav rezistence na kemoterapijo. Zaradi vloge, ki jo imajo nAChR pri razvoju in napredovanju raka pljuč, so raziskave uporabe antagonistov nAChR pri zdravljenju raka zelo aktualne. Na ta način bi radi preprečili stimulativni učinek nikotina in njemu podobnih snovi za nastanek in napredovanje pljučnega raka (Paleari in sod. 2009a, Mucchietto in sod. 2016, Mucchietto in sod. 2018). Pri uporabi antagonistov nAChR pa moramo zagotoviti, da le-ti delujejo prednostno na rakaste celice. Le tako se lahko izognemo različnim neželjenim stranskim učinkom. Uporaba nanodelcev, kot nosilcev zdravilnih učinkovin, med drugim omogoča tudi ciljano dostavo sicer neselektivnih učinkovin rakavim celicam (Cho in sod. 2008, Wolfram in Ferrari 2019).

Nikotin in pljučni rak

Nikotin je naravni alkaloid, ki ga najdemo v rastlinah iz družine razhudnikovk (zlasti v tobaku), kjer služi za obrambo rastlin pred rastlinojedci, zato so ga v preteklosti uporabljali tudi kot insekticid. Pri uporabi tobačnih izdelkov se nikotin lahko absorbira v organizem skozi pljučni epitel, skozi ustne in nosne sluznice ter skozi kožo. V krvi kadilca, ki pokadi 25 cigaret na dan, je od 0,025 do 0,444 µM nikotina (Sanner in Grimsrud 2015).

Glavni učinki nikotina v telesu so posledica njegovega delovanja na holinergični sistem. Nikotin stimulira delovanje nAChR, ki so v celicah osrednjega živčnega sistema, v ganglijih avtonomnega živčnega sistema in v parasimpatičnem avtonomnem živčnem sistemu (Sanner in Grimsrud 2015). Vezava nikotina na nAChR v možganih aktivira mezolimbični dopaminski sistem nagrajevanja, ki je odgovoren za nastanek odvisnosti in pojav odtegnitvenih simptomov (Wittenberg in sod. 2020, Picciotto in Kenny 2021).

Vloga holinergičnega sistema v živčevju je že dolgo znana, pomembno vlogo pa ima tudi v drugih organskih sistemih. Desetletja je veljalo, da so nAChR prisotni le na živčnih celicah v območju živčno-mišičnega stika, dokler niso odkrili, da se izražajo tudi v nekaterih drugih celicah, med drugim v celicah različnih oblik pljučnega raka. Schuller je leta 1989 prvič pokazal, da nAChR sodelujejo pri regulaciji rasti tumorjev (Schuller 1989). Eno leto za tem sta Maneckjee in Minna dokazala tudi vlogo nAChR v regulaciji apoptoze (Maneckjee in Minna 1990). Sledila je demonstracija izražanja teh receptorjev v številnih tkivih sesalcev (Wessler in Kirkpatrick 2008), ter njihove vključenosti v uravnavanje celične proliferacije, migracije in diferenciacije (Grando 2014). Številne *in vitro* ter *in vivo* raziskave, kakor tudi asociacijske študije na ravni celotnega genoma (angl. Genome-Wide Association Studies; GWAS), so pokazale na pomembno vlogo holinergične signalizacije pri razvoju in napredovanju raka pljuč. Celice pljučnega raka izražajo proteine, ki so potrebni za privzem holina, za sintezo, transport in razgradnjo acetilholina (ACh) (Friedman in sod. 2019). Izražajo pa tudi receptorje, katerih ligand je ACh: ionotropne nAChR in metabotropne muskarinske acetilholinske receptorje (mAChR). Od vseh komponent holinergične signalizacije se je največ dosedanjih raziskav povezanih z rakom pljuč usmerilo v preučevanje nAChR in z nAChR-povezanih signalnih poti, saj je znano, da so različni podtipi nAChR povezani s tveganjem za razvoj in napredovanje te bolezni.

Vpliv nikotina na nAChR

Raziskave so pokazale, da ima nikotin pomembno vlogo pri spodbujanju proliferacije celic pljučnega raka, pri pospeševanju angiogeneze in zasevanju rakavih celic. Preko delovanja na nAChR ščiti celice raka pljuč pred celično smrtjo (apoptozo), ki jo povzročajo kemoterapevtiki, ionizirajoče sevanje in oksidativni stres (Friedman in sod. 2019).

nAChR so ionotropni receptorji na celičnih membranah. Sestavlja jih pet podenot, ki so lahko α (izooblike 1-10), β (izooblike 1-4), γ, δ ali ε. Poznamo homopentamerne nAChR, npr. α7-nAChR, ki ga sestavlja pet identičnih α7 podenot, ali pa heteropentamerne receptorje, npr. α4β2-nAChR, ki ga sestavljata dve α4 in tri β2 podenote. Različni podtipi nAChR različno prepuščajo katione, Na^+, K^+ ali Ca^{2+}, in regulirajo različne procese, tudi v odvisnosti od tipa celic, v katerih se nahajajo (Schuller 2009). Vezava agonista, npr. fiziološkega nevrotransmiterja ACh, na nAChR izzove konformacijske spremembe receptorja, te pa omogočijo tok kationov skozi celično membrano (Hurst in sod. 2013). Poleg ACh, ki je endogeni agonist nAChR in deluje kot avtokrini ali parakrini rastni dejavnik pljučnih epitelnih celic, kakor tudi celic raka pljuč, aktivira nAChR tudi eksogeni nikotin in njegovi derivati (NNK: 4-(metilnitrozamino)-1-(3-piridil)-1-butanon in NNN: N'-nitrozonornikotin), ki nastajajo ob gorenju tobaka.

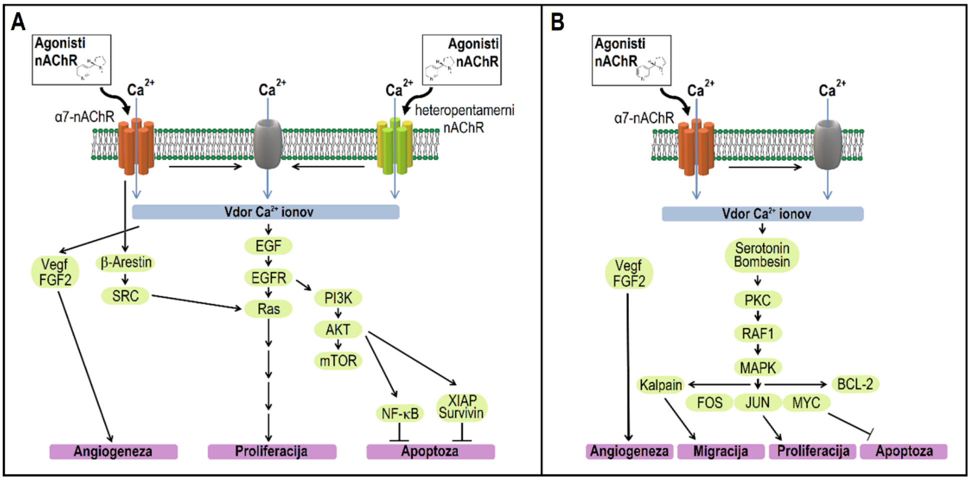
V celicah SCLC so najbolj izraženi homopentamerni α7-nAChR, ki selektivno prepuščajo Ca^{2+} ione (Chen in sod. 2019), v celicah NSCLC pa poleg α7-nAChR zasledimo tudi različne heteropentamerne nAChR (Schuller 2009). Vstop kationov v celico, zaradi vezave agonista na nAChR, zmanjša negativni potencial v notranjosti celice, kar pomeni depolarizacijo membrane, ta pa povzroči odprtje membranskih napetostno-odvisnih Ca^{2+} kanalčkov in še dodatnen dotok Ca^{2+} v celico. Dvig znotrajcelične koncentracije Ca^{2+} ionov ($[Ca^{2+}]_i$) sproži različne signalne poti, vključene v regulacijo celične proliferacije, apoptoze, migracije in diferenciacije (Sl. 1).

Za razliko od večine celičnih receptorjev, katerih izražanje se ob kronični izpostavitvi agonistu zmanjša, se izražanje nAChR ob kronični izpostavitvi nikotinu poveča (Schuller 2009). To poveča stimulacijo celic, tako z eksogenim nikotinom, kot z endogenim ACh, torej z avtokrino in parakrino zanko (Grando 2014). Ob kronični izpostavitvi celic nikotinu pride v centralnem živčevju (predvsem v delu možganov, imenovanem *nucleus accumbens*) do reverzibilne desenzitizacije α4β2-nAChR, medtem ko občutljivost α7-nAChR ostane nespremenjena (Kawai in Berg 2001). Pri kadilcih se tako poveča izražanje bioloških funkcij α7-nAChR, medtem ko se izražanje funkcij α4β2-nAChR zmanjša. Ker so α7-nAChR vključeni v stimuliracijo celic raka, α4β2-nAChR pa tudi v njihovo inhibicijo (Schuller 2009), se tako ustvarijo ugodne razmere za razvoj in napredovanje rakaste tvorbe. Pri celicah SCLC pride do povečanega izražanja ter do senzitizacije α7-nAChR tudi v hipoksičnem okolju (Schuller 2007), kar je eden izmed možnih vzrokov za visoko pojavnost raka pljuč pri bolnikih s kronično obstruktivno pljučno boleznijo (Carr in sod. 2018). Tako α7-nAChR kot tudi α4β2-nAChR stimulirata izločanje dopamina, ki ima poleg vloge ekscitatornega živčnega prenašalca v možganih tudi stimulativen učinek na proliferacijo celic nekaterih oblik raka, npr. raka prostate in raka dojke. Oba receptorja stimulirata izločanje stresnih živčnih hormonov (adrenalin, noradrenalin), ki prispevajo k razvoju številnih vrst raka, tudi pljučnega adenokarcinoma (Schuller 2009). α7-nAChR stimulira tudi izločanje serotonina in nevropeptida bombesina, oba rastna dejavnika SCLC (Cattaneo in sod. 1993, Jull in sod. 2001, Schuller 2009). α4β2-nAChR stimulira izločanje γ-aminomaslene kisline (GABA), inhibitornega nevrotransmiterja, ki deluje kot zaviralec adenokarcinoma pljuč (Schuller in sod. 2008).

Pri celicah SCLC so signalne poti regulirane zlasti preko α7-nAChR (Schuller 2009; Sl. 1A). Vezava agonista na α7-nAChR sproži vdor Ca^{2+} ionov v celico skozi α7-nAChR. Ta učinek se še ojača s posledičnim odprtjem napetostno-odvisnih Ca^{2+} kanalčkov. Povečana $[Ca^{2+}]_i$ sproži izločanje avtokrinih rastnih dejavnikov serotonina in bombesina, kot tudi dejavnikov angiogeneze (VEGF - vaskularni endotelni rastni dejanik; in FGF2 - fibroblastni rastni dejavnik 2). Serotonin in bombesin aktivirata signalno kaskado preko protein kinaze C (PKC), serin/treonin kinaze RAF1, z mitogenom aktiviranih protein kinaz (MAPK), regulatorja apoptoze BCL-2 ter transkripcijskih faktorjev FOS, JUN in MYC, kar privede do stimulacije proliferacije in inhibicije apoptoze. Od MAPK-odvisna fosforilacija kalpainov, s Ca^{2+}-reguliranih proteinaz, stimulira migracijo celic SCLC.

Pri celicah NSCLC je signalizacija povezana tako s homopentamernimi kot heteropentamernimi nAChR (Schuller 2009; Sl. 1B), razen pri kadilcih, kjer so signalne poti regulirane predvsem preko homopentamernih α7-nAChR, saj so heteropentamerni nAChR desenzitizirani. Povečana $[Ca^{2+}]_i$ sproži sproščanje dejavnikov angiogeneze, VEGF in FGF2. Vstop Ca^{2+} v celico sproži sproščanje epidermalnega rastnega dejavnika, EGF, in aktivacijo signalne poti preko njegovega receptorja (EGFR). α7-nAChR preko od β-arestin-odvisnega proto-onkogena SRC, aktivirajo regulatorni protein Ras, ki okrepi signalno pot EGFR, ta pa je ena izmed najpomembnejših poti regulacije rasti, proliferacije, preživetja in diferenciacije celic. EGFR aktivira signalno pot Akt in njene efektorje, inhibitorje apoptoze (XIAP in survivin), in jedrni faktor-κB (NF-κB).

Nedavne raziskave so pokazale, da pljučne celice ne izražajo nAChR le na zunanji celični membrani (plazmalemi), ampak izražajo funkcionalne nAChR tudi na zunanji membrani mitohondrijev (Friedman in sod. 2019). Izražanje mitohondrijskih nAChR je v celicah pljučnega raka povečano (Chernyavsky in sod. 2015). Aktivacija α7-nAChR v mitohondriju prepreči odprtje prehodnih por v mitohondrijski membrani (angl. mitochondrial permeability transition pore; mPTP), s tem pa sproščanje citokroma c in iniciacijo apoptoze celice (Grando 2014, Chernyavsky in sod. 2015, Skok in sod. 2016).



Slika 1: Signalne poti, ki jih sprožijo agonisti nAChR pri celicah drobnoceličnega karcinoma pljuč (SCLC) (**A**) in celicah nedrobnoceličnega karcinoma pljuč (NSCLC) (**B**). Vezava agonista na nAChR sproži vstop Ca^{2+} ionov v celico in signalno kaskado, ki vodi v povečano proliferacijo in migracijo celic, zmanjšano apoptozo celic ter v pospešeno angiogenezo. Okrajšave: AKT, AKT serin/treonin kinaza; BCL-2, B-celični CLL/limfoma 2 regulator apoptoze; EGF, epidermalni rastni dejavnik; SRC, SRC proto-onkogen, nereceptorska tirozin kinaza; EGFR, receptor epidermalnega rastnega dejavnika; FGF2, fibroblastni rastni dejavnik 2; FOS, transkripcijski faktor FOS; JUN, transkripcijski faktor JUN; MAPK, z mitogenom aktivirane protein kinaze; mTOR, tarča rapamicina pri sesalcih; MYC, transkripcijski faktor MYC; NF-κB, jedrni faktor–κB; PI3K, fosfoinozitid-3-kinaza; PKC, protein kinaza C; RAF1, Raf-1 proto-onkogen, serin/treonin kinaza; RAS, majhna regulatorna GTPaza; VEGF, vaskularni endotelni rastni dejanik; XIAP, X-vezani inhibitor apoptoze. Slika je povzeta po Schuller (2009).

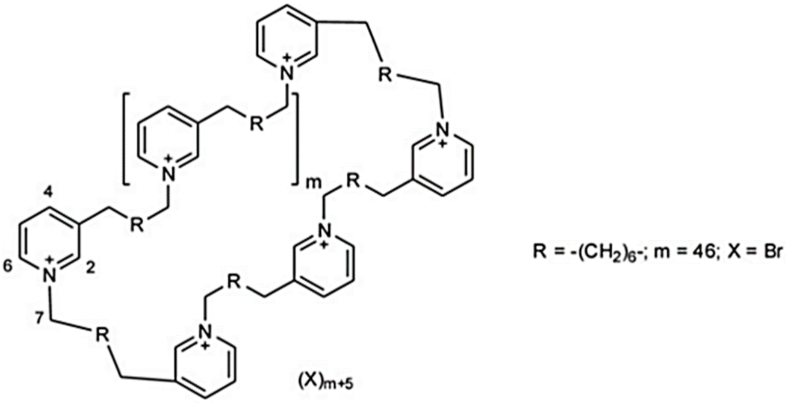
Figure 1: Signaling pathway triggered by nAChRs agonists in small-cell lung cancer cells (SCLC) (**A**) and non-small-cell lung cancer cells (NSCLC) (**B**). Agonist binding to nAChRs causes an Ca^{2+} influx and signal cascade leading to increased proliferation and migration, inhibition of apoptosis and stimulated angiogenesis. Abbreviations: AKT, AKT serine/threonine kinase; BCL-2, B-cell lymphoma 2 apoptosis regulator; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF2, fibroblast growth factor 2; FOS, transcription factor FOS; JUN, transcription factor JUN; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MYC, transcription factor MYC; NF-κB, nuclear factor-κB; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; RAF1, raf-1 proto-oncogene, serine/threonine kinase; RAS, small regulatory GTPase; SRC, SRC proto-oncogene, non-receptor tyrosine kinase; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis. The Figure is adapted according to original from Schuller (2009).

Antagonisti nAChR

Antagonisti nAChR z vezavo na receptor zmanjšajo ali celo preprečijo učinke agonista, npr. nikotina. Dasgupta in sod. (2006) so pokazali, da 1 µM koncentracija nikotina zavre apoptozo, ki jo sproži kemoterapevtik cisplatin, ta učinek pa je mogoče izničiti z uporabo heksametonijevega bromida, antagonista nAChR (Dasgupta in sod. 2006). Antagonisti so lahko kompetitivni, kar pomeni, da z agonisti tekmujejo za isto vezavno mesto na receptorju, lahko pa so nekompetitivni in se vežejo nekje drugje na receptorju ter posredno otežijo vezavo agonista na receptor (Hurst in sod. 2013). Večina naravnih antagonistov nAChR je nekompetitivnih in z vezavo na nAChR povežejo sosednji receptorski podenoti med seboj, ter tako preprečijo konformacijske spremembe receptorja, ki so potrebne za odprtje ionske pore (Samson in Levitt 2008). Nekateri nekompetitivni antagonisti se pri visokih koncentracijah vežejo tudi na mesto znotraj ionske pore in s tem fizično preprečijo prehajanje kationov (Liu in sod. 2008).

V preteklih raziskavah so se antagonisti, predvsem kompetitivni α-bungarotoksin in njegovi radioaktivni in fluorescenčni derivati, uporabljali za označevanje nAChR in kot molekularno orodje za preučevanje signalnih poti, povezanih z nAChR. V veliko pomoč so bili tudi pri strukturnih raziskavah, npr. za določitev aminokislinskih ostankov v nAChR, pomembnih za selektivno vezavo ligandov (Zhu in sod. 2020). Manj je znanja o tem, s katerimi signalnimi potmi je povezana vezava antagonistov na nAChR, to pa bi lahko bilo ključnega pomena za zdravljenje raka. Raziskave so nakazale, da bi se na ta način lahko sprožila celična apoptoza (Zovko in sod. 2013, Berne in sod. 2018).

Večina znanih naravnih antagonistov nAChR so toksini, npr. rastlinska toksina metililkakonitin in D-tubokurarin, in živalski toksini. Slednje so našli v kačjih strupih, npr. triprsta nevrotoksina, α-bungarotoksin in α-kobratoksin, ter sekretorne fosfolipaze A_2, kot vurtoksin. V strupih morskih polžev iz rodu *Conus* so t.i. konotoksini in primera dveh antagonistov nAChR sta ArIB in RgIA. Antagoniste nAChR najdemo tudi v algah. Iz enoceličnih dinoflagelatnih alg so tako izolirali fitotoksine s tako aktivnostjo, gimnodimine in spirolide (Wintersteiner in Dutcher 1943, Jennings in sod. 1986, Hu in sod. 2001, Haywood in sod. 2004, Whiteaker in sod. 2007, Utkin 2013, Vulfius in sod. 2014, Ren in sod. 2019). Omenjeni toksini za zdravljenje raka niso neposredno uporabni, saj bi zaradi preferenčne vezave na mišične nAChR v motoričnih sinapsah povzročili vrsto resnih neželenih (toksičnih) učinkov (Grozio in sod. 2008, Paleari in sod. 2009a, Paleari in sod. 2009b). Manj toksični antagonisti nAChR so zato dobrodošli. Taki so npr. cembranoidi, naravni diterpeni, izolirani iz koral, ki so že pokazali protirakavo delovanje (Ferchmin in sod. 2009). Precej raziskav poskuša z usmerjenim spreminjanjem strukture konotoksinov pripraviti mutante z večjo selektivnostjo vezave na podvrste nAChR, ki pomembno sodelujejo pri razvoju raka (Whiteaker in sod. 2007). Iz morske spužve *Reniera sarai* so že pred časom izolirali alkilpiridinijeve polimere (poli-APS), ki bi bili lahko uporabni za zdravljenje raka z antagonisti nAChR (Sepčić in sod. 1997, Turk in sod. 2007, Turk in sod. 2008). Uspeli so tudi s pripravo nekaterih sintetičnih analogov APS (Koss in sod. 2007, Houssen in sod. 2010) in pokazali, da je polimerna (1,3-oktilpiridinijeva) sol, APS8 (Sl. 2), močan nekompetitivni antagonist α7-nAChR. Delovanje α7 oblike receptorja blokira že pri koncentraciji 1-3 nM, precej manj učinkovit pa je na α4β2-nAChR. Dosedanje raziskave so pokazale, da APS8 pomembno zmanjša anti-apoptotske učinke nikotina na celice pljučnega adenokarcinoma (A549). Vezava APS8 na α7-nAChR sproži signalne poti, ki vodijo v apoptozo in hkrati zavre anti-apoptotske signale (Zovko in sod. 2013). APS8 so testirali tudi *in vivo* na ksenograftih humanega pljučnega adenokarcinoma na imunsko zavrtih miših (SCID). Medtem, ko je intratumorski vnos APS8 učinkovito zavrl rast tumorja (Berne in sod. 2018), pa je bilo zdravljenje s sistemskim intravenoznim vnosom manj učinkovito. Za nadaljnji razvoj v smeri tarčnega vnosa APS8 v rakave celice je pomembno, da toksičnih učinkov APS8 pri miškah ni bilo opaziti (Berne in sod. 2018).



Slika 2: Kemijska struktura polimerne različice 1,3-oktilpiridinijeve soli (APS8).
Figure 2: Chemical structure of 1,3-octylpyridinium salt (APS8).

Kljub nekaterim spodbudnim rezultatom je razumevanje vpliva antagonistov nAChR na celice in signalne poti, ki jih sprožijo ob vezavi na nAChR, še zelo omejeno. Poleg napredka v razumevanju molekularnega mehanizma delovanja antagonistov nAChR na celice, pa se veliko pozornosti posveča tudi zmanjševanju verjetnosti pojava stranskih učinkov zaradi delovanja na netarčne celice. Velik potencial na tem področju ima uporaba nanodelcev kot nanodostavnih sistemov.

Nanodostavni sistemi

Konvencionalni kemoterapevtiki se v telesu porazdelijo nespecifično, tako da vplivajo na rakave kot tudi na nerakave celice (Cho in sod. 2008). Velik izziv pri zdravljenju raka je vnos zdravilne učinkovine na način, da bi ta v zadostni koncentraciji dosegla želeno lokacijo, obenem pa ne delovala na sosedna tkiva in s tem povzročala neželene stranske učinke. Uporaba nanodelcev za ciljno dostavo zdravilnih učinkovin predstavlja obetaven pristop.

Nanodelci so vsi delci, ki imajo eno ali več dimenzij v velikostnem območju med 1 in 100 nm. Imajo posebne transportne, biološke, optične, magnetne, elektronske in termične lastnosti, drugačne od tistih na molekularnem kot tudi na makro nivoju. Z razvojem nanotehnologije se povečujejo možnosti načrtne priprave nanodelcev z želenimi lastnostmi, ki jih lahko uporabimo v medicini za diagnostiko, spremljanje bolezni ter njihovo zdravljenje (Murthy 2007). Nanodelce lahko uporabljamo tudi kot modulatorje imunskega odziva (Kononenko in sod. 2015). Pri zdravljenju rakavih obolenj lahko uporabimo magnetne nanodelce, s pomočjo katerih tumor uničujemo s segrevanjem - hipertermijo (Sadhukha in sod. 2013, Kononenko in sod. 2017a). V Evropi je že dovoljena uporaba zdravljenja možganskih tumorjev z nanodelci železovega oksida (NanoTherm), ki jih preko lokalne infuzije vnesemo na območje tumorja in nato izpostavimo izmeničnemu magnetnemu polju, ki sproži ablacijo tumorja s segrevanjem. Možna je tudi uporaba fotoreaktivnih nanodelcev, ki pri fotodinamični terapiji (ang. photodynamic therapy, PDT) prenesejo svetlobno energijo na kisik, s čimer nastanejo reaktivne kisikove zvrsti (ang. reactive oxygen species, ROS), ki delujejo fototoksično za rakave celice (Lucky in sod. 2015, Imani in sod. 2017, Kononenko in sod. 2017b). Enega izmed najbolj obetajočih produktov nanotehnologije pri zdravljenju rakavih obolenj pa predstavljajo nanodelci, ki jih lahko uporabimo za ciljno dostavo učinkovine. Z nanodostavnimi sistemi tako odpravljamo težavi slabe topnosti terapevtske učinkovine in nizke specifičnosti delovanja ter posledične sistemske toksičnosti. Za dostavo zdravil lahko uporabimo različne tipe nanodelcev, kot so liposomi, miceli, dendrimeri, polimerni nanodelci, virusni nanodelci, proteinski nanodelci, porozni nanodelci in kovinski nanodelci (Doroudian in sod. 2021).

Velikost nanodostavnega sistema omogoča vgradnjo več aktivnih in pomožnih komponent v isti nanodelec (Wolfram in Ferrari 2019). Posamezne komponente je mogoče načrtno oblikovati tako, da v čim večji meri prispevajo k učinkovitosti in varnosti zdravljenja. Pomožne komponente lahko služijo za izboljšanje topnosti aktivne komponente, kot zaščita pred njeno razgradnjo, kot pomoč pri kontroliranem in podaljšanem sproščanju v tumorskem mikrookolju, omogočajo pa tudi boljši prodor v tkiva in čez bariere, izogibanje imunskemu sistemu, izboljšajo specifičnost delovanja, služijo pa lahko tudi za označevanje rakavega tkiva na zajetih slikah (Blanco in sod. 2015, Wolfram in Ferrari 2019).

Konvencionalna zdravila so pogosto manjša od 1 nm in ko prispejo v krvožilni sistem se lahko porazdelijo po večini tkiv v telesu, relativno neodvisno od lokacijsko specifičnih lastnosti žilnega sistema. Difuzija omogoča prodor učinkovine do rakavih celic, hkrati pa prodre učinkovina tudi do zdravih celic, kar omejuje količino uporabljene doze, da se ne izzove preveč neželjenih stranskih učinkov. Transport in porazdelitev sistemsko apliciranih nanodelcev v telesu sta bolj odvisna od lastnosti žilja. Tako lahko pri uporabi nanodostavnih sistemov izkoristimo posebnosti žil, ki oskrbujejo tumorsko tkivo (Wolfram in Ferrari 2019). Lastnosti teh žil se močno razlikujejo od zdravih žil, saj je angiogeneza okoli hitro rastočega tumorskega tkiva zelo hitra, kar privede do prepleta krvnih žil s številnimi nepravilnostmi endotelija (Siemann 2011). Različne raziskave so pokazale, da se sistemsko aplicirani nanodelci v telesu kopičijo v tumorjih, kar se največkrat pripisuje učinku zadrževanja učinkovine na mestu tumorja zaradi pasivnega ciljanja (angl. enhanced permeability and retention effect, EPR). Učinek EPR je rezultat poškodovanosti krvnih žil, ki oskrbujejo tumorje, s tem pa njihove večje prepustnosti za nanodelce. Obenem je v tumorskem tkivu oslabljena limfna drenaža, kar podaljša zadrževalni čas nanodelcev (Wolfram in Ferrari 2019). Stopnja endocitoze je v rakavih celicah glede na zdrave pogosto večja, kar lahko izkoristimo za usmerjeno internalizacijo nanodelcev, polnih učinkovine, v rakave celice. To tudi prispeva k manjši toksičnosti učinkovine za normalne celice (Cho in sod. 2008, Ye in sod. 2018).

Nanodelci kot nosilci omogočajo tudi vnos zdravila na enostaven, učinkovit in neinvaziven način skozi pljuča, kar je zlasti primerno pri zdravljenju bolezni kot je pljučni rak (Paranjpe in Müller-Goymann 2014). Tako dosežemo lokalno dostavo in povečamo učinkovitost zdravila na želeni lokaciji (Shen in Minko 2020). Vnos nanodelcev z inhalacijo (pulmonalni vnos) predstavlja učinkovito alternativo drugim načinom vnosa. Pljuča predstavljajo veliko površino za vnos nanodelcev z zdravilom, ob katerem se le-to ne razgradi ali biotransformira, kot bi se lahko v prebavnem traktu oziroma v jetrih (Sadhukha in sod. 2013). Kot nosilci zdravil za inhalacijo se največkrat omenjajo različni biokompatibilni in biorazgradljivi nanodelci, kot so lipidni nanodelci (trdni lipidni nanodelci in liposomi) in polimerni nanodelci, sestavljeni iz polilaktične kisline (PLA), polilaktične-ko-glikolne kisline (PLGA), polikaprolaktona (PCL), alginata, hitozana in želatine (Paranjpe in Müller-Goymann 2014). Preučuje se tudi uporaba anorganskih nanodelcev za inhalacijo, ki imajo različne želene lastnosti (npr. magnetne, kontrastne, radiosenzibilne lastnosti). Povzročajo lahko tudi stranske učinke na pljučnem tkivu (npr. zaradi sprožitve oksidativnega stresa), ki pa se lahko kontrolirajo preko prilagajanja časa izpostavitve in doze (Anderson in sod. 2020).

Nanodostavna zdravila so v klinični uporabi za zdravljenje raka že skoraj 30 let. Leta 1995 je Doxil, kemoterapevtik doksorubicin v liposomu za zdravljenje Kaposijevega sarkoma, raka jajčnikov in različnih oblik mieloma, postalo prvo nanodostavno zdravilo, odobreno s strani ameriške Uprave za hrano in zdravila (Food and Drug Administration, FDA). Leta 2005 je FDA odobrila še eno zdravilo proti raku na bazi nanodelcev – Abraxane. To vsebuje kemoterapevtik paklitaksel, vezan na albuminske nanodelce. Sprva je bil Abraxane odobren le za zdravljenje raka dojke, zaradi dobrih rezultatov, pa je FDA leta 2012 odobrila njegovo uporabo tudi za zdravljenje pacientov z NSCLC. Čeprav je trenutno odobrenih še več nanoterapevtikov (Anselmo in Mitragotri 2019), pa je potencial nanotehnologije v medicini še vedno slabo izkoriščen.

Zaključek in pogled v prihodnost

Kljub vse boljšemu poznavanju patofiziologije rakavih obolenj, so te bolezni še vedno med glavnimi problemi in izzivi svetovnega zdravstva. Raziskave o vlogi nikotina pri razvoju pljučnega raka niso pomembne le za boljše razumevanje te bolezni pri kadilcih, ampak tudi za boljše razumevanje varnosti uporabe nikotinskih izdelkov brez tobaka, katerih uporaba se povečuje (npr. e-cigarete in izdelki, ki se uporabljajo pri nadomestnem zdravljenju z nikotinom).

Raziskave nAChR na celicah pljučnega raka nakazujejo, da ima nikotin pomembno vlogo pri razvoju in napredovanju bolezni. Ta spoznanja so pomembno vplivala na raziskave terapevtskega potenciala antagonistov nAChR za preprečevanje stimulativne vloge nikotina in drugih, tudi endogenih, agonistov nAChR pri razvoju raka.

Uporaba nanodostavnih sistemov, ki bi selektivno prenesli antagoniste nAChR oziroma kemoterapevtike do rakavih celic v pljučih, bi lahko bistveno zmanjšala pomanjkljivosti zdravljenja s konvencionalnimi kemoterapevtiki, predvsem pojav resnih stranskih učinkov. Raziskave, ki prinašajo nova znanja o molekularnih mehanizmih razvoja in poteh napredovanja raka pljuč, študije o snoveh, ki te procese zavirajo ter testiranje novih sistemov za dostavo zdravil, so ključnega pomena za razvoj novih pristopov zdravljenja te kompleksne bolezni.

Summary

Lung cancer is one of the most commonly diagnosed cancers in the world and is responsible for a large proportion of cancer related deaths. Tobacco smoking is the most important etiological factor for the development of all histological forms of lung cancer, because many carcinogenic chemicals are present in tobacco smoke. Nicotine is a component of tobacco smoke that is responsible for addiction, and although it is commonly not considered as a carcinogen, it can trigger a number of cancer-promoting effects. In addition to acetylcholine (ACh), which is an endogenous agonist of nicotinic acetylcholine receptors (nAChRs) and acts as an autocrine and paracrine growth factor for lung epithelial cells as well as lung cancer cells, also nicotine and its derivatives activate nAChRs. Chronic nicotine exposure increases the expression of different types of nAChRs in lung cells, leading to increased stimulation of especially α7-nAChRs by nicotine and physiological stimulation of cells by autocrine and paracrine ACh loops. Binding of an agonist (e.g. nicotine or ACh) to nAChRs leads to the increased intracellular concentration of Ca^{2+} ions and a cascade of different signaling pathways that result in the increased cell proliferation and migration, enhanced angiogenesis, and decreased apoptosis. Based on the findings of the role of nAChR agonists in the development and progression of lung cancer, research has been directed toward the use of nAChR antagonists, which prevent the stimulatory effects of nicotine and other nAChR agonists (including endogenous ones) and could be useful in cancer treatment. Furthermore, it was suggested that nAChR antagonists could induce apoptosis in some cases. So far they were mostly used as blocking agents for studying signalling pathways mediated by agonist binding. However, little is known about signalling pathways mediated by antagonist binding themselves. Moreover, the effects of nAChR antagonists on non-cancer cells can cause a number of side effects. Therefore, it is important to ensure that the antagonist acts predominantly on cancer cells, what can be achieved by nanodelivery systems that allow targeted drug delivery. Advances in nanotechnology are opening new opportunities for the use of nanoparticles as drug delivery systems in cancer cells, enabling the development of more effective and safer drugs for cancer treatment. The use of nanoparticles as drug carriers provides us with the ability to protect the drug from degradation, control and prolong drug release, achieve better and more controlled penetration of tissues and cells, incorporate multiple different active and supplementary compounds into the same nanoparticle, and achieve targeted drug delivery. Research that provides new insights into the mechanisms and pathways of lung cancer development, explorations of compounds that inhibit or stop these pathways, and testing of new drug delivery systems are critical to developing new and better approaches to treat this disease.

Zahvala

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Literatura

Anderson, C.F., Grimmett, M.E., Domalewski, C.J., Cui, H., 2020. Inhalable nanotherapeutics to improve treatment efficacy for common lung diseases. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 12 (1), e1586.

Anselmo, A.C., Mitragotri, S., 2019. Nanoparticles in the clinic: An update. Bioengineering & Translational Medicine, 4 (3), e10143.

Berne, S., Čemažar, M., Frangež, R., Juntes, P., Kranjc, S., Grandič, M., Savarin, M., Turk, T., 2018. APS8 Delays Tumor Growth in Mice by Inducing Apoptosis of Lung Adenocarcinoma Cells Expressing High Number of α7 Nicotinic Receptors. Marine Drugs, 16 (10), 367.

Blanco, E., Shen, H., Ferrari, M., 2015. Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nature Biotechnology, 33 (9), 941.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., Jemal, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians, 68 (6), 394–424.

Calles, A., Aguado, G., Sandoval, C., Álvarez, R., 2019. The role of immunotherapy in small cell lung cancer. Clinical and Translational Oncology, 21 (8), 961–976.

Carr, L.L., Jacobson, S., Lynch, D.A., Foreman, M.G., Flenaugh, E.L., Hersh, C.P., Sciurba, F.C., Wilson, D.O., Sieren, J.C., Mulhall, P., Kim, V., 2018. Features of COPD as predictors of lung cancer. Chest, 153 (6), 1326–1335.

Cattaneo, M.G., Codignola, A., Vicentini, L.M., Clementi, F., Sher, E., 1993. Nicotine stimulates a serotonergic autocrine loop in human small-cell lung carcinoma. Cancer Research, 53 (22), 5566–5568.

Chen, J., Cheuk, I.W., Shin, V.Y., Kwong, A., 2019. Acetylcholine receptors: key players in cancer development. Surgical Oncology, 31, 46–53.

Chernyavsky, A.I., Shchepotin, I.B., Galitovkiy, V., Grando, S.A., 2015. Mechanisms of tumor-promoting activities of nicotine in lung cancer: synergistic effects of cell membrane and mitochondrial nicotinic acetylcholine receptors. BMC Cancer, 15 (1), 152.

Cho, K., Wang, X.U., Nie, S., Shin, D.M., 2008. Therapeutic nanoparticles for drug delivery in cancer. Clinical Cancer Research, 14 (5), 1310–1316.

Dasgupta, P., Kinkade, R., Joshi, B., DeCook, C., Haura, E., Chellappan, S., 2006. Nicotine inhibits apoptosis induced by chemotherapeutic drugs by up-regulating XIAP and survivin. Proceedings of the National Academy of Sciences, 103 (16), 6332–6337.

Doroshow, D.B., Sanmamed, M.F., Hastings, K., Politi, K., Rimm, D.L., Chen, L., Ignacio, M., Kurt, A.S., Herbst, R.S., 2019. Immunotherapy in non–small cell lung cancer: facts and hopes. Clinical Cancer Research, 25 (15), 4592–4602.

Doroudian, M., Azhdari, M.H., Goodarzi, N., O'Sullivan, D., Donnelly, S.C., 2021. Smart Nanotherapeutics and Lung Cancer. Pharmaceutics, 13 (11), 1972.

El-Bayoumy, K., Stoner, G., 2022. Use of Freeze-dried Watercress for Detoxification of Carcinogens and Toxicants in Smokers: Implications of the Findings and Potential Opportunities. Cancer Prevention Research, 15 (3), 139–141.

Ferchmin, P.A., Pagán, O.R., Ulrich, H., Szeto, A.C., Hann, R.M., Eterović, V.A., 2009. Actions of octocoral and tobacco cembranoids on nicotinic receptors. Toxicon: official journal of the International Society on Toxinology, 54 (8), 1174–1182.

Friedman, J.R., Richbart, S.D., Merritt, J.C., Brown, K.C., Nolan, N.A., Akers, A.T., Lau, J.K., Robateau, Z.R., Miles, S.L., Dasgupta, P., 2019. Acetylcholine signaling system in progression of lung cancers. Pharmacology and Therapeutics, 194, 222–254.

Grando, S.A., 2014. Connections of nicotine to cancer. Nature Reviews Cancer, 14 (6), 419–429.

Grozio, A., Paleari, L., Catassi, A., Servent, D., Cilli, M., Piccardi, F., Paganuzzi, M., Cesario, A., Granone, P., Mourier, G., Russo, P., 2008. Natural agents targeting the α7-nicotinic-receptor in NSCLC: A promising prospective in anti-cancer drug development. International Journal of Cancer, 122 (8), 1911–1915.

Haywood, A.J., Steidinger, K.A., Truby, E.W., Bergquist, P.R., Bergquist, P.L., Adamson, J., Mackenzie, L., 2004. Comparative morphology and molecular phylogenetic analysis of three new species of the genus karenia (dinophyceae) from New Zealand. Journal of Phycology, 40, 165–179.

Houssen, W.E., Lu, Z., Edrada-Ebel, R., Chatzi, C., Tucker, S.J., Sepčić, K., Turk, T., Zovko, A., Shen, S., Mancini, I., Scott, R.H., 2010. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. Journal of Chemical Biology, 3 (3), 113–125.

Hu, T., Burton, I.W., Cembella, A.D., Curtis, J.M., Quilliam, M.A., Walter, J.A., Wright, J.L., 2001. Characterization of spirolides a, c, and 13-desmethyl c, new marine toxins isolated from toxic plankton and contaminated shellfish. Journal of Natural Products, 64 (3), 308–312.

Hurst, R., Rollema, H., Bertrand, D., 2013. Nicotinic acetylcholine receptors: from basic science to therapeutics. Pharmacology & Therapeutics, 137 (1), 22–54.

Imani, R., Dillert, R., Bahnemann, D.W., Pazoki, M., Apih, T., Kononenko, V., Repar, N., Kralj-Iglič, V., Boschloo, G., Drobne, D., Edvinsson, T., 2017. Multifunctional Gadolinium-Doped Mesoporous TiO_2 Nanobeads: Photoluminescence, Enhanced Spin Relaxation, and Reactive Oxygen Species Photogeneration, Beneficial for Cancer Diagnosis and Treatment. Small, 13 (20), 1700349.

Jennings, K.R., Brown, D.G., Wright, D.P., 1986. Methyllycaconitine, a naturally occurring insecticide with a high affinity for the insect cholinergic receptor. Experientia, 42, 611–613.

Jull, B., Plummer III, H.K., Schuller, H., 2001. Nicotinic receptor-mediated activation by the tobacco-specific nitrosamine NNK of a Raf-1/MAP kinase pathway, resulting in phosphorylation of c-myc in human small cell lung carcinoma cells and pulmonary neuroendocrine cells. Journal of Cancer Research and Clinical Oncology, 127 (12), 707–717.

Kawai, H., Berg, D.K., 2001. Nicotinic acetylcholine receptors containing the α7 subunits on rat cortical neurons do not undergo long lasting inactivation even when upregulated by chronic nicotine exposure. Journal of Neurochemistry, 78 (6), 1367–1378.

Kononenko, V., Narat, M., Drobne, D., 2015. Nanoparticle interaction with the immune system. Arhiv za Higijenu Rada i Toksikologiju, 66 (2), 97–108

Kononenko, V., Erman, A., Petan, T., Križaj, I., Kralj, S., Makovec, D., Drobne, D., 2017a. Harmful at non-cytotoxic concentrations: SiO_2-SPIONs affect surfactant metabolism and lamellar body biogenesis in A549 human alveolar epithelial cells. Nanotoxicology, 11 (3), 419–429.

Kononenko, V., Imani, R., Repar, N., Benčina, M., Lorenzetti, M., Erman, A., Drobne, D., Iglič, A., 2017b. Phototoxicity of Mesoporous TiO^{2+} Gd Microbeads With Theranostic Potential. In: Advances in Biomembranes and Lipid Self-Assembly, Academic Press, 26, 153–171.

Koss, D.J., Hindley, K.P., David, K.C., Mancini, I., Guella, G., Sepčić, K., Turk, T., Rebolj, K., Riedel, G., Platt, B., Scott, R.H., 2007. A comparative study of the actions of alkylpyridinium salts from a marine sponge and related synthetic compounds in rat cultured hippocampal neurones. BMC Pharmacology, 7 (1), 1.

Liu, Q., Yu, K.W., Chang, Y.C., Lukas, R.J., Wu, J., 2008. Agonist-induced hump current production in heterologously-expressed human alpha4beta2-nicotinic acetylcholine receptors. Acta Pharmacologica Sinica, 29 (3), 305–319.

Lucky, S.S., Soo, K.C., Zhang, Y., 2015. Nanoparticles in photodynamic therapy. Chemical Reviews, 115 (4), 1990-2042.

Maneckjee, R., Minna, J.D., 1990. Opioid and nicotine receptors affect growth regulation of human lung cancer cell lines. Proceedings of the National Academy of Sciences, 87 (9), 3294–3298.

Mao, Y., Yang, D., He, J., Krasna, M.J., 2016. Epidemiology of lung cancer. Surgical Oncology Clinics, 25 (3), 439–445.

Marques, P., Piqueras, L., Sanz, M.J., 2021. An updated overview of e-cigarette impact on human health. Respiratory Research, 22, 1–14.

Mucchietto, V., Crespi, A., Fasoli, F., Clementi, F., Gotti, C., 2016. Neuronal acetylcholine nicotinic receptors as new targets for lung cancer treatment. Current Pharmaceutical Design, 22 (14), 2160–2169.

Mucchietto, V., Fasoli, F., Pucci, S., Moretti, M., Benfante, R., Maroli, A., Di Lascio, S., Bolchi, C., Pallavicini, M., Dowell, C., McIntosh, M., 2018. α9-and α7-containing receptors mediate the pro-proliferative effects of nicotine in the A549 adenocarcinoma cell line. British Journal of Pharmacology, 175 (11), 1957–1972.

Murthy, S.K., 2007. Nanoparticles in modern medicine: state of the art and future challenges. International Journal of Nanomedicine, 2 (2), 129.

Onkološki inštitut Ljubljana, Rak pljuč, https://www.onko-i.si/za-javnost-in-bolnike/vrste-raka/rak-pljuc (4. 4. 2022)

Paleari, L., Negri, E., Catassi, A., Cilli, M., Servent, D., D'Angelillo, R., Cesario, A., Russo, P., Fini, M., 2009a. Inhibition of nonneuronal α7-nicotinic receptor for lung cancer treatment. American Journal of Respiratory and Critical Care Medicine, 179 (12), 1141–1150.

Paleari, L., Cesario, A., Fini, M., Russo, P., 2009b. Foundation review: α7-nicotinic receptor antagonists at the beginning of a clinical era for NSCLC and mesothelioma? Drug Discovery Today, 14, 822–836.

Paranjpe, M., Müller-Goymann, C., 2014. Nanoparticle-mediated pulmonary drug delivery: a review. International Journal of Molecular Sciences, 15 (4), 5852–5873.

Picciotto, M.R., Kenny, P.J., 2021. Mechanisms of nicotine addiction. Cold Spring Harbor Perspectives in Medicine, 11 (5), a039610.

Ren, J., Zhu, X., Xu, P., Li, R., Fu, Y., Dong, S., Zhangsun, D., Wu, Y., Luo, S., 2019. d-Amino Acid Substitution of α-Conotoxin RgIA Identifies its Critical Residues and Improves the Enzymatic Stability. Marine Drugs, 17 (3), 142.

Riley, R.S., June, C.H., Langer, R., Mitchell, M.J., 2019. Delivery technologies for cancer immunotherapy. Nature reviews Drug Discovery, 18 (3), 175–196.

Sadhukha, T., Wiedmann, T.S., Panyam, J., 2013. Inhalable magnetic nanoparticles for targeted hyperthermia in lung cancer therapy. Biomaterials, 34 (21), 5163–5171.

Samson, A.O., Levitt, M., 2008. Inhibition mechanism of the acetylcholine receptor by alpha-neurotoxins as revealed by normal-mode dynamics". Biochemistry, 47 (13), 4065–70.

Sanner, T., Grimsrud, T.K., 2015. Nicotine: carcinogenicity and effects on response to cancer treatment–a review. Frontiers in Oncology, 5, 196.

Schuller, H.M., 1989. Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. Biochemical Pharmacology, 38 (20), 3439–3442.

Schuller, H.M., 2007. Nitrosamines as nicotinic receptor ligands. Life sciences, 80 (24-25), 2274–2280.

Schuller, H.M., Al-Wadei, H.A., Majidi, M., 2008. Gammaaminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. Carcinogenesis, 29 (10), 1979–1985.

Schuller, H.M., 2009. Is cancer triggered by altered signalling of nicotinic acetylcholine receptors?. Nature Reviews Cancer, 9 (3), 195.

Schuller, H.M., 2019. The impact of smoking and the influence of other factors on lung cancer. Expert Review of Respiratory Medicine, 13 (8), 761–769.

Sepčić, K., Guella, G., Mancini, I., Pietra, F., Serra, M.D., Menestrina, G., Tubbs, K., Maček, P., Turk, T. (1997). Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge Reniera sarai in aqueous solutions. Journal of Natural Products, 60 (10), 991–996

Shen, A.M., Minko, T., 2020. Pharmacokinetics of inhaled nanotherapeutics for pulmonary delivery. Journal of Controlled Release, 326, 222–244.

Shiraishi, K., Kunitoh, H., Daigo, Y., Takahashi, A., Goto, K., Sakamoto, H., Ohnami, S., Shimada, Y., Ashikawa, K., Saito, A., Watanabe, S.I., 2012. A genome-wide association study identifies two new susceptibility loci for lung adenocarcinoma in the Japanese population. Nature Genetics, 44 (8), 900.

Siemann, D.W., 2011. The unique characteristics of tumor vasculature and preclinical evidence for its selective disruption by tumor-vascular disrupting agents. Cancer Treatment Reviews, 37 (1), 63–74.

Skok, M., Gergalova, G., Lykhmus, O., Kalashnyk, O., Koval, L., Uspenska, K., 2016. Nicotinic acetylcholine receptors in mitochondria: subunit composition, function and signaling. Neurotransmitter, 3, e1290

Thandra, K.C., Barsouk, A., Saginala, K., Aluru, J.S., Barsouk, A., 2021. Epidemiology of lung cancer. Contemporary Oncology, 25 (1), 45.

Turk, T., Frangež, R., Sepčić, K., 2007. Mechanisms of toxicity of 3-alkylpyridinium polymers from marine sponge Reniera sarai. Marine Drugs, 5, 157–167.

Turk, T., Sepčić, K., Mancini, I., Guella, G., 2008. 3-Akylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use. In: Studies in Natural Products Chemistry, Elsevier, 35, 355–397.

Utkin, Y.N., 2013. Three-finger toxins, a deadly weapon of elapid venom–milestones of discovery. Toxicon, 62, 50–55.

Vulfius, C.A., Kasheverov, I.E., Starkov, V.G., Osipov, A.V., Andreeva, T.V., Filkin, S.Y., Gorbacheva, E.V., Astashev, M.E., Tsetlin, V.I., Utkin, Y.N., 2014. Inhibition of nicotinic acetylcholine receptors, a novel facet in the pleiotropic activities of snake venom phospholipases A2. PloS One, 9 (12), e115428.

Wang, S., Hu, Y., 2018. α7 nicotinic acetylcholine receptors in lung cancer. Oncology Letters, 16 (2), 1375–1382.

Wessler, I., Kirkpatrick, C.J., 2008. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. British Journal of Pharmacology, 154 (8), 1558–1571.

Whiteaker, P., Christensen, S., Yoshikami, D., Dowell, C., Watkins, M., Gulyas, J., Rivier, J., Olivera, B.M., McIntosh, J.M., 2007. Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. Biochemistry, 46 (22), 6628–6638.

Wintersteiner, O., Dutcher, J.D., 1943. Curare alkaloids from Chondodendron tometosum. Science, 97, 467–470.

Wittenberg, R.E., Wolfman, S.L., De Biasi, M., Dani, J.A., 2020. Nicotinic acetylcholine receptors and nicotine addiction: A brief introduction. Neuropharmacology, 177, 108256.

Wolfram, J., Ferrari, M., 2019. Clinical cancer nanomedicine. Nano Today, 25, 85–98.

Ye, D., Li, Y., Gu, N., 2018. Magnetic labeling of natural lipid encapsulations with iron-based nanoparticles. Nano Research, 11 (6), 2970–2991.

Zhu, X., Pan, S., Xu, M., Zhang, L., Yu, J., Yu, J., Wu, Y., Fan, Y., Li, H., Kasheverov, I.E., Kudryavtsev, D.S., Tsetlin, V.I., Xue, Y., Zhangsun, D., Wang, X., Luo, S., 2020. High Selectivity of an α-Conotoxin LvIA Analogue for α3β2 Nicotinic Acetylcholine Receptors Is Mediated by β2 Functionally Important Residues. Journal of Medicinal Chemistry, 63 (22), 13656–13668.

Zovko, A., Viktorsson, K., Lewensohn, R., Kološa, K., Filipič, M., Xing, H., Kem, W.R., Paleari, L., Turk, T., 2013. APS8, a polymeric alkylpyridinium salt blocks α7 nAChR and induces apoptosis in non-small cell lung carcinoma. Marine Drugs, 11 (7), 2574–2594.

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Molecular characterization of *Escherichia coli* from dishwasher rubber seals

Molekularna opredelitev bakterij *Escherichia coli* z gumijastih tesnil pomivalnih strojev

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Abstract: In this study 35 *Escherichia coli* isolates obtained from sampled dishwashers rubber seals were analysed with polymerase chain reactions (PCR) using specific primers for phylogenetic group, multilocus sequence type (MLST) determination the presence of 43 virulence-associated genes (VAGs) linked with intestinal and extraintestinal *E. coli* infections and the presence of some typical *E. coli* virulence plasmids' sequences in order to assess their virulence potential and/or specific genes, associated with the adaption to a specific environment. It was found that all of the 35 *E. coli* isolates belonged to the commensal non-pathogenic phylogenetic group A and that the diversity of these *E. coli* isolates, according to MLST analysis, was relatively low. Further, the prevalence of virulence-associated genes among the dishwasher rubber seal *E. coli* isolates was also low. Only the following VAGs were detected: *fimH*, *crl*, *fluA*, *picU*, *irp*, *fyuA*, *sitA*, *aslA*. Of the five plasmid replication regions tested only RepFIA and RepFIIA were detected. The two sequences associated with conjugative plasmids namely *traJ* and *traT*, were detected in only one isolate. Based on the obtained results the studied isolates can be designated as commensal *E. coli* with low pathogenic potential. Due to the low diversity of sequence types, even among isolates obtained from dishwashers from different locations, there is a possibility that strains from certain clonal groups are more adapted to specific habitats outside warm-blooded hosts than strains of other sequence types.

Keywords: dishwasher, *E. coli*, genes, One Health, plasmids, virulence

Izvleček: V tej raziskavi smo analizirali 35 izolatov bakterije *Escherichia coli*, pridobljenih iz vzorčenih gumijastih tesnil kuhinjskih pomivalnih strojev, da bi ocenili njihovo potencialno patogenost in/ali prisotnost genov povezanih z adaptacijo na specifično okolje. Z verižno reakcijo s polimerazo (PCR), ob uporabi ustreznih oligonukleotidov, smo vse izolate uvrstili v filogenetske skupine, določili njihov sekvenčni tip na podlagi analize multilokusnega zaporedja (MLST), ugotavljali prisotnost 43 genov, povezanih z virulenco (VAG), ki so pomembni v črevesnih in zunajčrevesnih okužbah z *E. coli* ter ugotavljali prisotnost nekaterih značilnih zaporedij plazmidov, povezanih z virulenco *E. coli*. Ugotovili smo, da je vseh 35 izolatov *E. coli* pripadalo komenzalni nepatogeni filogenetski skupini A in da je bila raznolikost teh izolatov, glede na analizo MLST, relativno majhna. Poleg tega je bila razširjenost genov, povezanih z virulenco, med preučevanimi izolati *E. coli* nizka. Ugotovili smo le naslednje VAG: *fimH*, *crl*, *fluA*, *picU*, *irp*, *fyuA*, *sitA*, *aslA*. Od petih testiranih plazmidnih replikacijskih regij sta bili potrjeni samo RepFIA in RepFIIA. Zaporedji *traJ* in *traT*, povezani s konjugativnimi plazmidi, smo zasledili samo v enem izolatu. Na podlagi dobljenih rezultatov lahko preučevane izolate označimo kot komenzalne *E. coli* z nizkim patogenim potencialom. Glede na majhno diverziteto sekvenčnih tipov, tudi med izolati pridobljenimi iz pomivalnih strojev iz različnih krajev, pa obstaja možnost, da so sevi iz določenih klonalnih skupin bolj prilagojeni na specifične habitate izven toplokrvnih gostiteljev, kot sevi drugih sekvenčnih tipov.

Ključne besede: *E. coli*, Eno zdravje, geni, plazmidi, pomivalni stroj, virulenca

Introduction

Recently, the importance of the approach, known as "One Health" has been increasingly recognized in the world. This approach is based on the awareness that microbes, animals and humans share ecosystems and that research on the diversity and complexity of interactions is needed. Further, this approach recognizes that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent (Miao et al. 2022).

Escherichia coli (*E. coli*), which is one of the best studied microorganisms in the world and a well-known genetic model organism, is a typical example of a bacterium that can be found in the environment, in water, soil, as well as in animal and human hosts (Jang et al. 2017). It usually lives in a mutualistic relationship with the host, but can also be pathogenic and cause serious, even fatal, intestinal and extra-intestinal infections (Allocati at al. 2013). *E. coli* is excreted from the host by faeces into the environment, where it can spread and then be reintroduced into different animal and human hosts by different routes (Jang et al. 2017). It is known for its pronounced genetic diversity, reflected in many different strains (Chaudhuri and Henderson 2012). The *E. coli* pathogenic strains carry many different virulence factors genes including colonization and fitness factors, toxins, and other effectors intertwining with the host's physiology (Kaper et al. 2004). Furthermore, it is known that certain phylogenetic groups are associated with pathotypes, for example the B2 phylogenetic group is associated with *E. coli* strains causing extraintestinal infections (Čurová et al. 2020). On the other hand, commensal strains isolated from France and Sweden in the 2000s and from North America (USA), Japan, and Australia also mainly belong to the B2 group (43%), while commensal strains isolated from France and Croatia in the 1980s and from Africa (Mali and Benin), Asia (Pakistan), and South America (French Guiana, Colombia, and Bolivia) belong mainly to the A group (55%) (Tenaillon et al. 2010). The genetic diversity of *E. coli* is also reflected in the presence of different mobile genetic elements, among which plasmids, especially the conjugative ones, are notorious for their effect on the genetic diversity and for carriage of virulence-associated genes (Kaper et al. 2004).

Although *E. coli* can exhibit host-associated and free-living/environmental state, the data on environmental *E. coli* strains are particularly scarce as there is a huge bias towards data on pathogenic strains (Starčič Erjavec and Žgur-Bertok 2015). In order to understand the heterogeneity of *E. coli* strains, their ability to adapt and spread within and outside hosts and the interactions between these strains, including gene exchange with horizontal transfers, it is necessary to obtain more data on characteristics of strains from specific human-associated environments such as household dishwashers.

Zupančič et al. (2019) found that *E. coli* are relatively frequent members of dishwasher rubber seal bacterial communities. In their study 35 *E. coli* isolates were obtained from eight out of 30 sampled dishwashers rubber seals. The aim of the present study was to conduct molecular characterisation of these 35 isolates, including the determination of phylogenetic group and multilocus sequence type (MLST) and screening of virulence-associated genes and typical replications regions associated with *E. coli* virulence plasmids.

Materials and methods

Bacterial isolates and growth conditions

A total of 35 *E. coli* isolated in six out of 30 dishwashers randomly selected in kitchens inside private dwellings located in seven Slovenian cities (i.e., Ljubljana, Velenje, Žalec, Celje, Mislinja, Sežana, and Portorož) were obtained from the Mycosmo Microbial Culture Collection Ex at the Biotechnical Faculty in Ljubljana (more informations about the dishwashers are available in Zupančič et al. 2019, additional file S1). All isolates were grown in liquid LB or on LB plates at 37 °C. Liquid cultures were vigorously aerated by shaking at 180 rpm.

Determination of phylogenetic groups of E. coli *isolates*

The phylogenetic groups for all of the *E. coli* isolates were determined by PCR, as described by Clermont et al. (2000) and improved by Clermont et al. (2013). The phylogenetic groups obtained by the method of Clermont et al. (2000) were assigned to their phylogenetic (sub)groups, according to the interpretation of Escobar-Páramo et al. (2006).

Multilocus sequence typing of E. coli *isolates*

Multilocus sequence typing was carried out according to Wirth et al. (2006) using PCR primers and protocols specified on the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) to amplify housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*. The purified PCR products were sent to Microsynth AG (Switzerland) for DNA sequencing. The sequences were analysed for allelic profiles and sequence types through the *E. coli* MLST website.

Detection of virulence-associated genes (VAGs) and plasmid replication regions of the E. coli *isolates*

The presence of 43 VAGs, including adhesins (*fimH*, *crl*, *eae*, *bmaE*, *gaf*, *aaf*, *papG*I, *papG*II, *papG*III, *matA*, *sfa*/*foc*, *iha*, *afa*/*dra*, and *hra*), autotransporters (*sat*, *vat*, *hbp* (*tsh*), *picU*, and *fluA* (*Ag43*)), iron acquisition systems (*fyuA*, *irp*, *sitA*, *iutA*, *iucD*, and *iroN*), genes involved in increased serum survival and protectins (*iss*, $ompT_{APEC}$, *kpsMT*II, *neuCS*, *cvi)*, toxins (*stx1*, *stx2*, *astA*, *eltA*, *cnf2*, *hlyA*), invasins (*aslA*, *ibeA*, *gimB*, *tia*), and uropathogenic specific protein *usp,* was tested in all isolates. This was achieved using amplification procedures as described previously (Dozois et al. 1992, Yamamoto et al. 1996, Yamamoto and Echeverria 1996, Maurer et al. 1998, Paton and Paton 1998, Schubert et al. 1998, Hoffman et al. 2000, Johnson and Stell 2000, Vila et al. 2000, Janβen et al. 2001, Ruiz et al. 2002, Ewers et al. 2007, Runyen-Janecky et al. 2003, Starčič et al. 2003, Tóth et al. 2003, Watt et al. 2003, Parham et al. 2005, Vidal et al. 2005) and the instructions for the 'DEC Primer Mix'[Statens Serum Institut, Denmark]. *FluA* was amplified using the primer pair FluAF 5'-GCGGTGTACTGCTGGCCG-3' and FluAR 5'-CGTTGTGGCTGCCCAGAC-3', under the following cycling conditions: initial denaturation for 5 min at 95 °C, 30 cycles of 30 s at 94 °C and 30 s at 60 °C, followed by extension for 90 s at 72 °C. Likewise for *usp*, using UspDe-F 5'-ATGCTACTGTTTCCGGGTAGTGTGT-3' / UspDe-R 5'-CRTGTAGTCKGGGSGTAACAAT-3': initial denaturation for 5 min at 95 °C, 30 cycles of 30 s at 94 °C and 30 s at 55 °C, followed by extension for 2 min at 72 °C. Both final extensions were performed at 72 °C for 10 min. The plasmid replication regions were determined by PCR as described previously for RepFIA, RepFIIA (Starčič Erjavec et al. 2003), RepFIC (Carattoli et al. 2005) and IncP (Sobecky et al. 1997). For RepFIB, the following protocol was used: initial denaturation at 94 °C for 4.5 min, followed by 30 cycles, each consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 63 °C, and 1.5 min of elongation at 72 °C. Additionally, the *E. coli* isolates were screened for F-like plasmid conjugative transfer genes *traJ* (the main positive regulator of conjugal transfer) and *traT* (surface exclusion and serum resistance), as described previously (Starčič Erjavec et al. 2003, Johnson and Stell 2000).

Results

All tested dishwasher E. coli *isolates belong to the non-pathogenic phylogenetic group A*

Phylotyping revealed, that all of the 35 *E. coli* isolates from the dishwasher rubber seals belonged to the commensal non-pathogenic phylogenetic subgroup A_0 according to Clermont et al. (2000) and Escobar-Páramo et al. (2006), and to phylogenetic group A according to Clermont et al. (2013).

Multilocus sequence typing analysis of E. coli *dishwasher isolates reveals low strain diversity*

The diversity of the *E. coli* isolates according to MLST analysis was relatively low. Twenty-seven (77%) of the 35 *E. coli* isolates were assigned to four known sequence types: ST189 (12 isolates, 44%), ST216 (7 isolates, 26%), ST399 (7 isolates, 26%), and ST1316 (1 isolate, 4%). The remaining eight isolates belonged to a new sequence type (Tab. 1).

Prevalence of virulence-associated genes among the E. coli *isolates from dishwasher rubber seals is low*

The prevalence of virulence-associated genes among the dishwasher rubber seal *E. coli* isolates was low (Tab. 1, Fig. 1). All isolates carried the curli fimbriae regulator gene *crl* and, except for strain L-436, the type 1 fimbrial adhesion gene *fimH*. Isolates L-687 and L-748 were positive for the autotransporter gene *picU*. PicU has mucinolytic activity through which colonisation of the host intestine is facilitated, by helping the pathogenic enteric bacteria to penetrate the mucus layer that coats the intestinal epithelium (Bhullar et al. 2015). Among the genes associated with iron acquisition, *sitA* was detected in isolate L-436, and *irp* and *fyuA* in isolates L-595 and L-1071. Further, 14 isolates (L-439, L-437, L-591, L-1101, L-778, L-781, L-785, L-786, L-790, L-808, L-814, L-815, L-821, L-836) were positive for *aslA*, a gene that is associated with invasion of the blood–brain barrier (Hoffman et al. 2000).

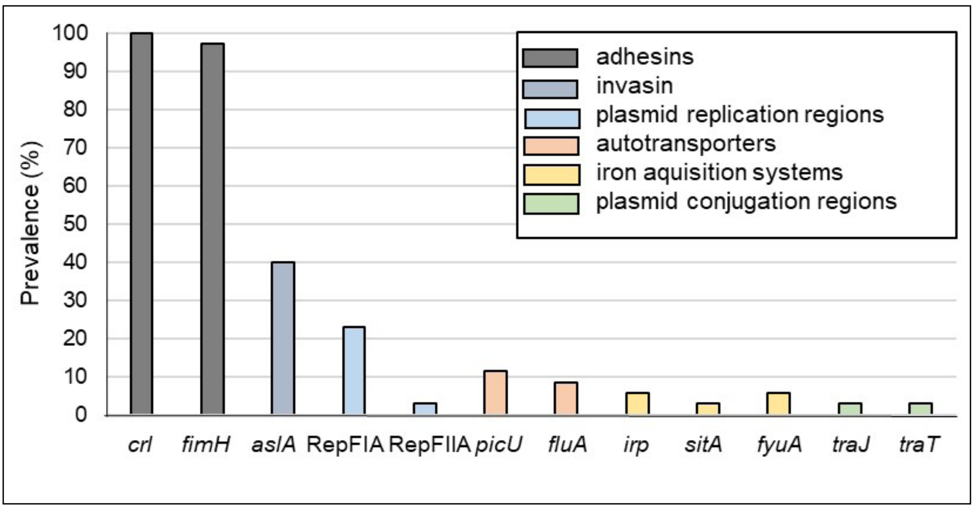

Figure 1: Prevalence of virulence-associated genes and plasmid replication regions in all *E. coli,* isolated from dishwasher rubber seals.

Slika 1: Prevalenca genov, povezanih z virulenco in plazmidnimi regijami za podvojevanje, v vseh izolatih *E. coli* z gumijastih tesnil pomivalnih strojev.

Prevalence of virulence plasmid associated sequences among the E. coli *isolates from dishwasher rubber seals is low*

In *E. coli*, plasmids can encode virulence factors and antibiotic resistance genes. Therefore, all of the isolates were analysed for plasmid-related characteristics; namely, the replication region and the *tra* region associated with conjugation. Of the five plasmid replication regions tested, only two were detected: RepFIA in eight isolates (L-396, L-434, L-431, L-433, L-594, L-736, L-983, L-982), and RepFIIA in one isolate (L-436) (Tab. 1). One isolate (L-429) was shown to have F-like plasmid *tra* region genes, *traJ* and *traT* (Tab. 1).

Table 1: MLST sequence type, virulence-associated genes and virulence plasmid associated sequences among the *E. coli* isolates from the dishwasher rubber seals. The following genes were not detected and are not included in the table: Adhesins: *eae*, *bmaE*, *gaf*, *aaf*, *papG*I, *papG*II, *papG*III, *matA*, *sfa/foc*, *iha*, *afa/dra* and *hra*, Protectins: *ompT, kpsMT*II, *neuCS, cvi* and *iss,* Autotransporters: *sat, vat* and *hpb/tsh,* Iron acquisition genes: *iutA, iucD* and *iroN*, Toxins: *stx1, stx2, astA, , cnf2, hlyA* and *elt,* Invasins: *ibeA, gimB* and *tia,* Uropathogenic specific protein: *usp*. The RepFIC, RepFIB and IncP plasmid replication regions were not present, and are not included in the table.

Tabela 1: Sekvenčni tip MLST, z virulenco povezani geni in zaporedja, značilna za plazmide, povezanih z virulenco pri izolatih *E. coli* z gumijastih tesnil kuhinjskih pomivalnih strojev. Naslednjih genov nismo zasledili pri preučevanih izolatih in zato niso vključeni v tabelo: Adhezini: *eae*, *bmaE*, *gaf*, *aaf*, *papG*I, *papG*II, *papG*III, *matA*, *sfa/foc*, *iha*, *afa/dra* in *hra*, Protektini: *ompT, kpsMT*II, *neuCS, cvi* in *iss,* Avtotransporterji: *sat, vat* in *hpb/tsh,* Sistemi za privzem železa: *iutA, iucD* in *iroN*, Toksini: *stx1, stx2, astA, , cnf2, hlyA* in *elt,* Invazini: *ibeA, gimB* in *tia,* Uropatogeni specifični protein: *usp*. Plazmidnih replikacijskih regij RepFIC, RepFIB in IncP prav tako nismo zasledili in niso vključene v tabelo.

Dish-washer	*E. coli* isolate	Sequence type	Adhesins		Autotrans-porters		Iron acquisition			Invasin	Plasmid replication regions		Plasmid conjugation regions	
			fimH	*crl*	*fluA*	*picU*	*irp*	*fyuA*	*sitA*	*aslA*	RepFIA	RepFIIA	*traT*	*traJ*
1	L-429	ST 399	+	+	+	–	–	–	–	–	–	–	+	+
	L-595	ST 399	+	+	+	–	+	+	–	–	–	–	–	–
	L-1071	ST 399	+	+	+	–	+	+	–	–	–	–	–	–
6	L-1101	ST 189	+	+	–	+	–	–	–	+	–	–	–	–
	L-790	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-396	ST 399	+	+	–	–	–	–	–	–	+	–	–	–
10	L-431	UN	+	+	–	–	–	–	–	–	+	–	–	–
	L-433	UN	+	+	–	–	–	–	–	–	+	–	–	–
	L-594	UN	+	+	–	–	–	–	–	–	+	–	–	–
	L-736	UN	+	+	–	–	–	–	–	–	+	–	–	–
	L-983	UN	+	+	–	–	–	–	–	–	+	–	–	–
	L-982	UN	+	+	–	–	–	–	–	–	+	–	–	–
13	L-434	ST 216	+	+	–	–	–	–	–	–	+	–	–	–

	L-589	ST 216	+	+	–	–	–	–	–	–	–	–	–	–
	L-590	ST 216	+	+	–	–	–	–	–	–	–	–	–	–
	L-592	ST 216	+	+	–	–	–	–	–	–	–	–	–	–
	L-745	ST 216	+	+	–	–	–	–	–	–	–	–	–	–
	L-747	ST 216	+	+	–	–	–	–	–	–	–	–	–	–
	L-748	ST 216	+	+	–	+	–	–	–	–	–	–	–	–
22	L-436	ST 1316	–	+	–	–	–	–	+	–	–	+	–	–
25	L-591	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-778	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-781	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-785	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-786	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-808	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-814	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-815	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-821	ST 189	+	+	–	–	–	–	–	+	–	–	–	–
	L-593	ST 399	+	+	–	–	–	–	–	–	–	–	–	–
	L-940	ST 399	+	+	–	–	–	–	–	–	–	–	–	–
	L-687	ST 399	+	+	–	+	–	–	–	–	–	–	–	–
	L-439	ST189	+	+	–	+	–	–	–	+	–	–	–	–
	L-437	UN	+	+	–	–	–	–	–	+	–	–	–	–
	L-836	UN	+	+	–	–	–	–	–	+	–	–	–	–

UN, unknown.

Discussion

The primary source of the studied dishwasher associated *E. coli* isolates might be both the household water supply system connected to the dishwasher and the contaminated vessels (Zupančič et al. 2019). In either case, it can be expected that studied *E. coli* strains are mainly commensal. Indeed, all of the *E. coli* isolates were assigned to phylogenetic group A_0, which is associated primarily with non-pathogenic commensal strains (Stoppe et al. 2017). This phylogenetic placement agrees with their low virulence potential and environmental adaptability. All of these isolates carried genes involved in adhesion; *crl*, and except one, *fimH*, the key factors in adhesion and biofilm formation on abiotic surfaces (Pratt and Kolter 1999). Furthermore, 14 isolates carried genes for AslA, a member of the arylsulphatase family of enzymes. In bacteria, the *asl* genes are expressed under conditions of sulphur starvation, in order to scavenge sulphate from exogenous substrates. In human meningitis strains of *E. coli* K1, AslA contributes to invasion of the blood–brain barrier (Hoffman et al. 2000). The prevalence of other tested virulence-associated genes was very low. Only three isolates carried one or two genes involved in iron acquisition. Three isolates carried *fluA* for autotransporter Ag43a, which mediates diffuse adherence and bacterial intercellular aggregation, thus promoting biofilm formation and persistence (Zude et al. 2013). *flu* genes are predominantly found in clinical isolates, although Restieri et al. (2007) reported that 56% of commensal isolates screened in their study were positive for Ag43 coding genes. Furthermore, four isolates carried the autotransporter gene *picU*, which has been associated with phylogenetic group B2 isolates. Pic has mucinolytic activity, promotes species-specific haemagglutination, host colonisation, and immune evasion, by direct cleavage of complement proteins. The potential role of Pic in commensal strains is unknown (Abreu et al. 2015, Bhullar et al. 2015).

Contrary to our initial expectations related to tap water as the environmental inoculum, MLST analysis indicated low diversity of *E. coli* isolates on dishwasher rubber seals. This might be partly due to isolation of the same strain from individual dishwashers, although isolates with the same sequence type isolated from the same dishwasher often carried different tested genes, e.g.: dishwasher 1 isolate L-429 (*traJ*, *traT*) and isolates L-595 and L-1071 (*irp*, *fyuA*) all belong to ST399. From dishwasher 25, isolates of three different sequence types were detected, namely ST189, ST399 and an as yet unknown sequence type. Again, isolates from the same ST also differed; e.g., ST189 strain L-439 was positive for *picU* and *aslA*, and isolate L-778 was positive only for *aslA*. The same applied to dishwasher 6, with isolates L-1101 (*picU*, *aslA*) and L-790 (*aslA*); both ST189. The same sequence type of isolated bacterial strain was found in different dishwashers (different time in use) with different frequency of use, which were even geographically located in different cities (e.g. Ljubljana and Celje), thus indicating the strong selective pressure for certain sequence type genetic background in such specific extreme environments.

Analysis of the traits characteristic for horizontal gene transfer (*traT*, *traJ* and IncP-replication region) showed that vast majority of the studied isolates does not possess conjugative F-like and IncP plasmids. In the light of the knowledge that virulence factor genes can be spread in bacterial populations via conjugative plasmids, the studied isolates are potential recipients which could have high acceptance for conjugative IncF or IncP group plasmids and hence might obtain additional virulence-associated genes via conjugation. Therefore, further studies revealing the ability of acquiring conjugative plasmids are needed.

Conclusion

To the best of our knowledge, this is the first molecular characterization of *E. coli* isolates from Slovenian dishwasher rubber seals. Based on the obtained results the studied isolates can be designated as commensal *E. coli* with low pathogenic potential. However, further studies should shed light on the genome traits enabling specific adaptation of some clonal linages to harsh environments such as the studied dishwasher rubber seals and the potential of horizontal gene exchange in such bacterial communities.

Povzetek

Koncept Enega zdravja vse bolj pridobiva na pomenu, saj smo spoznali, da je zdravje ljudi, domačih in divjih živali, rastlin in širšega okolja (vključno z ekosistemi) tesno povezano in soodvisno. *Escherichia coli* (*E. coli*), ki je eden najbolje raziskanih mikroorganizmov na svetu in dobro poznan genetski modelni organizem, je tipičen primer bakterije, ki jo najdemo v okolju, v vodi, zemlji, pa tudi v živalskih in človeških gostiteljih. *E. coli* sicer velja za komenzalno, nepatogeno bakterijo, ki s svojim gostiteljem živi v mutualističnem odnosu, a obstajajo sevi, ki zaradi nabora virulentnih dejavnikov lahko povzročajo okužbe. Za patogene seve *E. coli* so značilni številni različni dejavniki virulence, vključno z dejavniki, ki omogočajo kolonizacijo, sintezo toksinov in efektorskih molekul, ki se vpletajo v fiziologijo gostitelja. Nadalje je znano, da so določene filogenetske skupine povezane s patogenostjo sevov. Podatkov o okoljskih sevih *E. coli* in njihovih lastnostih je v literaturi malo. Namen te raziskave je

tako bil opredeliti molekularne značilnosti izolatov *E. coli* z gumijastih tesnil pomivalnih strojev in oceniti njihov patogeni potencial. V tej raziskavi smo 35 izolatov bakterije *E. coli*, osamljenih iz osmih vzorčenih gumijastih tesnil kuhinjskih pomivalnih strojev, analizirali z verižno reakcijo s polimerazo (PCR) z uporabo specifičnih začetnih oligonukleotidov za njihovo filogenetsko skupino in sekvenčni tip na osnovi multilokusnega zaporedja (MLST), za prisotnost 43 genov, povezanih z virulenco (VAG) *E. coli* pri črevesnih in zunajčrevesnih okužbah (geni adhezinov *fimH*, *crl*, *eae*, *bmaE*, *gaf*, *aaf*, *papG*I, *papG*II, *papG*III, *matA*, *sfa*/*foc*, *iha*, *afa*/*dra*, in *hra*; avtotransporterjev *sat*, *vat*, *hbp* (*tsh*), *picU*, in *fluA* (*Ag43*); sistemov za privzem železa *fyuA*, *irp*, *sitA*, *iutA*, *iucD*, in *iroN*; protektinov *iss*, *ompTAPEC*, *kpsMT*II, *neuCS*, *cvi*; toksinov *stx1*, *stx2*, *astA*, *eltA*, *cnf2*, *hlyA*; invazinov *aslA*, *ibeA*, *gimB*, *tia,* in uropatogenega specifičnega proteina *usp*) ter za prisotnost nekaterih značilnih zaporedij plazmidov, tudi povezanih z virulenco *E. coli* (zaporedja plazmidnih replikacijih regij RepFIA, RepFIB, RepFIIA, RepFIC, IncP ter zaporedji, povezani s konjugacijo, *traJ* in *traT)*. Ugotovili smo, da je vseh 35 izolatov *E. coli* pripadalo komenzalni, nepatogeni filogenetski skupini A po Clermontu in sod. (2013) in da je bila raznolikost teh izolatov glede na analizo MLST relativno majhna. Sedemindvajset izolatov smo uvrstili v enega izmed štirih sekvenčnih tipov: ST189 (12 izolatov), ST216 (7 izolatov), ST399 (7 izolatov), in ST1316 (1 izolat). Tudi razširjenost VAG med preučevanimi izolati *E. coli* je bila nizka. Potrdili smo le naslednje VAG: *fimH*, *crl*, *fluA*, *picU*, *irp*, *fyuA*, *sitA*, *aslA*. Vsi izolati so imeli genski zapis *crl*, *fimH* pa smo našli pri vseh, razen pri enem izolatu. Pri 14 izolatih smo potrdili prisotnost *aslA*, pri štirih izolatih *picU*, pri treh izolatih *fluA* ter pri dveh izolatih *irp* in *fyuA* (Tab. 1, Sl. 1). Od petih testiranih plazmidnih replikacijskih regij sta bili odkriti samo RepFIA in RepFIIA. Zaporedji *traJ* in *traT*, povezani s konjugativnimi plazmidi, sta bili odkriti samo pri enem izolatu (Tab. 1, Sl. 1). Majhna raznolikost sekvenčnih skupin (ST) ter podatka, da so bili iz istega pomivalnega stroja izolirani sevi iste ST z nekoliko drugačim naborom genov za dejavnike virulence, ter prisotnost sevov iz enake ST iz strojev na različnih lokacijah, bi lahko bila posledica na specifično okolje prilagojenih klonalnih skupin. Kolikor vemo, je to prva študija molekularne opredelitve izolatov *E. coli* z gumijastih tesnil pomivalnih strojev v Sloveniji. Na podlagi dobljenih rezultatov lahko preučevane izolate označimo kot komenzalne *E. coli* z nizkim patogenim potencialom.

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References

Abreu, A.G., Fraga, T.R., Granados Martínez, A.P., Kondo, M.Y., Juliano, M.A., Juliano, L., Navarro-Garcia, F., Isaac, L., Barbosa, A.S., Elias, W.P., 2015. The serine protease Pic from enteroaggregative *Escherichia coli* mediates immune evasion by the direct cleavage of complement. The Journal of Infectious Diseases, 212, 106-115.

Allocati, N., Masulli, M., Alexeyev, M.F., Di Ilio, C., 2013. *Escherichia coli* in Europe: an overview. International Journal of Environmental Research and Public Health, 10(12), 6235-6254.

Bhullar, K., Zarepour, M., Yu, H., Yang, H., Croxen, M., Stahl, M., Finlay, B.B., Turvey, S.E., Vallance, B.A., 2015. The serine protease autotransporter Pic modulates *Citrobacter rodentium* pathogenesis and its innate recognition by the host. Infection and Immunity, 83, 2636-2650.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. Journal of Microbiological Methods, 63, 219-228.

Chaudhuri, R.R., Henderson, I.R., 2012. The evolution of the *Escherichia coli* phylogeny. Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 12(2), 214-226.

Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Applied and Environmental Microbiology, 66, 4555-4558.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylotyping method revisited: improvement of specificity and detection of new phylo-groups. Environmental Microbiology Reports, 5, 58-65.

Čurová, K., Slebodníková, R., Kmeťová, M., Hrabovský, V., Maruniak, M., Liptáková, E., Siegfried, L., 2020. Virulence, phylogenetic background and antimicrobial resistance in *Escherichia coli* associated with extraintestinal infections. Journal of Infection and Public Health, 13(10), 1537-1543.

Dozois, C.M., Fairbrother, J.M., Harel, J., Bossé, M., 1992. Pap-and pil-related DNA sequences and other virulence determinants associated with *Escherichia coli* isolated from septicemic chickens and turkeys. Infection and Immunity, 60, 2648-2656.

Escobar-Páramo, P., Le Menac'h, A., Le Gall, T., Amorin, C., Gouriou, S., Picard, B., Skurnik, D., Denamur E., 2006. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environmental Microbiology, 11, 1975-1984.

Ewers, C., Li, G., Wilking, H., Kiessling, S., Alt, K., Antáo, E.M., Laturnus, C., Diehl, I., Glodde, S., Homeier, T., Böhnke, U., Steinrück, H., Philipp, H.C., Wieler. L.H., 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? International Journal of Medical Microbiology : IJMM, 297, 163-176.

Hoffman, J., Badger, J.L., Zhang, Y., Huang, S., Kim, K.S., 2000. *Escherichia coli* K1 *aslA* contributes to invasion of brain microvascular endothelial cells *in vitro* and *in vivo*. Infection and Immunity, 68, 5062-5067.

Jang, J., Hur, H.G., Sadowsky, M.J., Byappanahalli, M.N., Yan, T., Ishii, S., 2017. Environmental *Escherichia coli*: ecology and public health implications-a review. Journal of Applied Microbiology, 123(3), 570-581.

Janβen, T., Schwarz, C., Preikschat, P., Voss, M., Phillip, H.C., Wieler, L.H., 2001. Virulence associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. International Journal of Medical Microbiology : IJMM, 291, 371-378.

Johnson, J.R., Stell, A.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. The Journal of Infectious Diseases, 181, 261-272.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. Nature reviews. Microbiology, 2(2), 123-140.

Maurer, J.J., Brown, T.P., Steffens, W.L., Thayer, S.G., 1998. The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin tsh among avian *Escherichia coli*. Avian Diseases, 42, 106-118.

Miao, L., Li, H., Ding, W., Lu, S., Pan, S., Guo, X., Zhou, X., Wang, D., 2022. Research priorities on One Health: A bibliometric analysis. Frontiers in Public Health, 10, 889854.

Parham, N.J., Pollard, S.J., Chaudhuri, R.R., Beatson, S.A., Desvaux, M., Russell, M.A., Ruiz, J., Fivian, A., Vila, J., Henderson, I.R., 2005. Prevalence of pathogenicity island IICFT073 genes among extraintestinal clinical isolates of *Escherichia coli*. Journal of Clinical Microbiology, 43, 2425-2434.

Paton, A.W., Paton, J.C., 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfbO111*, and *rfbO157*. Journal of Clinical Microbiology, 36, 598-602.

Pratt, L.A., Kolter, R., 1999. Genetic analyses of bacterial biofilm formation. Current Opinion in Microbiology, 2, 598-603.

Restieri, C., Garriss, G., Locas, M.C., Dozois, C.M., 2007. Autotransporter-encoding sequences are phylogenetically distributed among *Escherichia coli* clinical isolates and reference strains. Applied and Environmental Microbiology, 73, 1553-1562.

Ruiz, J., Simon, K., Horcajada, J.P., Velasco, M., Barranco, M., Roig, G., Moreno-Martínez, A., Martínez, J.A., Jiménez de Anta, T., Mensa, J., Vila, J., 2002. Differences in virulence factors among clinical isolates of *Escherichia coli* causing cystitis and pyelonephritis in women and prostatitis in men. Journal of Clinical Microbiology, 40, 4445-4449.

Runyen-Janecky, L.J., Reeves, S.A., Gonzales, E.G., Payne, S.M., 2003. Contribution of the *Shigella flexneri* Sit, Iuc, and Feo iron acquisition systems to iron acquisition *in vitro* and in cultured cells. Infection and Immunity, 71, 1919-1928.

Schubert, S., Rakin, A., Karch, H., Carniel, E., Heesemann, J., 1998. Prevalence of the „high-pathogenicity island" of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. Infection and Immunity, 66, 480-485.

Sobecky, P.A., Mincer, T.J., Chang, M.C., Helinski, D.R., 1997. Plasmids isolated from marine sediment microbial communities contain replication and incompatibility regions unrelated to those of known plasmid groups. Applied and Environmental Microbiology, 63, 888-895.

Starčič, M., Žgur-Bertok, D., Jordi, B.J., Wösten, M.M., Gaastra, W., van Putten, J.P., 2003. The cyclic AMP-cyclic AMP receptor protein complex regulates activity of the *traJ* promoter of the *Escherichia coli* conjugative plasmid pRK100. Journal of Bacteriology, 185, 1616-1623.

Starčič Erjavec, M., Gaastra, W., van Putten, J., Žgur-Bertok, D., 2003. Identification of the origin of replications and partial characterization of plasmid pRK100. Plasmid, 50, 102-112.

Starčič Erjavec, M., Žgur-Bertok, D., 2015. Virulence potential for extraintestinal infections among commensal *Escherichia coli* isolated from healthy humans-the Trojan horse within our gut. FEMS Microbiology Letters, 362(5), fnu061.

Stoppe, N.C., Silva, J.S., Carlos, C., Sato, M.I.Z., Saraiva, A.M., Ottoboni, L.M.M., Torres, T.T., 2017. Worldwide phylogenetic group patterns of *Escherichia coli* from commensal human and wastewater treatment plant isolates. Frontiers in Microbiology, 8, 2512.

Tenaillon, O., Skurnik, D., Picard, B., Denamur, E., 2010. The population genetics of commensal *Escherichia coli*. Nature Reviews. Microbiology, 8, 207-217.

Tóth, I., Hérault, F., Beutin, L., Oswald E., 2003. Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV). Journal of Clinical Microbiology, 41, 4285-4291.

Vidal, M., Kruger, E., Durán, C., Lagos, R., Levine, M., Prado, V., Toro, C., Vidal, R., 2005. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. Journal of Clinical Microbiology, 43, 5362-5365.

Vila, J., Vargas, M., Henderson, I.R., Gascón, J., Nataro, J.P., 2000. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. The Journal of Infectious Diseases, 182, 1780-1783.

Watt, S., Lanotte, P., Mereghetti, L., Moulin-Schouleur, M., Picard, B., Quentin, R., 2003. *Escherichia coli* strains from pregnant women and neonates: intraspecies genetic distribution and prevalence of virulence factors. Journal of Clinical Microbiology, 41, 1929-1935.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Molecular Microbiology, 60, 1136-1151.

Yamamoto, S., Terai, A., Yuri, K., Kurazono, H., Takeda, Y., Yoshida, O., 1995. Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. FEMS Immunology and Medical Microbiology, 12, 85-90.

Yamamoto, T., Echeverria, P., 1996. Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene sequences in enterotoxigenic *E. coli* strains pathogenic for humans. Infection and Immunity, 64, 1441-1445.

Zude, I., Leimbach, A., Dobrindt, U., 2013. Prevalence of autotransporters in *Escherichia coli*: what is the impact of phylogeny and pathotype? International Journal of Medical Microbiology : IJMM, 304, 243-256.

Zupančič, J., Turk, M., Črnigoj, M., Ambrožič Avguštin, J., Gunde-Cimerman, N., 2019. The dishwasher rubber seal acts as a reservoir of bacteria in the home environment. BMC Microbiology, 19(1), 300.

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Study of temperature and food-grade preservatives affecting the *in vitro* stability of phycocyanin and phycoerythrin extracted from two *Nostoc* strains

Vpliv temperature in živilskih konzervansov na *in vitro* stabilnost fikocianina in fikoeritrina, ekstrahiranega iz dveh sevov vrste *Nostoc*

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Abstract: Cyanobacteria have many bioactive compounds. In the present study, we investigated the degree of purification and free radical scavenging ability of phycocyanin (PC) and phycoerythrin (PE), and compare their stability against selected preservatives at different temperatures with the aim of achieving the best and most stable preservative in increasing shelf life of PC and PE. After collecting and culturing *Nostoc* sp. strains FSN and ASN in BG-11_0 medium, the pigments phycocyanin and phycoerythrin were extracted and purified with 56% ammonium sulfate followed by dialysis. The antioxidant activity of pigments was evaluated by DPPH and ABTS assays. Their stability was compared with food-grade preservatives citric acid, sodium chloride, sucrose, and calcium chloride at two temperatures of 5 °C and 35 °C over time period from 3 to 30 days of cultivation. The results showed that the concentration and purity of the pigments increased after the dialysis, the pigments had antioxidant properties and were more stable at 5 °C. In addition, among different preservatives, citric acid caused more stability over time.

Keywords: bioactive compound, cyanobacteria, food-grade preservative, *Nostoc,* pigment

Izvleček: Cianobakterije vsebujejo mnoge bioaktivne spojine. V naši razskavi smo ekstrahirali barvili fikocianin (PC) in fikoeritrin (PE) in preučili njuno sposobnost odstranjevanja prostih radikalov ter primerjati njuno stabilnost v primerjavi z izbranim konzervansom pri različnih temperaturah z namenom doseganja najboljšega in najstabilnejšega konzervansa za podaljšanje roka uporabnosti PC in PE. Uporabili smo dva seva FSN in ASN vrste *Nostoc* sp., jih gojili v gojišču BG-110, ekstrahirali barvili in ju nadalje očistili s 56% amonijevim sulfatom in dializo. S testoma DPPH in ABTS smo ovrednotili antioksidativno aktivnost barvil. Njuno stabilnost smo primerjali z živilskimi konzervansi citronsko kislino, natrijevim kloridom, saharozo in kalcijevim kloridom pri 5 °C in 35 °C in v časovnem obdobju od 3 do 30 dni gojitve. Rezultati so pokazali, da sta se koncentracija in čistost barvil po dializi povečala, da imata barvili antioksidativne lastnosti in da sta bolj stabilni pri 5 °C. Izmed uporabljenih konzervansov je bila najbolj stabilna citronska kislina.

Ključne besede: bioaktivna spojina, modrozelene bakterije, *Nostoc*, barvilo, živilski konzervans

Introduction

Cyanobacteria are a diverse group of photosynthetic prokaryotes. They have the ability to photosynthesize oxygen, which is similar to ability of plants. However, instead of chloroplasts, cyanobacterial photosynthesis takes place in thylakoid membranes (Vothknecht and Westhoff 2001). Worldwide attention is drawn towards cyanobacteria for their possible use as food, feed, biopolymer, biofuel, and bio-fertilizer, for production of various secondary metabolites including vitamins, toxins, enzymes, pharmaceuticals, pharmacological probes, and pollution abatement (Righini et al. 2022). Phycobilisomes are light sensitive complexes in cyanobacteria that are located on the thylakoids and are essentially made up of protein pigment complexes known as phycobilia proteins (MacColl 1998). Phycobiliproteins (PBP) are divided into three groups based on their properties: phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC). They are also considered as environmentally friendly, non-toxic and have anti-cancer activities (Eghtedari et al. 2021). Among these pigments, PC and PE have been shown to have broad health properties that are used in food, pharmaceutical and cosmetic industries (Nowruzi and Porzani 2021). Recently, more attention is given to the application of PC and PE utilizing their biochemical and biophysical structural properties (Annibal et al. 2016). PC and PE are also used in fluorescent labelling of antibodies that are applied in diagnostic kits in immunology, cell biology and biomedical research (Sekar and Chandramohan 2008). PC, an accessory pigment to chlorophyll, is a pigment-protein complex of the phycobiliprotein family that occurs in many cyanobacteria and some red algae. Also, PC is a nutraceutical compound with antioxidant properties. It prevents oxidative damage and is used for human consumption as a colorant in food and milk shakes, and as an ingredient in cosmetic and pharmaceutical formulations (Mishra et al. 2010). PE is also good for human health, having antioxidant, radical scavenging, anti-inflammatory and anticancer properties. PE can be used as nutrient ingredient and natural dye for food and cosmetics besides, being potential therapeutic agent in oxidative stress-induced diseases and as a fluorescent marker in biomedical research (Mishra et al. 2010).

PC is formed by α- and β-subunits and has linear tetrapyrrole phytochromobilin covalently attached to its polypeptides via a thioether linkage to a conserved cystein residue (Stadnichuk et al. 2015). PC, exists as a complex interacting mixture of trimer, hexamer, and decamer aggregates which has been reported to be a function of pH and ionic strength of the medium, protein concentration, and algal origin (Mishra et al. 2008, Giannuzzi 2019). The stability of PC depends on its origin, pH, temperature, light, and some exogenous substances (Wu et al. 2016). Minor differences in the amino acid composition of PC affect the stability and structural properties of these proteins (Teng et al. 2010). Stabilizing agents are usually added to PC solutions to ensure long-term stability (Wu et al. 2016).

Antioxidants can prevent the production of reactive oxygen species (ROS) and scavenge them (He and Häder 2002). Excess formation and/ or insufficient removal of ROS causes oxidative stress, which is related to various diseases such as cancer, diabetes mellitus, inflammatory diseases, as well as neuro-degenerative diseases (Yang et al. 2011). Both the apoproteins and the prosthetic group, which are structural components of PBP, are involved in ROS stabilization. Apoproteins contributes to the removal of hydroxyl radical, as well as hypochlorous acid radical by reacting with its cysteine and methionine residues (Pleonsil et al. 2013). Other amino acids such as tryptophan, tyrosine and histidine can remove peroxyl radicals (Fernández-Rojas et al. 2014a). Fernandez-Rojas et al. (2014a) reported that phycocyanobilin removes most free radicals, the singlet oxygen is stabilized by the oxidation of double bonds of the tetrapyrrole. Phycocyanobilin can also scavenge peroxynitrite, hypochlorous, hydroxyl and peroxyl radicals (Fernández-Rojas et al. 2014a). Evidence is accumulating on the hydroxyl, and peroxyl free radical-scavenging properties of PC suggesting that its therapeutic effects are largely attributed to its antioxidant potentials (Farooq et al. 2014). It has been found that PC prevents apoptosis in mice with Cisplatin-induced nephrotoxicity. The protective effect of PC against CP-induced nephrotoxicity was associated with the ROS scavenging ability of phycocyanin (Fernández-Rojas et al. 2014c).

It is suggested that the human consumption of PC may be useful for the prevention and/or treatment of kidney diseases associated to oxidative stress (Fernández-Rojas et al. 2014b, Wu et al. 2016).

Although several methods have been developed for the separation and purification of PC and PE from cyanobacteria, the purity and recovery is relatively low because these pigments are highly sensitive to light, oxygen and moisture, hence, it is needed to process it along with efficient preservatives (Mishra et al. 2008). Sodium azide and dithiothreitol are commonly used as preservatives of PE for analytical purpose, but they are toxic (Mishra et al. 2010); so for developing process of food grade PE only edible preservatives with unique properties can be used (Mishra et al. 2010). It is therefore, desired to develop a simple, but more well-organized method for the separation, purification and stabilization of the food grade PE from cyanobacteria (Patil et al. 2006). Food additives have been used by mankind for centuries, e.g. salt; sugar and vinegar were among the first to be used as preservatives in food (Mishra et al. 2010). PE can be used as a food colorant which is not only safe but, has an extra advantage of being potential antioxidant. Preservative for the food grade PE is indispensable for making the process commercially viable due to its extreme sensitivity towards light, oxygen and temperature under aqueous condition (Mishra et al. 2010).

The main and the simplest way to improve the stability of PBP is by using additives. Most studies have focused on the use of additives to improve their thermal stability (Martelli et al. 2014; Gonzalez-Ramirez et al. 2014; Braga et al. 2016). This method of stabilization is easy to apply and does not require sophisticated or expensive equipment. However, low toxicity additives must be used because large amounts of additives may be necessary. Temperature and pH play an important role in the stability of PC (Freitas et al. 2022). Systematic investigations showed the maximum stability of PC was in the pH range of 5.5–6.0. Incubation at temperatures between 47 °C and 64 °C caused the concentration and half-life of PC in solution to decrease rapidly (Chaiklahan et al. 2012). In case of PE, the maximum stability was in the pH range of 4.0-10.0 (Gonzalez-Ramirez et al. 2014). In addition, all PBPs were thermostable up to 4-40 °C while their concentration decreased rapidly at 60-80 °C (Rastogi et al. 2015a).

Temperature is an important factor that strongly influences the oxygen-evolving activity of photosystem II and affects nutrients availability (Markou and Georgakakis 2011). Also, the fluidity of the cell membrane of microalgae is affected by temperature (Daliry et al. 2017). The optimum temperature for cyanobacterial cultivation depends on the species and strain (Mehnert et al. 2010). Increase in temperature above the optimum decreases the microalgae biomass primarily due to the denaturation of essential proteins and enzymes. In addition, if the temperature is below the optimum state, the growth of cyanobacterial cells slows down and enters the stationary phase (Hsieh-Lo et al. 2019). PC and PE are stable at low temperatures (4-5 °C) with addition of preservatives like citric acid (e.g., acidic or basic solutions) and therefore they could be utilized as a food colorant (chewing gum, jellies), beverages, and coloring agent in the sweet confectionery and cosmetics (Patel et al. 2004, Mishra et al. 2008, Eriksen et al., 2008).

The use of PC in food and other applications is limited due to its sensitivity to heat treatment, which results in precipitation and fading of the blue color. Sodium azide and dithiothreitol are commonly used as preservatives for PC for analytical purposes, but they are toxic and thus cannot be used for food-grade PC production (Mishra et al. 2008). Sugars and polyhydric alcohols have been used to stabilize proteins, and are being used widely at present as stabilizing agents in the food industry as well as in pharmaceutical formulations since they are safe for consumption (Petersen et al. 2004). Moreover, many studies have reported that the modification of the protein conformation itself can improve the stability of proteins (Deller et al. 2016). Fukui et al. (2004) reported that dithio- bis (succinimidyl propionate)) DSP) modified phycocyanin was resistant to bleaching by urea treatment because the amino groups of modified lysine residues were cross-linked, thus maintaining the protein's high-order structure (Fukui et al. 2004). Another method used to improve stability was reported by Li et al. (2009). The study of Chaiklahan et al. (2012) showed that the measured photodamage rate constant of PC entrapped in a silica matrix (immobilized biomolecules of protein with silica) was 25-times lower than that of PC in a buffer solution (Chaiklahan et al. 2012, Li et al. 2009).

Although a number of reports are available for PE and PC purification and characterization from different cyanobacterial and red algal strains, exploitation and optimization of PE and PC production from the genus *Nostoc* extracted from paddy fields with different pigments are limited (Mishra et al. 2010; Tan et al. 2016). However, the main challenges for PE and PC commercialization and implementation in food and cosmetic applications are their low yield during production and limited chemical instability (Freitas et al. 2022). For this reason, the present study aimed isolation and purification of the PE and PC from *Nostoc* sp. strains FSN and ASN, and evaluation of their antioxidant activity for biotechnological applications. In addition, no research has been done on the optimum temperature. So, in this study, two temperatures of 5 °C and 35 °C degrees were studied during 35 days of growth. Also, shelf times of two pigments with or without additives were evaluated.

Materials and methods

Cyanobacterial isolation and growth conditions

Nostoc sp. strain FSN and *Nostoc* sp. strain ASN were isolated from paddy fields of Golestan province, Iran (36° 54′ 41″ N, 54° 47′ 25″ W). In order to obtain a cyanobacterial monoculture, soil samples were spread into sterile Petri dishes containing liquid BG-11_0 (two 10-days-old cyanobacterial strains) medium (Allen 1968), without a nitrogen source, pH 7.1, and incubated in a growth chamber (Merck, Germany) for two weeks at 28 ± 1 ºC under constant cool white fluorescent light (100-150 μE/m^2s). After 14 days of growth, selected colonies were transferred to a fresh solid BG-11_0 medium. For bacteria-free cultures, colonies were tested for bacterial contamination in dextrose-peptone broth and caseinate-glucose agar media according to Rajabpour et al. (2019). The selected bacteria-free colony was maintained on different agar slants media. After 20 days, the isolate was washed with sterile deionized water, and transferred to 1L of freshly prepared liquid BG-11_0 medium.

Morphological characterization of the studied strains

Cyanobacterial strains were examined under a light microscope (Leica DM750). After cultivation, the morphological characteristics were investigated according to the classification system devised by Komárek (2013). The strain FSN was green, and the strain ASN was brown. The reason for the color change is due to the higher concentration of one pigment than the other and it is common among cyanobacterial strains. When there is more PE, the strain is brown and when the level of PC is higher, the strain is green.

Molecular analysis

Because it is not possible to use the morphological characteristics of cyanobacteria to accurately identify the genus, the most reliable method to identify the genus is to use the genetic sequences. To do this, genomic DNA was extracted from living cells using the EZNA SP Plant DNA mini kit (Omega Bio-tek) according to the manufacturer's instructions. PCR reactions were performed on the genomic DNA using the oligonucleotide primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and B23S (5'-CTTCGCCTCTGTGTGCCTAGGT-3') (Stoyanov et al. 2014) that target the 16S rRNA gene sequence. Reactions were cycled with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C, and final extension step at 72 °C for 5 min. Amplicons were verified by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. PCR products were purified using the Geneclean® Turbo kit (Qbiogene, Inc.) prior to sequencing.

Sequencing reactions were carried out using the refined PCRs products in a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Life Technologies). A total volume of 10 μL of the PCR master mix included 1 μL of forward or reverse primers (10 μM), 1 x sequencing buffer, 1 μL of Big dye and 100 ng (1μL) of DNA. The cycle sequencing reaction was carried out using 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 minutes, followed by storage overnight at 4 °C. After the completion of the sequencing reactions, the sequenced products were precipitated by adding 40 µL of 0,125 M NaCl and 2.5 x volume of cold 100% ethanol, followed by vortexing and centrifugation at 13,000 rpm for 10 min at 4 °C. Once the supernatant was removed, a 5 x volume of 70% ethanol was added, and the sample centrifuged at 13,000 rpm for 5 minutes (4 °C). The supernatant was removed, and the pellet was dried at 37 °C. The purified reactant was resuspended in 12 µL of HiDi-formamide, the mixture was spun down, denatured for 2 min at 94 °C and subjected to sequencing. The runtime for each reaction was 45 min with a running voltage of 15 kV at a temperature of 50 °C and the polymer used was POP-6™ (Applied Biosystems, Life Technologies). The 16S rRNA gene sequences obtained in this study was used to construct a consensus sequence in BioEdit version 7.0. Positions with gaps, as well as undetermined and ambiguous sequences were removed. BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) of the partial 16S rRNA gene sequence were used to identify similar sequences available in the GenBank database of National Center for Biotechnology Information (NCBI).

Chemicals

All chemicals and protein molecular weight marker used in this study were of analytical grade, purchased from the Hi-Media, Merck and Sigma manufacturers. All buffers and reagents used were prepared in double distilled water.

Extraction and purification of PC and PE

As two *Nostoc* strains have different colors, we assumed that they have PC and PE. PE and PC were extracted from 500 mL of homogenized log phase (14 days old) culture after centrifuging at 4,000 rpm to obtain a pellet. The pellet was suspended in 100 mL of 20 mM acetate buffer (pH 5.1). Extraction was carried out by repeated freezing (-20°C) and thawing (room temperature) method for 4 days until cell biomass became dark purple according to Afreen and Fatma (2018). Cell debris was removed by centrifugation at 5,000 rpm for 10 min, and a crude extract was obtained.

Purification was carried out according to Afreen and Fatma (2018) (Afreen and Fatma 2018). Solid ammonium sulphate was added to the crude extract slowly for achieving 65% saturation by continuous stirring. The resulting solution was allowed to stand for 12 h under cold room, and centrifuged at 4,500 rpm for 10 min. The pellets were resuspended in a small volume of 50 mM acetic acid–sodium acetate buffer (pH 7.1), and dialyzed overnight. The extract was recovered from the dialysis membrane and filtered through 0.45 µm filter (Chakdar and Pabbi 2012, Tiwari et al. 2015).

The absorption spectrum was determined by scanning the sample in a range of 300-750 nm wavelengths by Specord 200 spectrophotometer (Analytik Jena, Germany) (Mishra et al. 2008, 2010).

The amounts of PE, PC and APC in different extracts and biliprotein (PBPs) containing solutions were calculated from measurements of the absorbance at 565 nm, 620 nm and 650 nm using the following equations below (Nowruzi et al., 2020). Purity of PE and PC were calculated at each step as purity ratio (A555/A280) and (A620/A280), respectively.

$$\text{PE } (\mu\text{g mL}^{-1}) = \frac{(\text{OD } 565\text{nm} - 2.8[\text{R-PC}] - 1.34[\text{APC}]}{12.7})$$

$$\text{PC } (\mu\text{g mL}^{-1}) = \frac{(\text{OD } 620\text{nm} - 0.7 \text{ OD } 650\text{nm})}{7.38})$$

$$\text{APC } (\mu\text{g mL}^{-1}) = \frac{(\text{OD } 650\text{nm} - 0.19 \text{ OD}620\text{nm})}{5.65})$$

Stability studies of purified pigments

Stability of purified PE and PC were obtained by adding additives including citric acid, sucrose, sodium chloride, and calcium chloride as preservatives (0.193 and 0.653 mg/mL, respectively) at different temperatures (5 and 35 °C) by recording its absorption spectrum for 30 days (Setyoningrum and Nur 2015, Gonzalez-Ramirez et al. 2014, Rastogi et al. 2015b).

Antioxidant activity of purified PE and PC

DPPH assay

This test was conducted following the method described by Shanab et al. (2012) with modifications. An amount of 710 µg/mL purified PE was mixed with 1 mL of DPPH reagent. After incubating for 30 min in the dark at room temperature, the absorbance was measured at 517 nm. Ascorbic acid (100 µg/mL) was used as a positive control.

$$\text{Activity (\%)} = Ac - At/Ac \times 100$$

where At was the absorbance of sample, and Ac the absorbance of DPPH.

ABTS assay

$ABTS^+$ radicals (7 mM) were produced by adding 2.45 mM potassium persulphate in the dark for 12–16 h. The resulting solution was diluted with ethanol up to an absorbance of 0.5 at 734 nm. An aliquot of 3 mL of $ABTS^+$ solution was added to 50 µL of the PE sample (710 µg/mL) and standards, and the absorbance was recorded at 734 nm against ethanol as blank (Re et al. 1999). $ABTS^+$ solution was taken as positive control and BHT as standard.

$$\text{Activity (\%)} = Ac - At/Ac \times 100$$

where At was the absorbance of sample, and Ac the absorbance of ABTS.

Results

Morphological and molecular characterization

Nostoc sp. strains FSN and ASN were grown rapidly on liquid media culture, and changed the colour to dark brown and dark green after 15 days (Fig. 1). The unicyanobacterial culture and dry material were deposited into ALBORZ herbarium and Cyanobacteria Culture Collection (CCC) of Science and Research Branch, Islamic Azad University, Tehran, respectively. The phylogenetic position of the two *Nostoc* strains in relation to *Nostoc-carneum*-BF2 is shown in full circle (Fig. 2). Numbers near nodes indicate bootstrap values over 50% for NJ analyses.

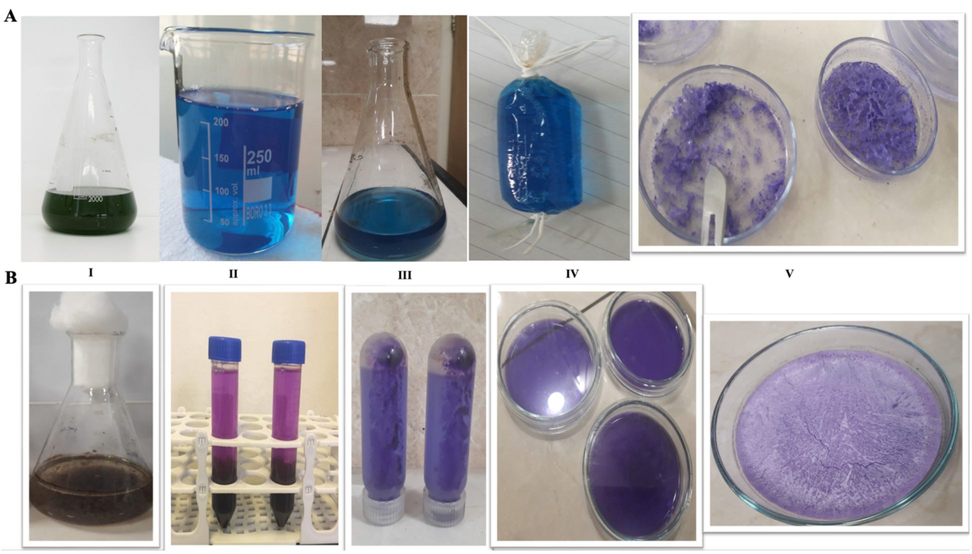


Figure 1: Pigment production process of (**A**) phycocyanin and (**B**) phycoerythrin. I, Primary cultivation; II, Preparation of the crude extract; III, Ammonium sulphate precipitation; IV, Dialysis; V, Freeze-drying.
Slika 1: Proces pridobivanja barvila (A) fikocianina in (B) fikoeritrina. I, Primarna kultura; II, Priprava grobega ekstrakta; III, Precipitacija z amonijevim sulfatom; IV, Dializa; V, Zamrznjeno sušenje.

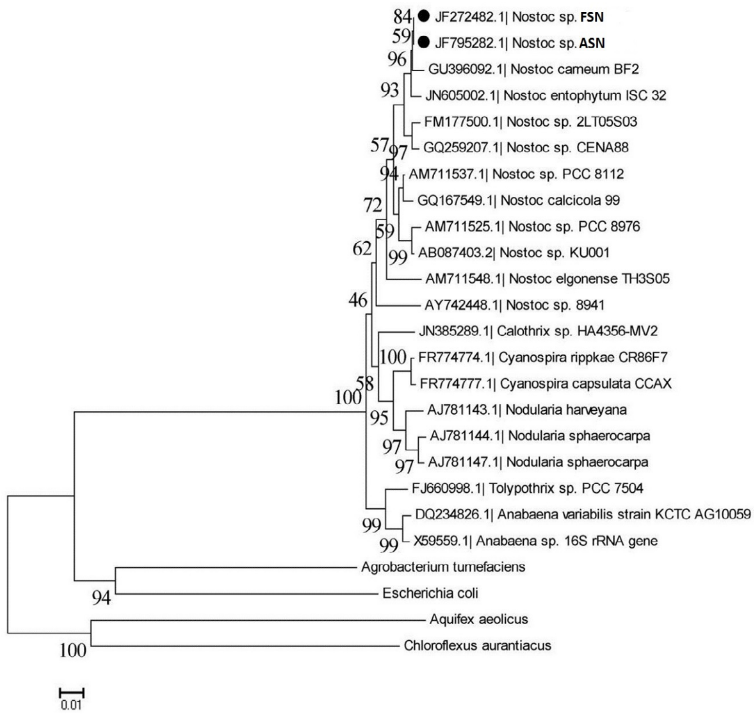


Figure 2: Consensus bootstrap tree on the basis of neighbour-joined distances of 1815 bp long full-length 16S rRNA genes sequences and sequences that were taken from the GenBank. Bar = 0.01 change per sequence position.

Slika 2: Filogenetsko drevo po metodi skupnega vezenja na osnovi 1815 bp dolgega genskega zaporedja za 16S rRNA in zaporedij iz baze GenBank. Merilce predstavlja spremembo 0,01 v legi zaporedja.

Extraction, purification and characterization of PE and PC

At each purification step, concentration and purity of PE and PC were checked as shown in Table 1. During successive steps of purification, the purity absorption rate increased from 0.797 nm up to 3.200 nm for PE and from 0.209 nm up to 0. 251 nm for PC. Purity ratio was found to be enhanced after each purification step. From crude extract to purified PE, purity was increased by almost 4-times for PE and almost 1.2-times for PC, which showed the efficiency of the method using successive steps of purification (solid ammonium sulphate and dialysis) to obtain high purity PE.

Table 1: Stepwise purification of phycoerythrin (PE) and phycocyanin (PC) from two *Nostoc* strains.
Tabela 1: Koraki čiščenja fikoeritrina (PE) in fikocianina (PC) iz dveh sevov vrste *Nostoc*.

Nostoc **sp. strain**	**Step**	**Peak**	**PE (µg/mL)**	**Purity of PE (OD555/OD280)**
FSN	Crude extract	566.2 - 616.9	0.108	0.797
	Ammonium sulphate precipitation	565.5 - 617.4	0.152	1.559
	Dialysis	567.6 - 617.7	0.193	3.20
	Step	**Peak**	**PC (µg/mL)**	**Purity of PC (A620/A280)**
ASN	Crude extract	619/8	0.058	0.209
	Ammonium sulphate precipitation	620.1	0.063	0.239
	Dialysis	621.9	0.653	0. 251

Effects of different temperatures and preservatives on PE and PC

The absorption spectrum was recorded with or without preservative at 5 °C and 35 °C. The visible absorption spectra of PE and PC showed that the loss of color was slightly lower at 5°C and very high at 35°C in 30 days (Tab. 2). The maximum rate of decrease in color was found with control (Fig. 3 and 4) at both temperatures, while the use of additives increased the absorbance of PE and PC. Among the additives, with citric acid at both temperatures, the absorbance increased (Fig. 5 and 6).

Table 2: Absorbance of purified phycoerythrin (PE) and phycocyanin (PC) at various days and temperatures of cultivation.
Tabela 2: Aborbanca očiščenega fikoeritrina (PE) in fikocianina (PC) ob različnih dnevih in temperaturah gojenja.

	Day 3 / 5 °C	**Day 3 / 35 °C**	**Day 5 / 5 °C**	**Day 5 / 35 °C**	**Day 10 / 5 °C**	**Day 10 / 35 °C**	**Day 15 / 5 °C**	**Day 15 / 35 °C**	**Day 20 / 5 °C**	**Day 20 / 35 °C**	**Day 30 / 5 °C**	**Day 30 / 35 °C**
Nostoc **sp. FSN (PE)**	0.20	0.13	0.19	0.12	0.14	0.06	0.10	0.042	0.10	0.04	0.08	0.03
Nostoc **sp. ASN (PC)**	0.63	0.33	0.37	0.33	0.27	0.22	0.26	0.20	0.24	0.19	0.21	0.16

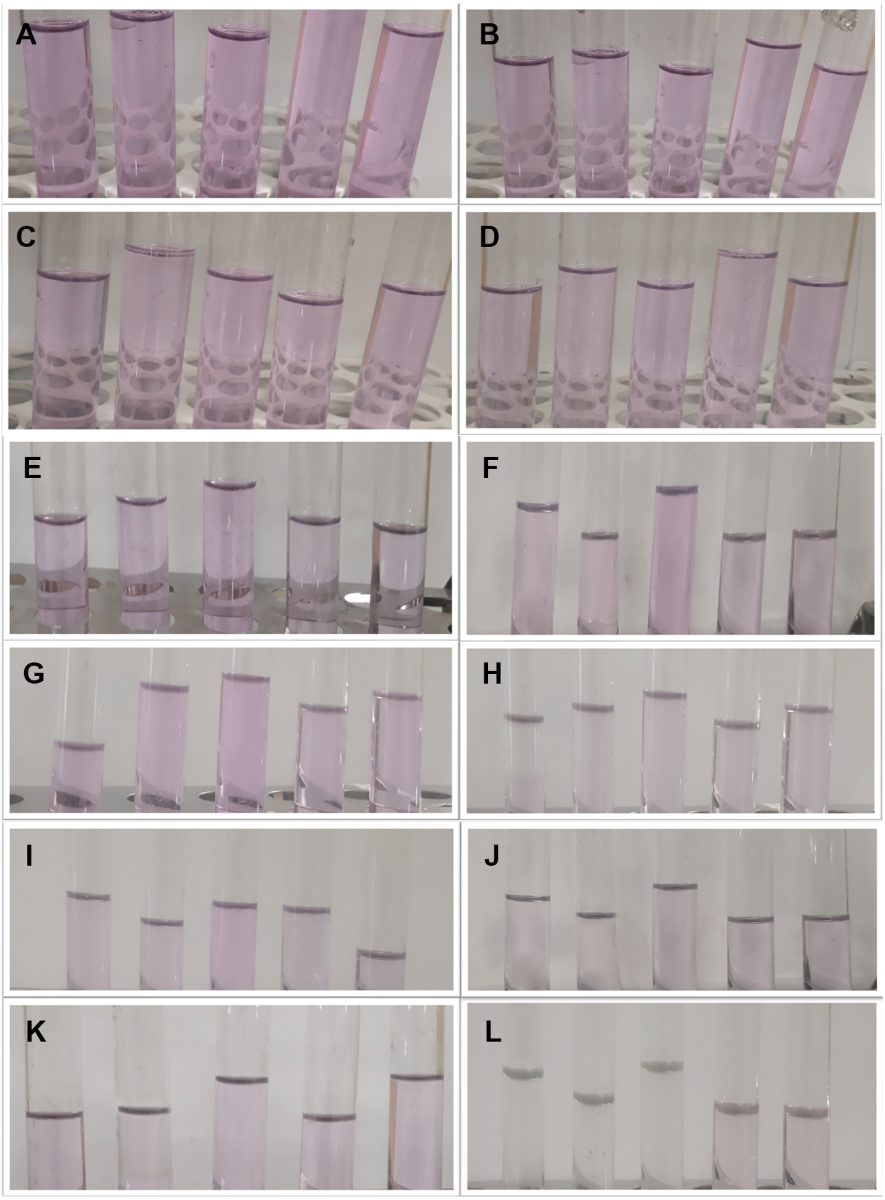


Figure 3: Coloured eluates of phycoerythrin at different days and temperatures. A, day 3 / 5 ◦C; B, day 3 / 35 ◦C; C, day 5 / 5 ◦C; D, day 5 / 35 ◦C; E, day 10 / 5 ◦C; F, day 10 / 35 ◦C; G, day 15 / 5 ◦C; H, day 15 / 35 ◦C; I, day 20 / 5 ◦C; J, day 20 / 35 ◦C; K, day 30 / 5 ◦C; L, day 30 / 35 ◦C.

Slika 3: Obarvani eluati fikoeritrina ob različnih dnevih in temperaturah. A, dan 3 / 5 °C; B, dan 3 / 35 °C; C, dan 5 / 5 °C; D, dan 5 / 35 °C; E, dan 10 / 5 °C; F, dan 10 / 35 °C; G, dan 15 / 5 °C; H, dan 15 / 35 °C; I, dan 20 / 5 °C; J, dan 20 / 35 °C; K, dan 30 / 5 °C; L, dan 30 / 35 °C.

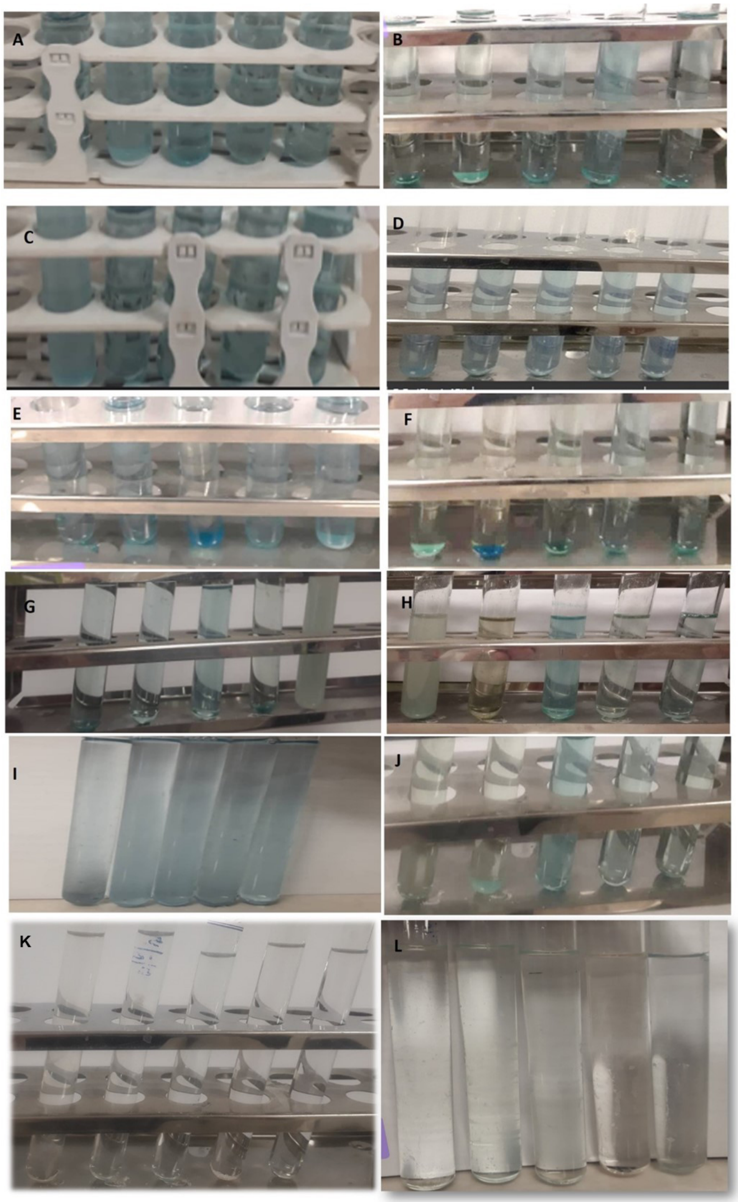
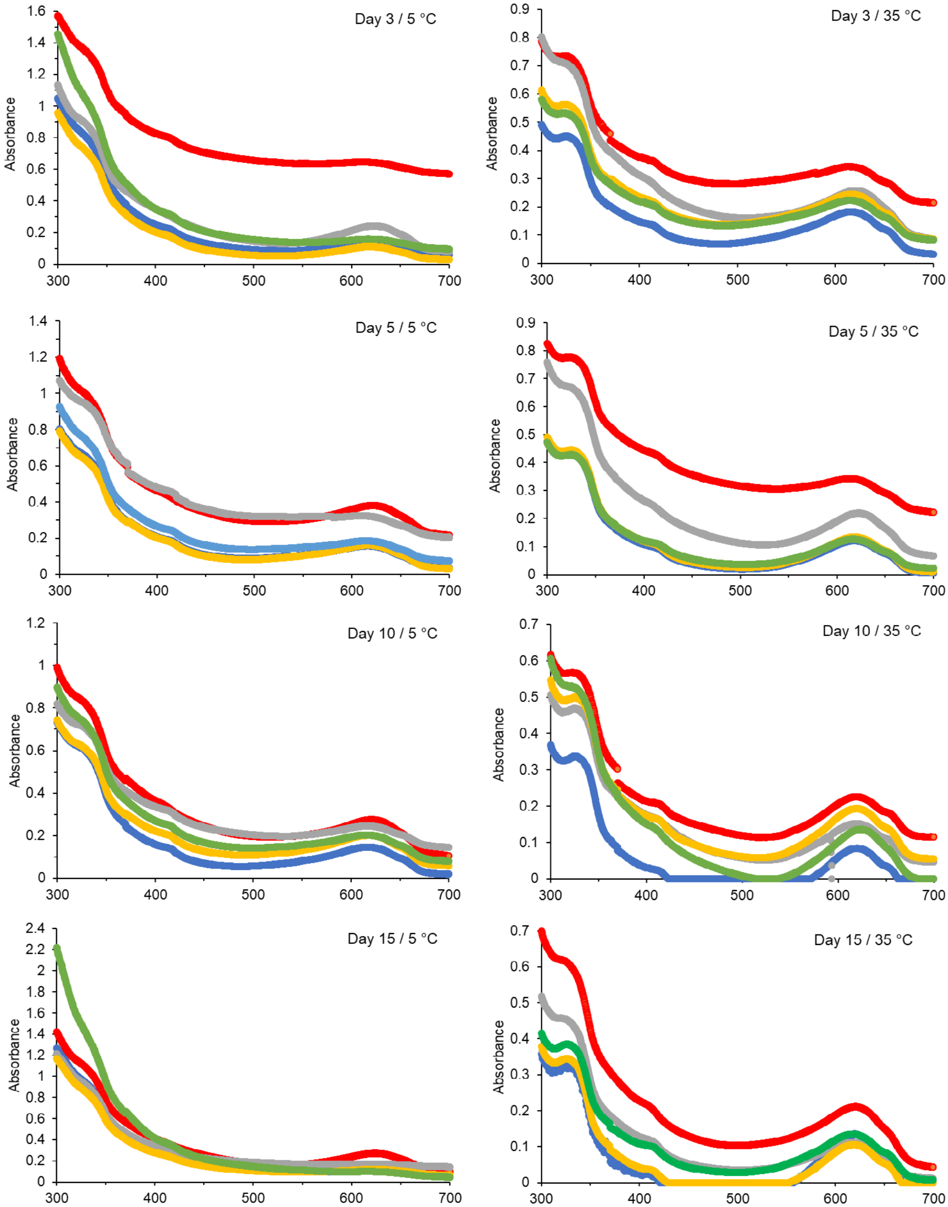


Figure 4: Coloured eluates of phycocyanin at different days and temperatures. A, day 3 / 5 °C; B, day 3 / 35 °C; C, day 5 / 5 °C; D, day 5 / 35 °C; E, day 10 / 5 °C; F, day 10 / 35 °C; G, day 15 / 5 °C; H, day 15 / 35 °C; I, day 20 / 5 °C; J, day 20 / 35 °C; K, day 30 / 5 °C; L, day 30 / 35 °C.

Slika 4: Obarvani eluati fikocianina ob različnih dnevih in temperaturah. A, dan 3 / 5 °C; B, dan 3 / 35 °C; C, dan 5 / 5 °C; D, dan 5 / 35 °C; E, dan 10 / 5 °C; F, dan 10 / 35 °C; G, dan 15 / 5 °C; H, dan 15 / 35 °C; I, dan 20 / 5 °C; J, dan 20 / 35 °C; K, dan 30 / 5 °C; L, dan 30 / 35 °C.


Day 3 / 5 °C
Day 3 / 35 °C
Day 5 / 5 °C
Day 5 / 35 °C
Day 10 / 5 °C
Day 10 / 35 °C
Day 15 / 5 °C
Day 15 / 35 °C
Absorbance

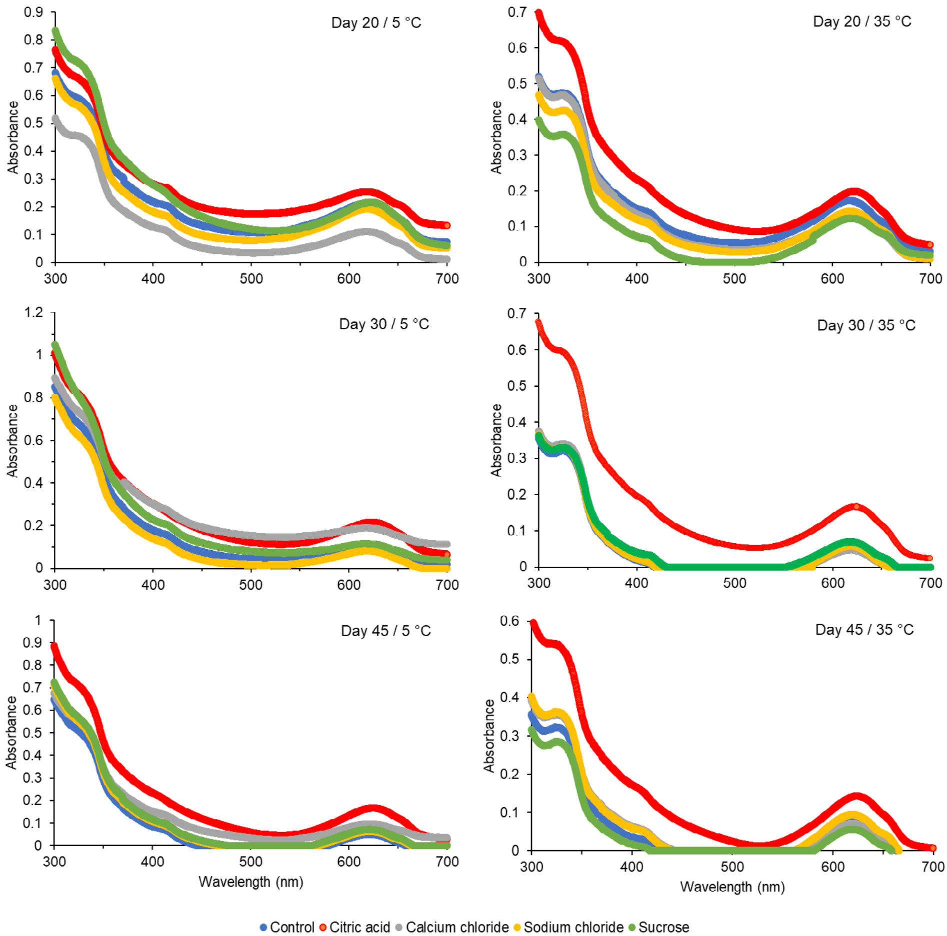
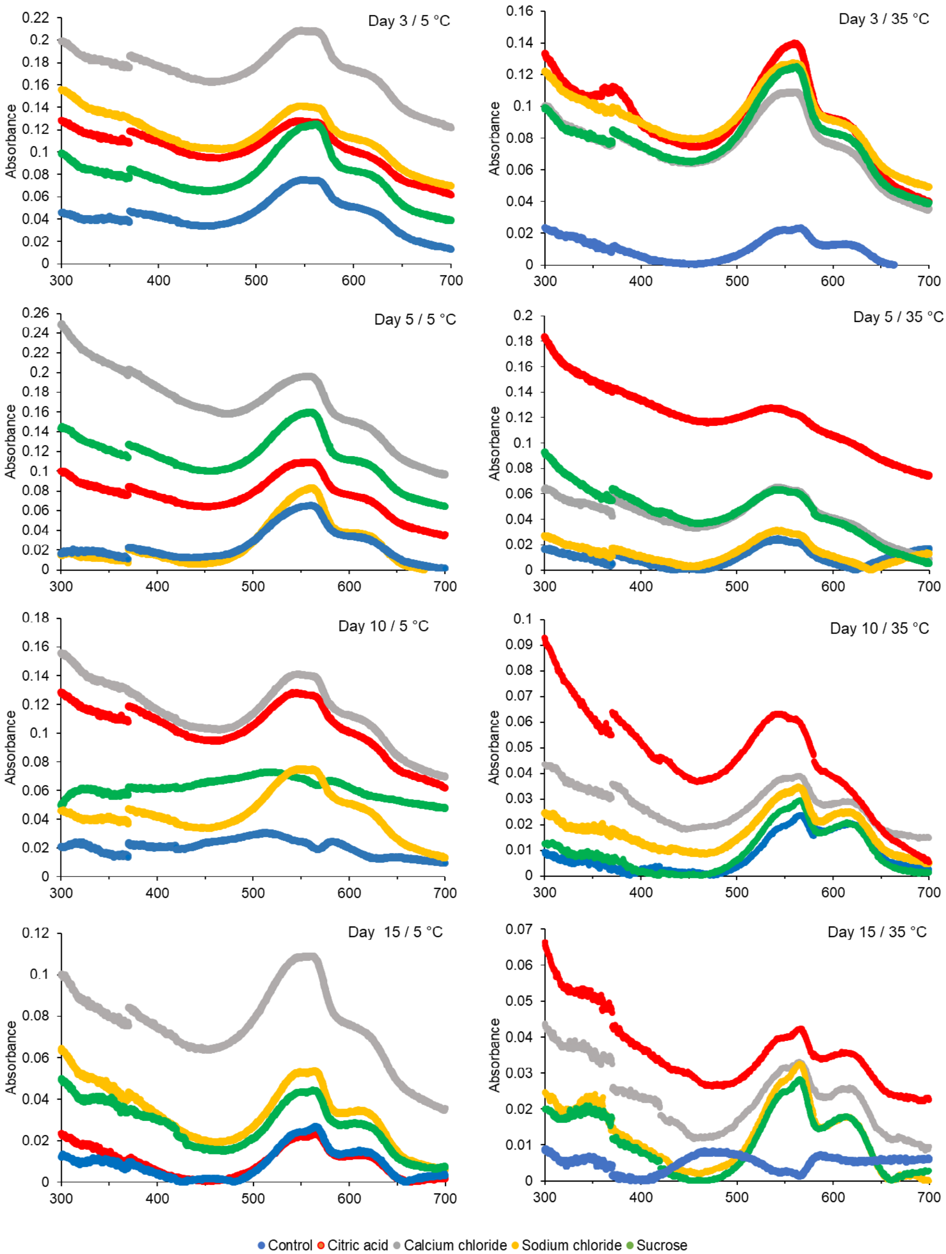


Figure 5: Absorbance spectra of purified phycocyanin at different days and temperatures.
Slika 5: Absorpcijski spektri fikocianina ob različnih dnevih in temperaturah.


Day 3 / 5 °C
Day 3 / 35 °C
Day 5 / 5 °C
Day 5 / 35 °C
Day 10 / 5 °C
Day 10 / 35 °C
Day 15 / 5 °C
Day 15 / 35 °C
Absorbance
Control Citric acid Calcium chloride Sodium chloride Sucrose

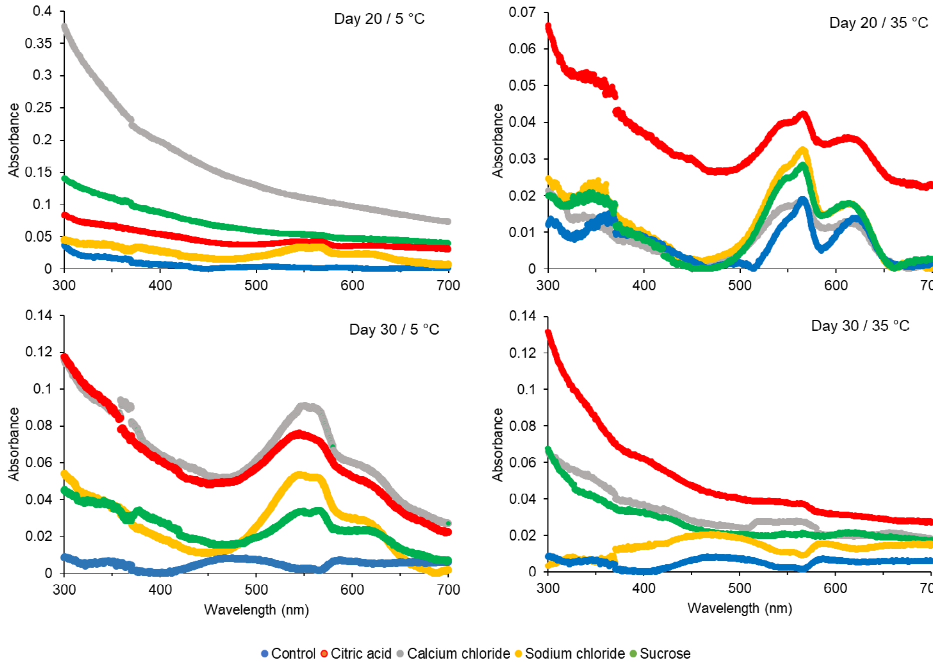


Figure 6: Absorbance spectra of purified phycoerythrin at different days and temperatures.
Slika 6: Absorpcijski spektri fikoeritrina ob različnih dnevih in temperaturah

Antioxidant activity of purified PE and PC

Free radical scavenging potential of two *Nostoc* strains in two methods was found to be concentration dependent. IC_{50} value in DPPH and ABTS method were 0.03 and 0.04 mg/mL for FSN and ASN strain, and 0.02 mg/mL for ascorbic acid (vitamin C) and butylated hydroxyl toluene (BHT), that were used as standard (Fig. 7).

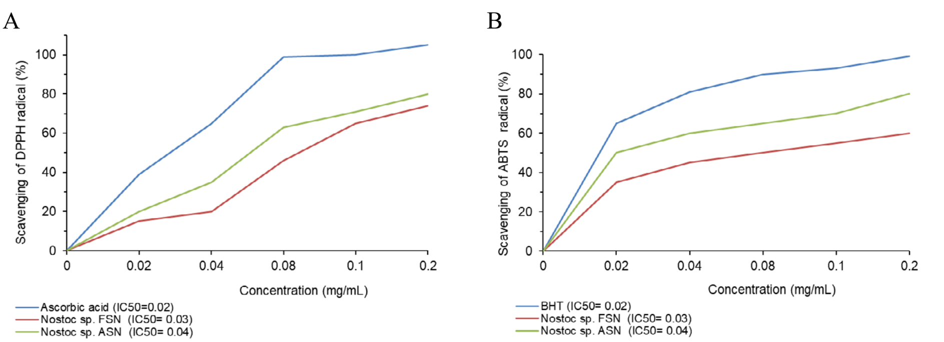


Figure 7: Free radical scavenging potential of two *Nostoc* strains FSN and ASN with DPPH method (**A**) and ABTS method (**B**).
Slika 7: Potencial odstranjevanja prostih radikalov sevov FSN in ASN vrste *Nostoc* po metodi DPPH (**A**) in ABTS (**B**).

Discussion

PC and PE are photosynthetic pigments extracted from microalgae with great biotechnological potential due to their intense colors, fluorescent properties and potential health benefits (Hsieh-Lo et al. 2019). Their principal applications are as nutritional supplements, natural colorants in foods and cosmetics, and as a reagent for immunological assays (Hsieh-Lo et al. 2019). Different physicochemical factors such as pH, temperature, light, affect PC and PE stability (Johnson et al. 2014). Several authors suggest the main factors affecting PBP stability are pH and temperature (Chaiklahan et al. 2012, Wu et al. 2016). The optimum pH range for PC is slightly more acidic than for the PE. The values of pH is the main factor that affects the aggregation and dissociation of PC in monomers, trimers, hexamers and other oligomers in solution. The hexameric form predominates in pH near 7.0. This is the most stable structure and avoids the denaturation of the PBP (Chaiklahan et al. 2012). At higher or lower pH values, this structure dissociates easily, decreasing stability. According to Chaiklahan et al. (2012) at pH 6.0, 77% of PC was aggregated in its hexameric form, while at pH 7.0 only 18% was aggregated. Therefore, it is recommended to handle the pigments at their optimal pH to avoid degradation. On the other hand, it has been reported by Liu et al. (2009) and González-Ramírez et al. (2014) that PE is stable in a wide range of pH, from 4.0 to 10.0. They showed that the secondary structure of PE could adopt a stable conformation in that pH range. The stability is maintained by the formation of hexameric structures (Liu et al. 2009). Providing stability at a wide pH compared to other PE molecules can facilitate its application in the food industry (Gonzalez-Ramirez et al. 2014). In general, it is preferable to handle and preserve PBP at a low temperature. As these molecules are protein pigments, their primary cause of degradation is denaturation. Munier et al. (2014) mentioned that when the temperature increases, the amount of alpha helix decreases, resulting in the loss of stability. The optimum temperature for PC and PE is around 4 °C; however, it can be stable up to 40–45 °C but slow degradation still occurs. It is not recommended to preserve PBP at a higher than room temperature because of their susceptibility to degradation by microorganisms (Chaiklahan et al. 2012). Using preservative for PE and PC is indispensable due to high sensitivity to temperature, if the process should be commercially viable. Results from the present study showed that without adding additives, PE and PC absorbance decreased as the temperature changed. At 5 °C with the presence of acid citric, PE and PC stability was found to be better. Moreover, we have demonstrated that almost complete loss of PE and PC content occurred at 35 **°C. However,** Galland-Irmouli et al. (2000) have demonstrated the thermostability of PE from *Palmaria palmate* (Rhodophyta) up to 60 °C. Moreover, the purified PE from *Nostoc* sp. FSN exhibited more stability after 30 days in comparison to purified PC.

Wu et al. (2016) and Munier et al. (2014) reported that PC and PE are sensitive to light (Wu et al. 2016, Munier et al. 2014). Wu et al. (2016) observed that PC showed a higher level of degradation after being exposed to a light intensity of 100 μmol m^{-2} s^{-1} compared to the treatment exposed to 50 μmol m^{-2} s^{-1} (Wu et al. 2016). When PBP are exposed to light for long periods, they tend to lose their chromophores, thus losing their color and stability (Munier et al. 2014). Nevertheless, although some physicochemical factors can significantly affect the stability of PBP, there are alternatives for improving their stability such as the use of additives, encapsulation and other methods (Hsieh-Lo et al. 2019).

According to Chaiklahan et al. (2012), the addition of sorbic acid or sodium azide did not significantly increase the stability of PC compared to glucose, sucrose and sodium chloride (Chaiklahan et al. 2012). High concentrations of sugar can improve stability. Martelli et al. (2014) also reported that the high concentration of sugar strongly increases the thermal stability of the PBP and its stabilization depends on the final concentration of the added sugar rather than type. Sugars and salts such as glucose, sucrose and sodium chloride could act as protein stabilizing agents. They can cover the surface of PC, and maintain and protect its chemical structure. By adding sugars, the water surface tension increases, and consequently the thermal stability of the proteins increases (Chaiklahan et al. 2012). Likewise, Braga et al. (2016) recommend the use of glucose to improve the thermal stability of the pigment and other additives such as 6% polyethylene oxide and 50% sorbitol. Furthermore, they reported that the addition of nanofibers could improve the thermal stabilization of the pigment by lowering the enthalpy of the system. Nanofibers can reduce the protein denaturation by forming multiple unions to the polymer, preventing conformational changes of the proteins in adverse environments. Another interesting additive to consider in order to improve PBP stability is benzoic acid. This additive can act as an antimicrobial agent inhibiting bacterial growth and has shown antioxidant activity, being able to preserve and increase pigments stability (Kannaujiya and Sinha 2016).

PE and PC can be largely useful as food colorant (Galland-Irmouli et al. 2000). Although its security has been questioned, it is a strong antioxidant, which highlights its advantage. Sonani et al. (2015) have reported the anti-oxidant based anti-ageing activity and anti-Alzheimer potential of PE isolated from *Lyngbya* sp. A09DM in wild type and transgenic *Caenorhabditis elegans* (Sonani et al. 2015). However, little investigation about the effect of PE and PC antioxidant activity is available in the literature.

Our results of antioxidant surveys suggest that PE and PC have efficient scavenging effects (IC50 = 0.03 and 0.04 for DPPH and ABTS respectively) compared to ascorbic acid and BHT (IC50=0.02) and is a potent free radical scavenger acting as antioxidant molecule. Similarly, Afreen and Fatma (2018) reported IC50 = 0.043 mg/mL using the extract of *Michrochaete* for DPPH test. Furthermore, it is demonstrated that extracts of *Nostoc linckia* (70% inhibition of ABTS radical) at 5 mg/L and ABTS radical scavenging capacity increased with the increasing phycobiliproteins concentration. Extracts of *Nostoc spheroids* showed considerable superoxide radical inhibiting activity according to Kuriakose (2014).

Conclusions

PE and PC produced by *Nostoc* strains FSN and ASN were successfully extracted, purified, characterized and evaluated for *in vitro* stability under different temperatures and additives. Significant antioxidant activities of PE and PC shown its feasibility for future applications as colorant in food industries and for pharmaceutical purposes. However, toxicological studies must be carried out for commercial production, since *Nostoc* is known as a toxin-producing genus. Furthermore, studies on its conformational behaviour under high stress conditions will be necessary to explore and enhance its molecular stability for industry applications. In addition, *Nostoc*'s PE and PC also shown significant stability 35% over 30 days. This highlights the need to explore microorganisms from unfavourable environmental conditions due to their potential for a wide variety of biological activities.

Summary

Cyanobacteria have a wide variety and richness in terms of bioactive metabolites, including cytotoxic, antifungal and antiviral compounds. Over the past few years, the isolation and detection of several new and diverse cyanobacterial metabolites with pharmacological activities have appeared, and the importance of the use of cyanobacteria became more apparent in the pharmaceutical industry. The aim of this study was to investigate the degree of purification and free radical scavenging ability of phycocyanin and phycoerythrin, and compare the stability of these pigments against selected preservatives at different temperatures with the aim of achieving the best and most stable preservative in increasing shelf life of PC and PE. In this study, after collecting and culturing *Nostoc* sp. strains FSN and ASN in BG-11_0 medium, the natural pigments phycocyanin and phycoerythrin were extracted by sequential melting and freezing the cyanobacterial cells. Then, purification of photosynthetic pigments was performed in two stages: extraction with 56% ammonium sulfate followed by dialysis. After that, antioxidant activity of photosynthetic pigments was evaluated by DPPH and ABTS assays. The purified pigments were then fermented and by adding sodium acetate buffer to the obtained powder, the resulting solution was used to measure the stability with the preservatives citric acid, sodium chloride, sucrose, and calcium chloride on the pigment at two temperatures of 5 °C and 35 °C in an aqueous solution. The results obtained after extraction and purification of natural pigments showed that the concentration and purity of the pigment increased after the dialysis step. Examination of spear antioxidant activity of extracted pigments showed that they had antioxidant properties. The stability of phycocyanin and phycoerythrin pigments was higher at 5 °C than at 35 °C. In addition, among different preservatives, citric acid caused more stability over time.

Povzetek

Cianobakterije so bogate z bioaktivnimi spojinami, vključno s citotoksičnim, protiglivnim in protivirusnim delovanjem. V zadnjih nekaj letih je bilo izoliranih in identificiranih več novih in raznolikih spojin iz cianobakterij s farmakološkim delovanjem, pomen uporabe cianobakterij pa je postal očitnejši v farmacevtski industriji. Namen te študije je bil raziskati stopnjo čiščenja fikocianina in fikoeritrina, njuno sposobnost odstranjevanja prostih radikalov ter primerjati njuno stabilnost v primerjavi z izbranim konzervansom pri različnih temperaturah z namenom doseganja najboljšega in najstabilnejšega konzervansa za podaljšanje roka uporabnosti PC in PE. V tej študiji smo po zbiranju in gojenju sevov FSN in ASN vrste *Nostoc* sp. v gojišču BG-110 ekstrahirali naravni barvili fikocianin in fikoeritrin s zaporednim taljenjem in zamrzovanjem cianobakterijskih celic. Nato je bilo izvedeno čiščenje fotosintetskih barvil v dveh stopnjah: ekstrakcija s 56% amonijevim sulfatom, ki ji je sledila dializa. Nato smo s testoma DPPH in ABTS ovrednotili antioksidativno aktivnost barvil. Očiščeni barvili smo nato fermentirali in z dodajanjem natrijevega acetatnega pufra dobljenemu prahu pripravili raztopino, ki smo jo uporabili za merjenje stabilnosti v primerjavi s konzervansi citronsko kislino, natrijevim kloridom, saharozo in kalcijevim kloridom v vodni raztopini pri dveh temperaturah, 5 °C in 35 °C. Rezultati, dobljeni po ekstrakciji in čiščenju naravnih barvil so pokazali, da sta se koncentracija in čistost barvila po koraku dialize povečala. Preiskava antioksidativne aktivnosti ekstrahiranih barvil je pokazala, da imata antioksidativne lastnosti. Stabilnost fikocianina in fikoeritrina je bila višja pri 5 °C kot pri 35 °C. Poleg tega je izmed različnih uporabljenih konzervansov citronska kislina povzročila večjo stabilnost skozi čas.

References

Afreen, S., Fatma, T., 2018. Extraction, purification and characterization of phycoerythrin from Michrochaete and its biological activities. Biocatalysis and Agricultural Biotechnology, 13, 84-89.

Allen, M.M., 1968. Simple conditions for growth of unicellular blue-green algae. Journal of General Microbiology, 51, 199-202.

Annibal, A., Riemer, T., Jovanovic, O., Westphal, D., Griesser, E., Pohl, E. E., Schiller, J., Hoffmann, R., Fedorova, M., 2016. Structural, biological and biophysical properties of glycated and glycoxidized phosphatidylethanolamines. Free Radical Biology and Medicine, 95, 293-307.

Braga, A. R. C., Figueira, F. d. S., Silveira, J. T. d., Morais, M. G. d., Costa, J. A. V., Kalil, S. J., 2016. Improvement of thermal stability of c-phycocyanin by nanofiber and preservative agents. Journal of Food Processing and Preservation, 40, 1264-69.

Chaiklahan, R., Chirasuwan, N. Bunnag, B. 2012. Stability of phycocyanin extracted from *Spirulina* sp.: influence of temperature, pH and preservatives. Process Biochemistry, 47, 659-64.

Chakdar, H., Pabbi, S., 2012. Extraction and purification of phycoerythrin from *Anabaena variabilis* (CCC421). Phykos, 42, 25-31.

Daliry, S., Hallajisani, A., Mohammadi, R. J., Nouri, H. & Golzary, A., 2017. Investigation of optimal condition for *Chlorella vulgaris* microalgae growth. Global Journal of Environmental Science and Management, 3, 217-230.

Deller, M. C., Kong, L., Rupp, B., 2016. Protein stability: a crystallographer's perspective. Acta Crystallographica Section F Structural Biology Communications, 72, 72-95.

Eghtedari, M., Porzani, S. J., Nowruzi, B., 2021. Anticancer potential of natural peptides from terrestrial

and marine environments: A review. Phytochemistry Letters, 42, 87-103.
Eriksen, N. T., 2008. Production of phycocyanin–a pigment with applications in biology, biotechnology, foods and medicine. Applied Microbiology and Biotechnology, 80(1), 1-14.
Farooq, S. M., Boppana, N. B., Asokan, D., Sekaran, S. D., Shankar, E. M., Li, C., Gopal, K., Bakar, S. A., Karthik, H. S., Ebrahim, A. S., 2014. C-phycocyanin confers protection against oxalate-mediated oxidative stress and mitochondrial dysfunctions in MDCK cells. PloS one, 9, e93056.
Fernández-Rojas, B., Hernández-Juárez, J., Pedraza-Chaverri, J., 2014a. Nutraceutical properties of phycocyanin. Journal of Functional Foods, 11, 375-92.
Fernández-Rojas, B., Medina-Campos, O. N., Hernández-Pando, R., Negrette-Guzmán, M., Huerta-Yepez, S., Pedraza-Chaverri, J., 2014b. C-phycocyanin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress. Food and Function, 5, 480-90.
Fernández-Rojas, B., Medina-Campos, O. N., Hernández-Pando, R., Negrette-Guzmán, M., Huerta-Yepez, S., Pedraza-Chaverri, J., 2014c. C-phycocyanin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress. Food and Function, 5, 480-90.
Freitas, M. V., Pacheco, D., Cotas, J., Mouga, T., Afonso, C., Pereira, L., 2022. Red Seaweed Pigments from a Biotechnological Perspective. Phycology, 2, 1-29.
Fukui, K., Saito, T., Noguchi, Y., Kodera, Y., Matsushima, A., Nishimura, H., Inada, Y., 2004. Relationship between color development and protein conformation in the phycocyanin molecule. Dyes and Pigments, 63, 89-94.
Galland-Irmouli, A., Pons, L., Lucon, M., Villaume, C., Mrabet, N., Guéant, J., Fleurence, J., 2000. One-step purification of R-phycoerythrin from the red macroalga *Palmaria palmata* using preparative polyacrylamide gel electrophoresis. Journal of Chromatography B: Biomedical Sciences and Applications, 739, 117-23.
Giannuzzi, L., 2019. Cyanobacteria growth kinetics. Repositorio Institucional Conicet Digital.
Gonzalez-Ramirez, E., Andujar-Sanchez, M., Ortiz-Salmeron, E., Bacarizo, J., Cuadri, C., Mazzuca-Sobczuk, T., Ibáñez, M. J., Camara-Artigas, A., Martinez-Rodriguez, S., 2014. Thermal and pH stability of the B-phycoerythrin from the red algae *Porphyridium cruentum*. Food Biophysics, 9, 184-92.
He, Y.-Y., Häder, D.-P., 2002. Reactive oxygen species and UV-B: effect on cyanobacteria. Photochemical, Photobiological Sciences, 1, 729-36.
Hsieh-Lo, M., Castillo, G., Ochoa-Becerra, M. A., Mojica, L., 2019. Phycocyanin and phycoerythrin: Strategies to improve production yield and chemical stability. Algal Research, 42, 101600.
Johnson, E. M., Kumar, K., Das, D., 2014. Physicochemical parameters optimization, and purification of phycobiliproteins from the isolated Nostoc sp. Bioresource Technology, 166, 541-47.
Kannaujiya, V. K., Sinha, R. P., 2016. Thermokinetic stability of phycocyanin and phycoerythrin in food-grade preservatives. Journal of Applied Phycology, 28, 1063-70.
Komárek, J., 2013. Süßwasserflora von mitteleuropa, Bd. 19/3: cyanoprokaryota. 3. Teil/3rd part: heterocytous genera. Süßwasserflora von Mitteleuropa. Spektrum Academischer Verlag, Heidelberg, 19, 414.
Kuriakose, G. C., 2014. Biomodulatory effects of *Nostoc* sphaeroides. Mahatma Gandhi University, xxvi, 256p. http://hdl.handle.net/10603/22412
Li, Y., Yang, H., Cao, F. M., 2009. Effect of ultraviolet irradiation on photostability of C-phycocyanin in a silica matrix. Research on Chemical Intermediates, 35, 607-13.
Liu, L.-N., Su, H.-N., Yan, S.-G., Shao, S.-M., Xie, B.-B., Chen, X.-L., Zhang, X.-Y., Zhou, B.-C., Zhang, Y.-Z., 2009. Probing the pH sensitivity of R-phycoerythrin: Investigations of active conformational and functional variation. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1787, 939-46.
MacColl, R., 1998. Cyanobacterial phycobilisomes. Journal of Structural Biology, 124, 311-34.
Markou, G., Georgakakis, D., 2011. Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: a review. Applied Energy, 88, 3389-401.
Martelli, G., Folli, C., Visai, L., Daglia, M., Ferrari, D., 2014. Thermal stability improvement of blue

colorant C-Phycocyanin from *Spirulina platensis* for food industry applications. Process Biochemistry, 49, 154-59.

Mehnert, G., Leunert, F., Cirés, S., Jöhnk, K. D., Rücker, J., Nixdorf, B., Wiedner, C., 2010. Competitiveness of invasive and native cyanobacteria from temperate freshwaters under various light and temperature conditions. Journal of Plankton Research, 32, 1009-21.

Mishra, S. K., Shrivastav, A., Mishra, S., 2008. Effect of preservatives for food grade C-PC from *Spirulina platensis*. Process Biochemistry, 43, 339-45.

Mishra, S. K., Shrivastav, A., Pancha, I., Jain, D., Mishra, S., 2010. Effect of preservatives for food grade C-Phycoerythrin, isolated from marine cyanobacteria *Pseudanabaena* sp. International Journal of Biological Macromolecules, 47, 597-602.

Munier, M., Jubeau, S., Wijaya, A., Morancais, M., Dumay, J., Marchal, L., Jaouen, P., Fleurence, J., 2014. Physicochemical factors affecting the stability of two pigments: R-phycoerythrin of *Grateloupia turuturu* and B-phycoerythrin of *Porphyridium cruentum*. Food Chemistry, 150, 400-07.

Nowruzi, B., Fahimi, H., Lorenzi, A. S., 2020. Recovery of pure C-phycoerythrin from a limestone drought tolerant cyanobacterium *Nostoc sp.* and evaluation of its biological activity. Anales de Biología. Servicio de Publicaciones de la Universidad de Murcia, pp. 115-28.

Nowruzi, B., Porzani, S. J., 2021. Toxic compounds produced by cyanobacteria belonging to several species of the order Nostocales: A review. Journal of Applied Toxicology, 41, 510-48.

Patel, A., Pawar, R., Mishra, S., Sonawane, S., Ghosh, P., 2004. Kinetic studies on thermal denaturation of C-phycocyanin. Indian Journal of Biochemistry and Biophysics, 41, 254-257.

Patil, G., Chethana, S., Sridevi, A., Raghavarao, K., 2006. Method to obtain C-phycocyanin of high purity. Journal of Chromatography A, 1127, 76-81.

Petersen, S. B., Jonson, V., Fojan, P., Wimmer, R., Pedersen, S., 2004. Sorbitol prevents the self-aggregation of unfolded lysozyme leading to an up to 13 C stabilisation of the folded form. Journal of Biotechnology, 114, 269-78.

Pleonsil, P., Soogarun, S., Suwanwong, Y., 2013. Anti-oxidant activity of holo-and apo-c-phycocyanin and their protective effects on human erythrocytes. International Journal of Biological Macromolecules, 60, 393-98.

Rastogi, R. P., Sonani, R. R., Madamwar, D., 2015a. Physico-chemical factors affecting the in vitro stability of phycobiliproteins from *Phormidium rubidum* A09DM. Bioresource Technology, 190, 219-26.

Rastogi, R. P., Sonani, R. R., Madamwar, D., 2015b. Physico-chemical factors affecting the in vitro stability of phycobiliproteins from *Phormidium rubidum* A09DM. Bioresource Technology, 190, 219-26.

Rajabpour N, Nowruzi B, Ghobeh M., 2019. Investigation of the toxicity, antioxidant and antimicrobial activities of some cyanobacterial strains isolated from different habitats. Acta Biologica Slovenica, 62, 3-14.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26, 1231-37.

Righini, H., Francioso, O., Martel Quintana, A., Roberti, R., 2022. Cyanobacteria: a natural source for controlling agricultural plant diseases caused by fungi and oomycetes and improving plant growth. Horticulturae, 8, 58.

Sekar, S., Chandramohan, M., 2008. Phycobiliproteins as a commodity: trends in applied research, patents and commercialization. Journal of Applied Phycology, 20, 113-36.

Setyoningrum, T. M., Nur, M. A., 2015. Optimization of C-phycocyanin production from *S. platensis* cultivated on mixotrophic condition by using response surface methodology. Biocatalysis and Agricultural Biotechnology, 4, 603-07.

Shanab, S., Mostafa, S., Shalaby, E., Mahmoud, G., 2012. Aqueous extracts of microalgae exhibit antioxidant and anticancer activities. Asian Pacific Journal of Tropical Biomedicine, 2, 608–15.

Sonani, R., Rastogi, R., Madamwar, D., 2015. Antioxidant potential of phycobiliproteins: Role in anti-

aging research. Biochemistry, Analytical Biochemistry, 4, 2161-1009.
Stadnichuk, I., Krasilnikov, P., Zlenko, D., 2015. Cyanobacterial phycobilisomes and phycobiliproteins. Microbiology, 84, 101-11.
Stoyanov, P., Moten, D., Mladenov, R., Dzhambazov, B., Teneva, I., 2014. Phylogenetic relationships of some filamentous cyanoprokaryotic species. Evolutionary Bioinformatics, 10, 13748.
Tan, H., Gao, S., Zhuang, Y., Dong, Y., Guan, W., Zhang, K., Xu, J., Cui, J., 2016. R-Phycoerythrin induces SGC-7901 apoptosis by arresting cell cycle at S phase. Marine Drugs, 14, 166.
Teng, S., Srivastava, A. K., Schwartz, C. E., Alexov, E., Wang, L., 2010. Structural assessment of the effects of amino acid substitutions on protein stability and protein protein interaction. International Journal of Computational Biology and Drug Design, 3, 334-49.
Tiwari, O. N., Devi, W. I., Silvia, C., Devi, A. T., Oinam, G., Singh, O. A., Singh, K. O., Indrama, T., Sharma, A. S., Khangembam, R., 2015. Modulation of phycobiliprotein production in Nostoc muscorum through culture manipulation. Journal of Applied Biology and Biotechnology, 3, 0-1.
Vothknecht, U. C., Westhoff, P., 2001. Biogenesis and origin of thylakoid membranes. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1541, 91-101.
Wu, H.-L., Wang, G.-H., Xiang, W.-Z., Li, T., He, H., 2016. Stability and antioxidant activity of food-grade phycocyanin isolated from *Spirulina platensis*. International Journal of Food Properties, 19, 2349-62.
Yang, H., Jin, X., Lam, C. W. K., Yan, S.-K., 2011. Oxidative stress and diabetes mellitus. Clinical Chemistry and Laboratory Medicine, 49, 1773-82.

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One year spread and insight into ecology of invasive *Impatiens glandulifera* in Ljubljansko barje area (Central Slovenia)

Enoletna dinamika širjenja in vpogled v ekologijo invazivne tujerodne vrste *Impatiens glandulifera* na območju Ljubljanskega barja (osrednja Slovenija)

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Abstract: *Impatiens glandulifera* Royle (Himalayan balsam) is an annual plant, native to humid parts of the Himalayas. Brought to Europe in the XIX century, it has since successfully naturalized and spread throughout the continent, becoming one of the best-known invasive plants. Even though it has been thoroughly studied by many authors, some aspects of its biology and ecology remain unclear and debatable, such as its spreading dynamics, negative impacts in invaded ecosystems and ecological adaptability regarding moisture, nutrients, and light. This 2019 field study from the Ljubljana Marsh (Slovenia) has proved that Himalayan balsam successfully develops under mesophilic conditions, where it easily compensates moderate deviations from its ecological optimum. The species thrives in riparian zones, out-competing native vegetation, trait shown as potentially the biggest negative impact of chosen species in this context. This study additionally gave us a different insight into - usually highly emphasized - dynamics of species' spreading. Spreading was not either dependent on hydrochory or as drastic as mentioned in literary sources, giving it a secondary role in species' invasiveness on the chosen area. Lastly, we proposed rough estimates of eradication expenses for given area, based on our field results of species' abundance and distribution on chosen area.

Keywords: ecology, Himalayan balsam, *Impatiens glandulifera*, invasive species, plant invasions

Izvleček: *Impatiens glandulifera* Royle (žlezava nedotika) je enoletna rastlina, domorodna v vlažnih predelih območja Himalaj. Vrsta je bila vnešena v Evropo v XIX. stoletju, kjer je od takrat, kot ena izmed najbolj znanih tujerodnih vrst, postala uspešno naturalizirana in invazivna, ter razširjena po vsej celini. Nekatere njene biološke lastnosti še vedno ostajajo precej nejasne in vprašljive. To se nanaša predvsem na njeno invazivnost in morebitni vpliv v ekosistemih, dinamiko širjenja in možnost prilagajanja na različne okoljske dejavnike, kot so vlaga, hranila in svetloba. Terenska raziskava iz 2019, opravljena na območju Ljubljanskega barja v osrednji Sloveniji, je pokazala da žlezava nedotika uspeva v zmernih pogojih, kjer uspešno prenaša zmerna odstopanja od svojega ekološkega optimuma. Vrsta je najbolj uspešna v obrežnem pasu, kjer je precej bolj kompetitivna kot domorodna vlagoljubna vegetacija, kar bi v tem kontekstu lahko definirali kot največji negativni vpliv izbrane tujerodne vrste. V tej študiji smo tudi ugotovili, da ima dinamika širjenja sekundarni pomen v njeni invazivnosti na Ljubljanskem barju, ter da širjenje ni odvisno od hidrohorije, niti ni toliko uspešno kot navajajo nekateri drugi literaturni viri. Na podlagi rezultatov o razširjenosti in velikosti lokalnih populacij žlezave nedotike na Ljubljanskem barju smo pripravili ocene stroškov odstranjevanja vrste in predlog monitoringa stanja v prihodnjih letih.

Ključne besede: ekologija, *Impatiens glandulifera*, invazivne tujerodne rastlinske vrste, žlezava nedotika

Introduction

Short introduction to biological and plant invasions

Biological invasions are one of the most covered topics in modern science and an important negative consequence of globalization (Lambdon et al. 2008, Perglová et al. 2009, Vilà et al. 2010, Vilà et al. 2011, Bieberich et al. 2020). Invasive species are diverse and known for their specific biological traits, which make them stronger competitors in comparison to native species and facilitate their success in newly invaded habitats (Čuda et al. 2014, Enders et al. 2020). Many studies have proven negative impacts of invasive species on environment and society, which are derived from their disruption of ecosystem services. The disruption comes from changes in native species' diversity, trophic networks, biogeochemical cycles, and habitat structure in invaded ecosystems, all of which are confirmed consequences of biological invasions (Hejda et al. 2009, Vilà et al. 2011, Pyšek et al. 2012, Blackburn et al. 2014). Impacts of invasive species are however context-dependent and determined by various aspects of individual case of species' invasion, such as its residence time and naturalization status, biological traits of given species and biotic and abiotic conditions found in ecosystem they invade (Richardson and Pyšek 2006, Pyšek et al. 2012, Blackburn et al. 2014). Different ecosystems show different levels of vulnerability regarding biological invasions. Generally, it is proposed that ecosystems with higher number of endemic species are more susceptible to the negative impacts of biological invasions. On the other hand, ruderal vegetation is often predominantly composed of neophytes, to which frequent disturbances found in urban areas are suitable (Zelnik 2012). Due to both natural disturbances and human pressures put on them - such as building dams, flow regulations, degradation, eutrophication or draining, riparian habitats are exceptionally sensitive to biological invasions (Schnitzler et al. 2006, Richardson et al. 2007, Nobis et al. 2017). Additionally, permanent presence of water is reducing drought stress, which is an important limiting factor for some alien plants from more humid climates.

General traits of Himalayan balsam

One of the invasive species commonly found in riparian habitats, where it triggers conservation issues, is Himalayan balsam, *Impatiens glandulifera* Royle (Hejda and Pyšek 2006). Himalayan balsam is a summer therophyte, up to 2.5 (4) meters tall and known as the tallest annual plant in Europe. It is easily distinguished by its zygomorphic, pinkish-purple flowers and opposite, lanceolate-shaped, serrated leaves. Complex, multicellular, dark-purple tipped glands develop at the leaf base and serve as extrafloral nectaria, to which the species owns its scientific name. In Europe, seeds of Himalayan balsam start germinating in early spring, usually in March (Balogh, 2008), and according to the experimental study by Perglová et al. (2009), they require stratification for germination. Seedlings are very vulnerable to late frosts, snails, and fungal infections, as seen in a study by Prowse (1998), in which only about 5-10% of observed young plants survived. Flowering begins 13 weeks after the germination and it is the most intense in July and August, even though it can last until October or November. Individual flowers last for 2-3 days. Fruit and seed development end approximately 13 weeks after the flowering and are followed by plant maturation and seed dispersal, which take place in autumn and last until November. Fruit type is a capsule which can contain up to 16 seeds, so whole plant can produce up to 2500 seeds. Upon maturation, capsules burst explosively, shooting seeds up to 7 meters away from the parent-plant (Balogh, 2008). This mechanism is known as ballistochory (Elst et al. 2016). By this natural mechanism, local populations spread their range by 3-5 meters per year on average, even though spreading is often enhanced by human activities, animals, or waters (Balogh 2008). Often cited study by Perrins et al. (1993) reports on Himalayan balsam's spread by rivers in England. Authors have calculated the maximum downstream spreading rate of 38 km/year, although they note that the realistic enhancement by hydrochory is around 2.6 km/year. Čuda et al. (2017a) additionally state that flooding might have an important role in species' spreading. In the soil seed bank, some seeds can survive for 4 (Skalová et al. 2019) or even 6 years (Schuldes 1995).

Himalayan balsam is often found in humid and half-shaded habitats, in both its native and non-native range. In Europe, it usually inhabits temperate areas with rainfall values of at least 250 mm/year, optimally 1000 mm/year, with an average annual temperature between 6 and 12 ˚C. Species avoids direct sunlight, unless provided with adequate sources of water, since it is defined as hydrolabile. It is commonly found in riparian zones and damp ruderal communities (Balogh 2008), but it can also be found in other types of ecosystems, like forest fringes, arable fields, and meadows (Čuda et al. 2017a). Species is found growing in alluvial soils, loam, and humic clay, avoiding both acidic and alkaline soils (Balogh 2008). Species is often regarded as nitrophilous (Hejda and Pyšek 2006). According to Beerling and Perrins (1993), optimum soil nitrate values for Himalayan balsam range from 0.5 ± 0.3 µg/g to 5.5 ± 1.3 µg/g. Andrews et al. (2005) have confirmed that species has an ability of nitrates accumulation, what possibly stimulates species' extensive growth in shaded habitats, whereas Andrews et al. (2009) report on positive effect of higher nitrates value on seed germination, later confirmed again by Skalová et al. (2019).

Often-used methodology used for describing species' ecological preferences are indicator values (see Bartelheimer and Poschlod 2016, Chytrý et al. 2018). In this study, we present and use modified, Ellenberg-type indicator values (shortened as EV in continuation) by Chytrý et al. (2018), which describe Himalayan balsam as transitional between the plants of half-shade and half-light, rarely found on less than 20% of full light intensity (EV for light 6), transitional between mesothermic and thermophilous plant species (EV for temperature 6), transitional between indicators of wet and moist soils (EV for moisture 8), indicator of neutral soils, avoidant of extremely acid soils (EV for pH 7) and the favourer of fertile soils (EV for nutrients 7).

Introduction of Himalayan balsam in Europe and its current invasiveness status

Native to the Western Himalayas, species was introduced to Europe in 1839 as an ornamental plant, whose seeds were sent to Kew Garden from Kashmir by dr. J. F. Royle. Soon after the introduction in 1848, Himalayan balsam escaped the cultivated area and was proclaimed as successfully naturalized in United Kingdom by 1855. In the continental Europe, first records on naturalization date from 1897, although the species probably didn't become invasive in Europe until the last three decades of previous century (Balogh 2008). Today, Himalayan balsam is widely distributed across the Northern Hemisphere, in zone between 40˚ and 65˚ of geographic latitude, which includes most parts of temperate Europe and North America, along with some parts of Asia where it's not native, such as Russia and Japan (Drescher and Prots 2003). Results of DAISIE project report on its presence in more than 30 European countries, out of which 25 consider it as at least naturalized allochthonous species, what puts Himalayan balsam on the list of 150 mostly widespread alien plants in Europe (Lambdon et al. 2008). Due to its progressive spreading and impacts on biodiversity and subsequently economy, it was included in the first edition of the List of invasive species of Union concern (European Commission 2017). Therefore, all member states of the European Union must act towards preventing its further introductions and spreading, while simultaneously working on adequate management and monitoring methodology (ibid.; Commission, 2017). In Slovenia, it was first reported in 1935 (Petkovšek 1966), whereas oldest findings on northern margins of capital city Ljubljana date from Zalokar, from 1939 (data from University of Ljubljana's Herbarium). Species has been successfully spreading since, mostly along main water bodies, as seen in older publication by Prekoršek (1964), who claimed its presence by rivers Sava, Savinja, Drava and Mura, and is today widespread across the country (Jogan et al. 2001). Zelnik (2012) lists it as one of the most invasive alien plants in Slovenia, found in an array of habitat types, such as riparian zones, floodplains and swamps, forests, urban areas, and managed ecosystems.

Studies of Himalayan balsam's invasiveness

Although much is known about the species, some aspects of its ecology and invasiveness are still insufficiently understood and sometimes debatable. For instance, while some authors report on its negative impact in invaded habitats (Hulme and Bremner 2006, Kiełtyk and Delimat 2019), other studies deny it (Hejda and Pyšek 2006, Hejda et al. 2009, Diekmann et al. 2016, Čuda et al. 2017b, Bieberich et al. 2020). Notably, studies were carried out in different habitats and biogeographic regions, so the impact should be interpreted as context-dependent. For instance, Hulme and Bremner (2006) found that Himalayan balsam's worst negative impact was on the native heliophytes in its undergrowth in riparian zones, whose development was disturbed due to Himalayan balsam's rapid growth and height. Kiełtyk and Delimat (2019) confirmed species' impact on reduced native biodiversity in temperate meadows, while its impact was negligible in mesophilic forests (Čuda et al. 2017b). Apart from invasiveness, other questionable traits of the species are its nitrophily - considering the fact that it survives in wider range of nutrients availability in soil (Beerling and Perrins 1993, Hejda and Pyšek 2006, Clements et al. 2008), and its dependence on moisture and riparian zones. Some authors hypothesise that latter might be an evolutionary adaptation which would enhance spreading along the watercourses (Čuda et al. 2014). Results of study by Elst et al. (2016) have shown that Himalayan balsam could be pre-adapted to invasions, since both native and non-native population express phenotypic plasticity, regardless of environmental conditions they grew in.

Aims of the study

Aim of this study was to get a clearer insight into dynamics of species' seasonal spreading in chosen area, since it corresponds with its described ecological optimum and it might show potential impact of water bodies in seed spreading. Additionally, due to differing literary sources on ecological traits of chosen species, we studied its ecology throughout its accompanying flora, for which we hypothesised that it would include widespread, ruderal species and neophytes. Due to the state of Himalayan balsam's widespread and continuously growing populations in Europe and stance of European conservation politics on it, we proposed simplified eradication expenses estimation, based on field findings, along with an adequate eradication plan for initial and following upcoming management actions.

Materials and methods

Study area

Field sampling was performed during the summer of 2019, in area of Nature Park Ljubljana Marsh (in continuation: Ljubljana Marsh; in Slovenian: Krajinski park Ljubljansko barje), which holds the basic level of nature protection as a nature park. Almost the entire area of Ljubljana Marsh was included in Natura 2000 network in 2004 and it has had a status of a protected area since 2008. It is a floodplain of river Ljubljanica and some of its tributaries, noted for its irregular, seasonal flooding and mosaic of different habitats including wetland meadows, riparian forests, and remains of peat bogs. The area has been under heavy human pressures, which include river flow regulation, peat excavation, intense agriculture, and prolonged draining in recent times (Zorn and Šmid Hribar 2012). Human activities have also contributed to spreading of invasive species. Based on official reports on the distribution of Himalayan balsam in the previous season (Lozej 2018), we designed a systematic census of Himalayan balsam's population in 2019, to compare states of populations between the two consecutive seasons and to see, whether theoretical claims of species' spreading could be applied to chosen area. Our methodologies differed slightly: our inventory distinguished each locality its by size and shape, followed by partial or complete inventory of accompanying flora, whereas previous report focused mostly on geographic mapping of entire population (Lozej 2018).

Field work

We sampled throughout July, August, and September of 2019, at the peak of the species' development. Field data (geographical coordinates) from previous report (Lozej 2018) were used, which were all reviewed for presence of Himalayan balsam, along with the other potential localities near them, or elsewhere considered suitable for the species based on its biological traits. Entire road and cart-track network of the study area has been systematically monitored. Every confirmed locality was marked as either a point - when less than 50 meters long, or as either linear or polygon – both longer/wider than 50 m, typified according to the spatial traits (shape) of a given local stand. Two stands (localities, populations *s.l.*) were distinguished as separated, if there was approximately 10 meters between them without any flowering individuals of Himalayan balsam. Size of each stand was roughly estimated on a scale which consisted of 4 size classes: l-10, 10-100, 100-1000 and over 1000 fully developed plants. Fully developed, flowering individuals were considered for localities' distinguishment and size estimations, since Himalayan balsam is an annual therophyte which spreads primarily by its seeds. Therefore, flowering individuals represent adequate basis for population size estimations and stand as a reference for discussions regarding population dynamics in forthcoming vegetation seasons. To get a better insight in species' ecology, the general habitat type was also determined, based on the predominant vegetation type, proximity to agricultural areas, travel infrastructure and water bodies. No detailed analysis (mapping) of habitat types were carried out, due to the complexity of study area, interconnectedness of its many habitat types and changes in their structure, whose causation goes beyond this specific study. Each locality was described as accessible, inaccessible, or partially accessible, for purposes of further monitoring propositions and calculations of potential eradication expenses. Qualitative inventory of Himalayan balsam's accompanying flora was carried out on 67.5% (370 of 548 in total) of the locations (following nomenclature in Martinčič et al. 2007), whereas no cover/abundance examination was carried out for those species. Inventories of accompanying flora were carried out based on accessibility and the general state of given stand (for example, overgrown by Himalayan balsam, or small plant stands where only few plant individuals of any species were present). In case of presence of any other invasive alien plant species within stands of Himalayan balsam, those were always documented, regardless of whether the inventory of the entire accompanying flora was carried out or not.

Data analysis

Spatial data was processed in QGIS programme, version 3.18.1 (QGIS.org 2022), which was used to create maps of species' current distribution and to compare Himalayan balsam's population dynamics between two consecutive seasons. Distribution map in form of population hotspots was created using the built-in function in QGIS, which considers both the density of stands on a certain area, as well as their maximum size, as described above in the field work methodology. For ecological analyses, we used modified Ellenberg-type indicator values (Chytrý et al. 2018), applied to significantly abundant taxa, found at 11 or more locations where qualitative vegetation surveys were carried out ($f \geq 11$). Species which occurred sporadically in Himalayan balsam's accompanying flora ($f \leq 10$) were omitted from analysis, due to their low abundance in microsites where Himalayan balsam was growing, so their co-existence wasn't considered a possible indicator of Himalayan balsam's ecological preferences. Average values for Ellenberg-type indicator values in Himalayan balsam's accompanying flora for light (L), temperature (T), moisture (M), pH reaction (R) and nutrients (N) were calculated, along with percentages of each individual value for each ecological condition. Average values were then compared to the Himalayan balsam's theoretical optimum for given ecological factor, following Chytrý et al. (2018). Observed average values in Himalayan balsam's accompanying flora were then compared to the average values for each ecological condition for dataset of entire flora Ljubljana Marsh flora (data from Jogan et al. 2001). Comparisons between Himalayan balsam's stands and Ljubljana Marsh flora were carried out to get a robust estimations of species' possible ecological niche profiling on chosen area, since no similar studies of Himalayan balsam's accompanying flora ecology were carried out previously in Slovenia. Additionally, general ecology of Ljubljana Marsh fits into basic ecology of Himalayan balsam (described above), so its distribution and co-existence with specific species might provide additional information on species' biology. Due to those reasons, all species in Ljubljana Marsh flora dataset were included in analysis. Species without modified Ellenberg values were left out of calculations. Distribution of significantly abundant accompanying flora on Ljubljana Marsh was used to create a simple model of Himalayan balsam's prospective distribution in Slovenia. Occurrence of those species in floristic quadrants at Ljubljana Marsh was extrapolated to entire Slovenia, using data from Slovenian Centre for fauna and flora cartography, available at Bioportal.si (2022). Suitability of conditions was simply estimated as portion of present taxa of accompanying flora in quadrants of Slovenia.

Eradication expenses were estimated from our field results, based on values calculated from combinations of local populations' accessibility and sizes. We assumed that partially accessible and inaccessible populations would require solely manual eradication work, whilst accessible ones could be managed by machines. Hypothetical wage of 10 EUR/hour was used in both cases, fair for the work effort needed for theoretical hand-eradication of one plant per minute. For accessible stands, we made a simple calculation by multiplying their frequency with hypothetical wage of 10 EUR/hour. Partially accessible and inaccessible stands were analysed in more detail, taking into consideration their frequencies combined with Himalayan balsam's stands size for each stand category, multiplying both minimum, maximum sizes of stands (10, 100, 1000 and 10 000 for stands for over 1000), along with combinations of minimum and maximum sizes in intermediate stands. Minimum stand size wasn't considered only for populations of size range 1-10, due to low probability of finding single individual of Himalayan balsam in a stand, whilst stands with over 1000 were defined with hypothetical upper stand size of 10000. Those numbers were then divided with 60, resulting in hypothetical hours needed for manual eradication, then multiplied with hypothetical 10 EUR/hour wage. Numbers were added to the value of eradication expenses for accessible stands, resulting in price ranges. To achieve the completely effective prevention of future flowering of a local population, consecutive approaches are needed in the following years, due to soil seed-bank persistency (Schuldes 1995, Skálová et al. 2019). We predicted the rough estimate needed to cover those expenses in future growing seasons, which would in practice depend on the success of the previous eradication and should be significantly lower than the starting price point.

Results

Distribution and estimation of spreading

In 2019, Himalayan balsam was found on 548 localities in Ljubljana Marsh. Most of the localities were situated in the northern part of area, by the water bodies - usually streams, creeks and drainage ditches. Some of the biggest stands (written as numbered on Fig. 1) were found in villages Vnanje Gorice and Notranje Gorice by the stream Drobtinka (1), in Plešivica by the stream Veliki Graben (2), in Brezovica by the stream Radna (3), in area of Rakova Jelša by the stream Curnovec (4) and in village Črna vas, by the rivers Iška and Ižica, and streams Lahov graben and Prošca (5). On the southern parts of Ljubljana Marsh, Himalayan balsam occurrence is sparse, in form of scattered localities by rivers Borovniščica and Iška, by the the stream Draščica and in the Draga area, by the pond Veliki Ribnik. Due to uneven distribution and highlighted concentrated in the northern part of the area, we have decided to present species' distribution in forms of both common map and hotspots map, as shown below on Fig. 1 (locations are numbered as written above). We would additionally emphasise local stands 6*, located near the old riverbed of Ljubljanica river (in Slovenian: Stara struga Ljubljanice), and 7*, situated at the very border of Ljubljana Marsh, by the waste collecting and management centre Barje (in Slovenian: Zbirni center Barje). Those aren't shown as true hotspots though, due to their smaller size compared to other stands which expand over larger area.

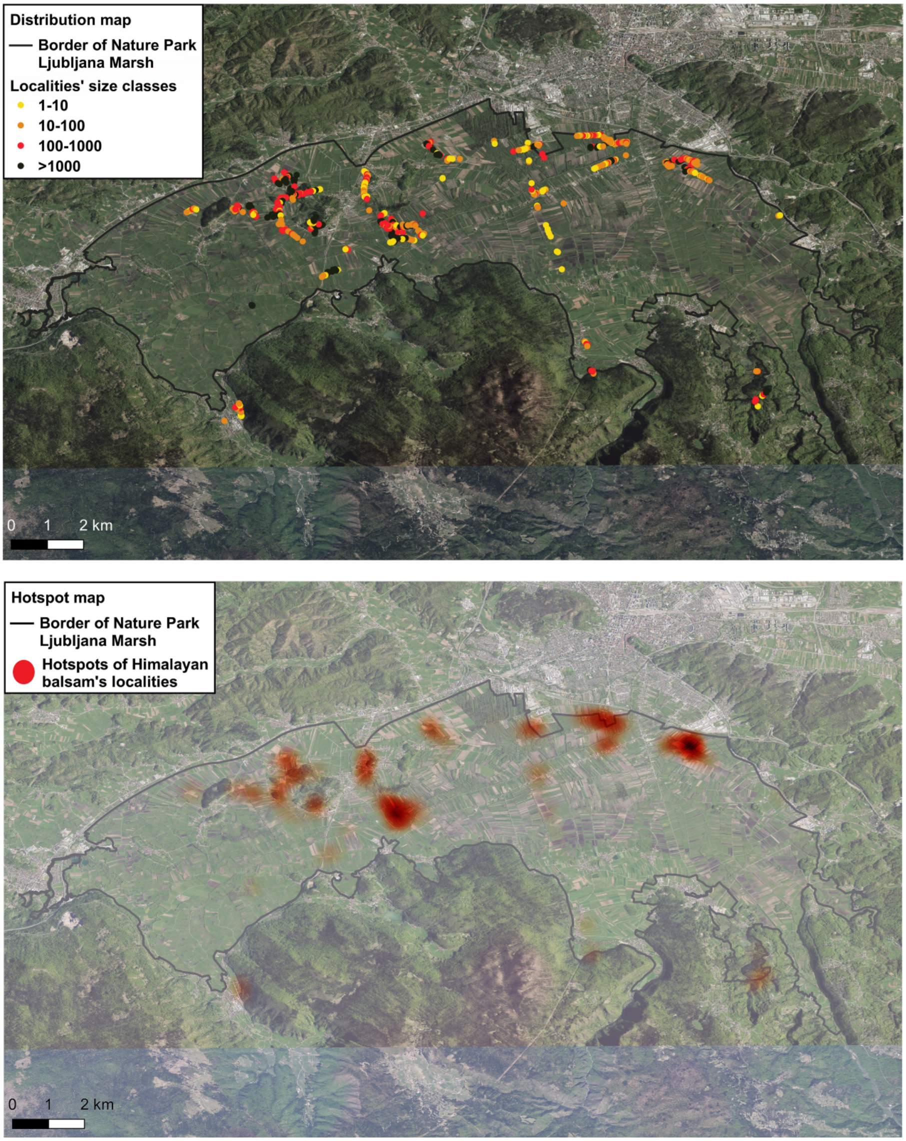

Figure 1: Distribution of Himalayan balsam on Ljubljana Marsh (bordered with black) in season 2019, given in form of individual population locations, categorized by size classes (**A**) and hotspots (**B**). Hotspots are numbered as: Notranje and Vnanje Gorice (1), Podplešivica (2), Brezovica (3), Rakova Jelša (4) and Črna vas (5). Additional mention goes to smaller areas with densely distributed populations, by the oldriver bed of Ljubljanica river (6*), and by the waste collecting and management center Barje (7*).

Slika 1: Razširjenost žlezave nedotike na območju Ljubljanskega barja (omejeno s črnim poligonom) v letu 2019, prikazana v obliki posamičnih nahajališč populacij, razdeljenih po velikostnih razredih (**A**) in vročih točk (**B**). Vroče točke so označene kot: Notranje in Vnanje Gorice (1), Podplešivica (2), Brezovica (3), Rakova Jelša (4) in Črna vas (5). Poudarjamo še dve manjši lokaliteti z večjo gostoto lokalnih populacij, kot sta Stara struga Ljubljanice (6*) in Zbirni center Barje (7*).

Majority of the localities – 442, (80.7%) were marked as points - stands smaller than 50 meters. We recorded 82 linear ones (15%) and 24 polygons (4.3%). Around 90% of all localities had up to 1000 individuals, whereas the most frequent size class was the second one (10-100), with 223 localities (40.7%), followed by the first one (1-10), with 152 (27.7%) and the third one (100-1000) with 122 localities (22.2%). Fifty-one localities counted more than 1000 individuals (9.4%). We found Himalayan balsam in 9 different habitats, listed according to their frequency: roads, ditches, fields, hedges, forests, grasslands, riparian zones, banks, and embankments. About a half (49.8%) of the stands were located by roads, followed by drainage ditches, where around a quarter (26.8%) of stands were found. Other common habitat types included fields (8%), where we found species as either a weed growing among crops or at the field margins, and hedges (7.1%), fragments of bush vegetation specific for study area, which usually divide individual parcels. Himalayan balsam grew in other habitat types on Ljubljana Marsh only sporadically.

Comparison of 2018 and 2019 spatial data showed that the general pattern of Himalayan balsam's population on study area in two consecutive seasons was very similar, without observed long-distance spreading. Our study has shown that Himalayan balsam can successfully spread up to 150 meters in one year (Fig. 2), whereas the average distance of newly recorded populations from the nearest localities known in 2018 was 122 meters.

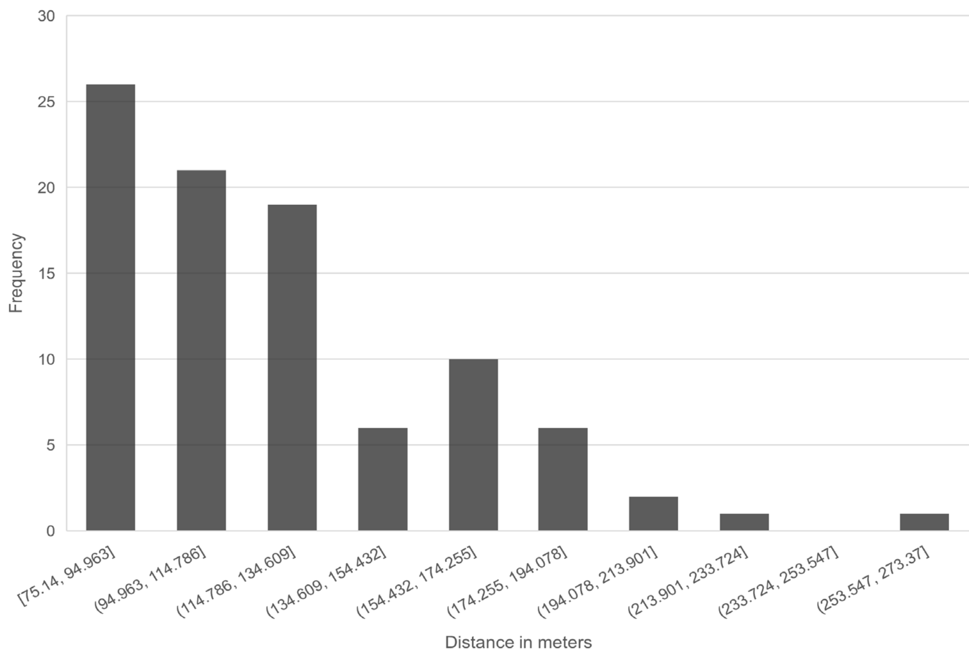

Figure 2: Frequency distribution of measured distances between the newly recorded locations and the nearest previously known populations, regarding the spreading of Himalayan balsam in 2018-2019 period.

Slika 2: Porazdelitev pogostosti izmerjenih razdalj med na-novo zabeleženimi populacijami v letu 2019 in njim najbližjimi populacijami, zabeleženimi v letu 2018, s ciljem ugotavljanja dinamike širjenja žlezave nedotike v obdobju 2018-2019.

Accompanying flora, statistical and ecological analysis

Inventory of Himalayan balsam's accompanying flora resulted in presence of 238 other plant taxa. Identification was to the species-level, unless specimens were unreachable or underdeveloped. On average, there were 11 other taxa in communities beside Himalayan balsam, whereas the maximum number was 53. Five of the most common accompanying species were European dewberry *Rubus caesius* L. (on 250 locations), common nettle *Urtica dioica* L. (221), cock's-foot *Dactylis glomerata* L. [s.str.] (200), giant goldenrod *Solidago gigantea* Aiton (139) and large-flowered hemp-nettle *Galeopsis speciosa* Mill. (114). The most common woody species were common alder *Alnus glutinosa* (L.) Gaertn. (93), white willow *Salix alba* L. (76), European ash *Fraxinus excelsior* L. (49), brittle willow *Salix fragilis* L. (36), goat willow *Salix caprea* L. (17) and black poplar *Populus nigra* L. (14). Alongside Himalayan balsam, on 285 of its stands (52 % in total), at least one of 26 other alien plants species was present (27 taxa, if we include inconclusively determined goldenrods, due to inacessibility). Apart from the already mentioned *S. gigantea* – which was the most common allochthonous species, other significantly abundant alien plants were Canadian goldenrod *S. canadensis* L. (80), small balsam *Impatiens parviflora* DC. (62), Japanese knotweed *Fallopia japonica* (Houtt.) Ronse Decr. (56) and annual fleabane *Erigeron annuus* (L.) Pers. (52).

Ecological analysis of Himalayan balsam's accompanying flora showed that average values of four of the observed environmental variables (temperature, pH reaction, light, and nutrients) were approximate to the given theoretical optimum (Ellenberg-type ecological indicator value) of Himalayan balsam for each variable (according to Chytrý et al., 2018), as seen in Tab. 1 below. Values for temperature and pH reaction had the lowest variance of all the results (0.45 in both cases), whereas the variance was the highest for moisture (2.15). In Tab. 1 are also presented values of Ellenberg-type indicator values for entire flora of Ljubljana Marsh.

Table 1: Analysis of Ellenberg-type indicator values for temperature, pH reaction, light, nutrients, and moisture, for Himalayan balsam's accompanying flora and Ljubljana Marsh flora (Jogan et al., 2001), following Chytrý et al. (2018). Corresponding values of Himalayan balsam are highlighted with an asterisk (*).

Tabela 1: Analiza modificiranih Ellenbergovih indikatorskih vrednosti za temperaturo, pH reakcijo, svetlobo, količino hranil in vlago v spremljevalni flori žlezave nedotike na Ljubljanskem barju in za celotno floro Ljubljanskega barja (Jogan in sod., 2001), prirejena po Chytrý in sod. (2018). Vrednosti za žlezavo nedotiko so v preglednici označene z zvezdico (*).

Short description of Ellenberg-type indicator values for temperature	**Himalayan balsam's accompanying flora**		**Ljubljana Marsh flora**	
	Numerus of observations	Percentage (%)	Numerus of observations	Percentage (%)
1 – cold indicator (alpine and nival belt)	0	0	0	0
2 – between 1 and 3	0	0	0	0
3 – cool indicator (mostly in subalpine areas)	0	0	5	0.6
4 – between 3 and 5	0	0	55	6.9
5 – moderate heat indicator	41	59.4	321	39.9
6* – between 5 and 7 (lowland and colline species)	23	33.3	323	40.2
7 – heat indicator	4	5.8	83	10.3

8 – between 7 and 9	1	1.5	16	2.0
9 – extreme heat indicator	0	0	1	0.1
Average value	5.5	/	5.5	/
Variance	0.45	/	0.74	/

Short description of Ellenberg-type indicator values for pH reaction	**Himalayan balsam's accompanying flora**		**Ljubljana Marsh flora**	
	Numerus of observations	Percentage (%)	Numerus of observations	Percentage (%)
1 – indicator of strong acidity	0	0	4	0.5
2 – between 1 and 3	0	0	12	1.5
3 – acidity indicator	0	0	29	3.6
4 – between 3 and 5	1	1.4	48	6.0
5 – indicator of moderate acidity	3	4.4	74	9.2
6 – between 5 and 7	32	46.4	205	25.5
7* – indicator of slightly acidic to slightly basic conditions	32	46.4	325	40.4
8 – between 7 and 9	1	1.4	104	12.9
9 – base and lime indicator	0	0	3	0.4
Average value	6,4	/	6.2	
Variance	0.45	/	1.9	

Short description of Ellenberg-type indicator values for light	**Himalayan balsam's accompanying flora**		**Ljubljana Marsh flora**	
	Numerus of observations	Percentage (%)	Numerus of observations	Percentage
1 – deep shade plants	0	0	0	0
2 – between 1 and 3	0	0	2	0.2
3 – shade plant	0	0	29	3.6
4 – between 3 and 5	5	7.3	68	8.5
5 – semi-shade plant	7	10.1	66	8.2
6* – between 5 and 7	19	27.5	151	18.8
7 – half-light plant	32	46.4	296	36.8
8 – light plant	6	8.7	164	20.4
9 – full light plant	0	0	28	3.5
Average value for accomp. flora	6.4	/	6.5	
Variance	1.06	/	2.02	

Short description of Ellenberg-type indicator values for nutrients	**Himalayan balsam's accompanying flora**		**Ljubljana Marsh flora**	
	Numerus of observations	Percentage (%)	Numerus of observations	Percentage (%)
1 – occurring on nutrient-poorest sites	0	0	5	0.6
2 – between 1 and 3	0	0	57	7.1
3 – occurring on nutrient-poor sites more frequently than at average sites	0	0	112	13.9
4 – between 3 and 5	2	2.9	109	13.6
5 – occurring at moderately nutrient-rich sites	10	14.5	141	17.5
6 – between 5 and 7	20	29.0	180	22.4
7* – occurring at nutrient-rich sites more often than on average sites	25	36.2	141	17.5
8 – pronounced nutrient indicator	11	15.9	55	6.8
9 – concentrated at very nutrient-rich sites	1	1.5	4	0.5
Average value for accomp. flora	6.5	/	5.1	
Variance	1.13	/	3.03	

Short description of Ellenberg-type indicator values for moisture	**Himalayan balsam's accompanying flora**		**Ljubljana Marsh flora**	
	Numerus of observations	Percentage (%)	Numerus of observations	Percentage (%)
1 – strong drought indicator	0	0	1	0.1
2 – between 1 and 3	0	0	7	0.9
3 – missing on damp soil	1	1.45	64	8.0
4 – between 3 and 5	6	8.7	165	20.5
5 – indicator of average moisture and fresh soils	25	36.2	225	28.0
6 – between 5 and 7	16	23.2	105	13.0
7 – humidity indicator	5	7.3	53	6.6
8* – between 7 and 9	13	18.8	69	8.6
9 – wetness indicator	2	2.9	64	8.0
10 – aquatic plant which survives long periods without flooding	1	1.45	25	3.1
11 – aquatic plant rooted under water	0	0	10	1.2
12 – (almost) permanently submerged aquatic plant	0	0	16	2.0
Average value for accomp. flora	6.0	/	5.8	
Variance	2.15	/	4.6	

Values for Himalayan balsam's accompanying flora from Tab. 1 are summarized graphically on box and whisker plot, seen on Fig. 3.

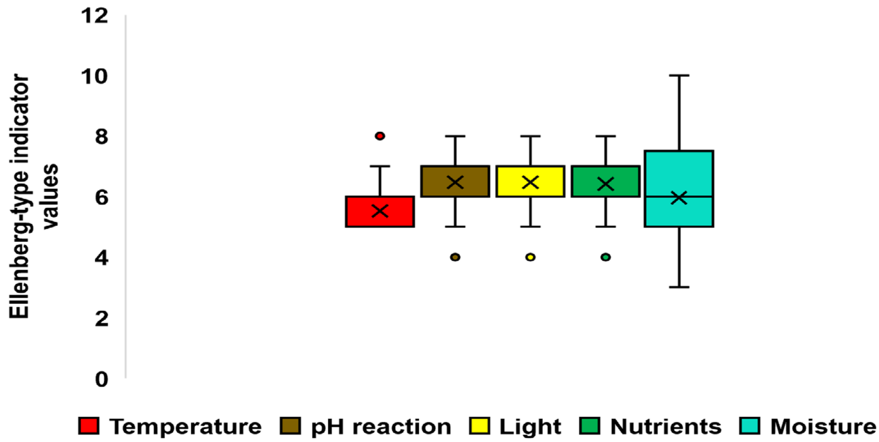

Figure 3: Ellenberg-type indicator values of Himalayan balsam's accompanying flora on Ljubljana Marsh, shown with outliers for each ecological condition. x represents average value.

Slika 3: Ellenbergove indikatorske vrednosti za spremljevalno floro žlezave nedotike na Ljubljanskem barju, s prikazom odstopajojočih vrednosti. x predstavlja povprečno vrednost.

Similarly, for accompanying flora, variance across Ljubljana Marsh flora dataset was the highest for moisture, but the difference between average values between accompanying flora and entire dataset was the highest for nutrients (Tab. 1 above). Comparisons between observed values of Ellenberg-type indicator factors for accompanying flora and Ljubljana Marsh flora are shown graphically on box and whisker plot below, on Fig. 4., showing that the species primarily fell into middle, mesophilic range for many conditions on large-scale area, except regarding nutrients.

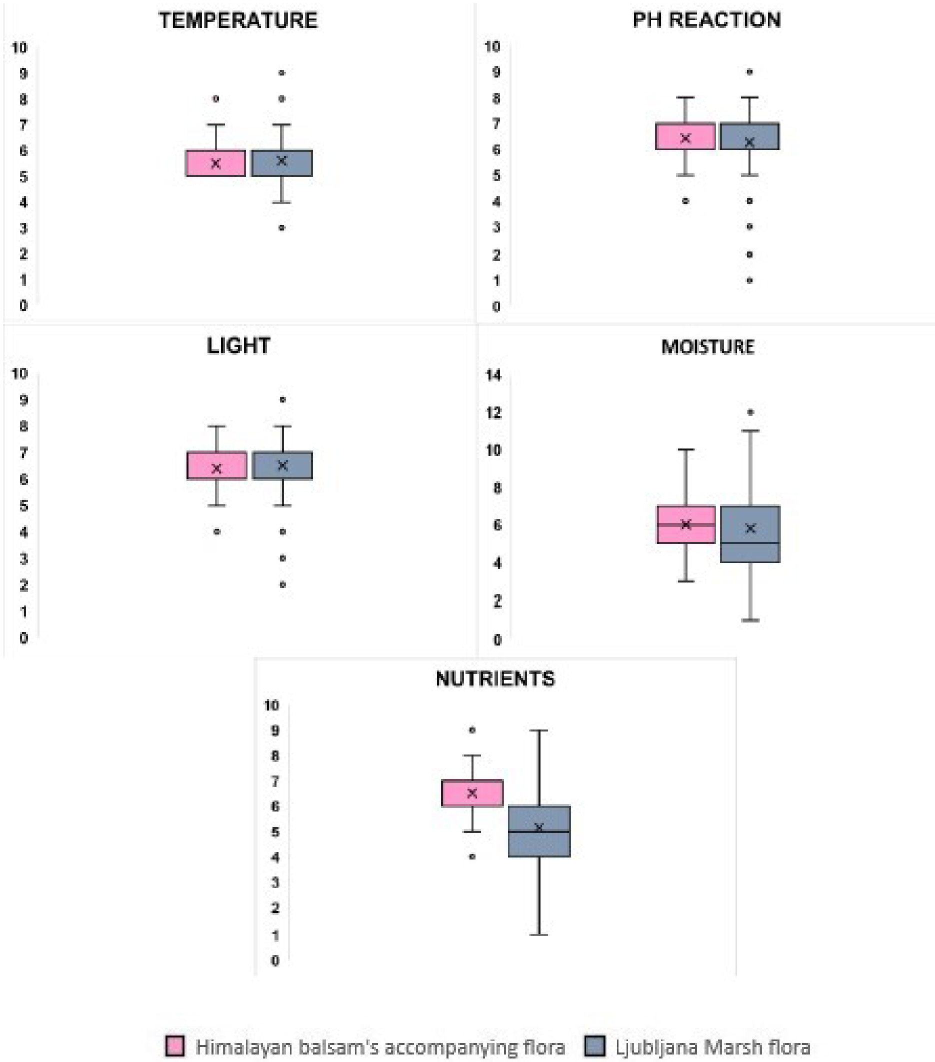

Figure 4: Box and whisker plots with comparisons between Ellenberg-type indicator values for temperature, pH reaction, light, nutrients and moisture between Himalayan balsam's accompanying flora and Ljubljana Marsh flora. x represents average value.

Slika 4: Grafična primerjava Ellenbergovih modificiranih indikatorskih vrednosti za temperaturo, pH, svetlobo, nutriente in vlago med spremljevalno floro žlezave nedotike in floro Ljubljanskega barja.

Analysis of species' potential future spreading, based on existing data on distribution of its accompanying flora has shown that it is highly likely for Himalayan balsam to continue spreading across temperate regions of Slovenia. The only parts which are ecologically unsuitable are Alps, Dinarides (both with higher altitudes) and Sub-Mediterranean region (with higher temperatures and longer summer drought period) (below on Fig. 5).

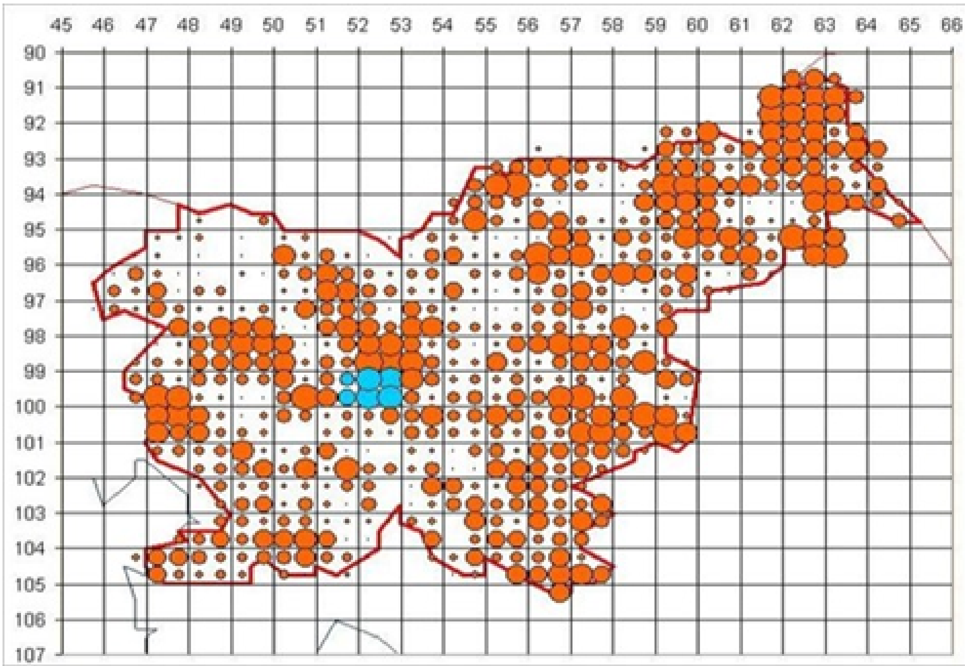

Figure 5: Simplified model of possible Himalayan balsam's distribution in Slovenia, based on distribution of its accompanying flora on Ljubljana Marsh. Color code: blue, area of Ljubljana Marsh; orange, predicted distribution.

Slika 5: Poenostavljen model predvidenega areala žlezave nedotike v Sloveniji, narejen na podlagi razširjenosti spremljevalne flore vrste na Ljubljanskem barju. Barvna shema: modro, območje Ljubljanskega barja; oranžno, predviden ustrezen areal vrste v prihodnosti.

Eradication expenses

According to our calculations (Tab. 2), expenses for eradication on the entire area would run around 20.000 EUR per year. Eradication on accessible localities costs less since they would – theoretically - require only machine work. In our case, that cost was estimated approximately to 3.300 EUR, evaluated as number of accessible sites (330), multiplied by hypothetical wage of 10 EUR/h. Larger effort and budget will be needed for eradication of the inaccessible populations hidden in deep drainage ditches, on steep banks, in thickets, below hedgerows and on private properties, which would require manual processing and therefore. Final costs depend on stands' sizes and density - number of individuals that need to be pulled out, cut down and properly handled. Combining different possibilities of sizes (minimum, minimum and maximum and just maximum sizes), expected prices range from ~ 5.000 EUR to ~ 50.000 EUR, which are both extremes with smallest possible and highest possible number of individuals included. Intermediate value, with the highest probability of combination of smaller and bigger populations (as observed on field), comes to a price point of 16.000 EUR, which combined with mechanical work, gives a final estimate of ~ 20.000 EUR per year. If the first-year eradication approach was proven as effective, repetition of the same approach in the consecutive years would be less expensive and after about 6 years, when the soil seedbank of existing populations would be exhausted, only regular monitoring of sites and hand pulling of individual plants (when needed) would suffice.

Table 2: Himalayan balsam's eradication expenses' estimation for Ljubljana Marsh, calculated on the basis of field results from 2019, by methodology explained in Methods.
Tabela 2: Ocena stroškov odstranjevanja žlezave nedotike na Ljubljanskem barju, narejena na podlagi rezultatov terenske študiji po metodologiji opisani v zgornjem delu besedila, v poglavju Metod.

Stands classification by size classes and accessibility					
Stand size classes	**Number of accessible stands**	**Number of partially accessible stands**	**Number of inaccessible stands**	**∑ of stands by size classes**	
1-10	97	9	46	152	
10-100	151	13	59	223	
100-1000	58	34	30	122	
Over 1000	27	14	10	51	
	333	70	145	548	
x10 EUR/h	3330				
	∑ partially accessible and inaccessible stands	Minimum sizes of stands considered in calculations	Min. and max. stand sizes combined in calculations	Min. and max. stand sizes combined (2) in calculations	Maximum stand sizes considered in calculations
1-10	55	550	550	550	550
10-100	72	720	7200	7200	720
100-1000	64	6400	6400	64000	64000
Over 1000	24	24000	24000	24000	240000
∑	215	31670	38150	95750	311750
	In hours	527.83	635.83	1595.83	5195.83
	x10 EUR/h	5278.33	6358.33	15958.3	51958.3
	Total estimated cost in EUR (+ acc.)	8608.33	9688.33	19288.33	55288.33

Discussion

Spreading dynamic and species' ecology

Scattered distribution of Himalayan balsam in the given area is very likely a result of many circumstances, such as area's geographical and hydrological structure, complex conservational system, intensive agriculture, and several other human activities. Dominance of point-type stands is not surprising, since those included an array of different sized stands, including either handful of individuals or up to 1000. Those smaller stands were found in a range of different habitats - predominantly dispersed alongside the roads, but were also located in fields, meadows, by the forest edges or along the waters. Linear and polygon-type stands were mostly located on larger, often less accessible areas, such as alongside drainage ditches, where linear stands were predominant, or on abandoned fields or properties, dominated by polygon-type stands with over 1000 individuals. Those are likely older stands, which successfully maintain their size by local spreading. Between the two seasons, Himalayan balsam has spread across the Ljubljana Marsh only moderately and to relatively short distances: mostly up to 150 meters away of existing populations, what confirms its hypothesised successful spreading capabilities by water only partially. Based on our results and our field observations, we assume that water bodies of Ljubljana Marsh are not the main vector of species' spreading on chosen area. Firstly, there were only few populations located near the biggest water course - river Ljubljanica, while the rest were mostly found by the streams or along ditches. Secondly, their main hydrological characteristic: slow water flow due to minimum elevation differences, could not carry out long-distance spreading as seen in some other studies (Perrins et al. 1993), which report on spreading dynamics in British Isles of 38 km/year. We did confirm successful short distance spreading in the nearby area of the parent plants, which reaches up to 7 meters by the ballistochory mechanism (Balogh 2008). The main spreading mechanism in these instances was more effective than just ballistochory, but situation is not clear-cut. We can assume that various anthropogenic factors on Ljubljana Marsh have had a significant role in species' spreading, along with limited hydrochory. Thus, we presume that the primary role of water bodies in drainage ditch network of Ljubljana Marsh might be maintenance of local populations, by enabling favourable moist conditions, regular opening of bare soils due to ditch maintenance and reduced accessibility for the agricultural activities (mowing, pasturing, trampling). Čuda et al. (2014) hypothesized that existence of Himalayan balsam in riparian zones represents an evolutionary adaptation which would reinforce the hydrochory. Our results cannot confirm that either. Another note was the absence of typical winter floods in given period. Floods were reportedly mentioned as a contributing factor in invasiveness of the species (Čuda et al. 2017a). For additional insight into spreading dynamic, we checked Flood Warning map of Ljubljana Marsh (data from Slovenian INSPIRE metadata system, 2022). The southwest part of Ljubljana Marsh is the one primarily exposed to regular flooding (Fig. 6), Himalayan balsam is mostly absent there, as seen in Fig. 1, except for parts of Podplešivica and some parts of Notranje Gorice and Vnanje Gorice. Hereof, we conclude that hydrochory should not be considered as the main pathway in spreading of given species on Ljubljana Marsh, especially in such short time interval. Further research, which would incorporate the possible impact of flooding, is therefore highly appreciated.

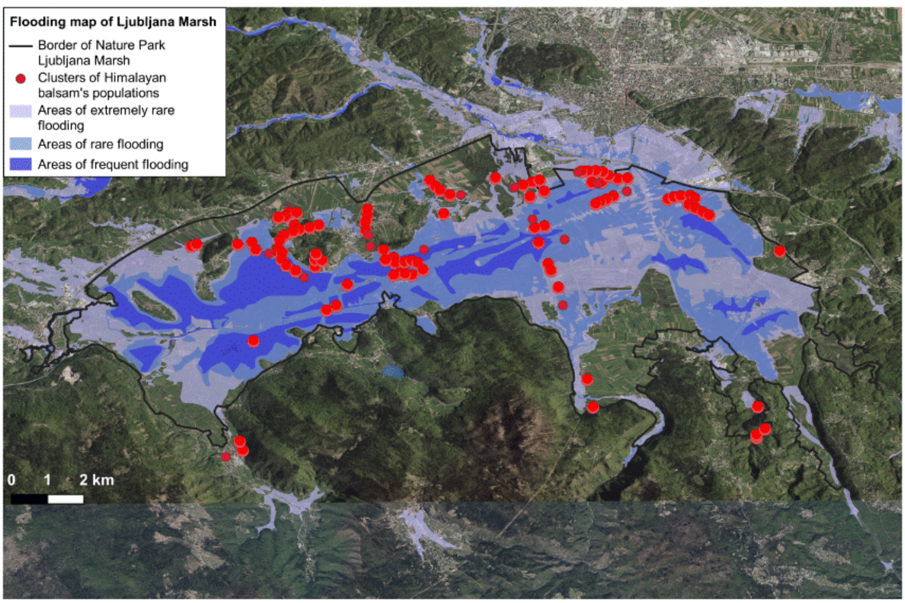

Figure 6: Flooding areas of Ljubljana Marsh (bordered with black line), which are divided into areas of extremely rare flooding (lightest blue on map), which include floods with return period of 50 years and more, areas of rare flooding (medium blue on map), which include floods with return period between 10 and 20 years, and areas of frequent flooding (darkest blue on map), which include floods with return period of 2 to 5 years. GIS layers and correspoding legend descriptions are found at publicly available server of Slovenian INSPIRE metadata system, as Flood warning map (2022). Red points represent clusters of Himalayan balsam's populations found at Ljubljana Marsh, grouped by built-in function Point Cluster in QGIS 3.18.1.

Slika 6: Območja poplavljanja na Ljubljanskem barju (omejeno s črno linijo), razdeljena v območja zelo redkih poplav (svetlo modra na karti), ki vključujejo poplave s povratno dobo 50 ali več let, območja redkih poplav (srednje temno modra na karti), ki vključujejo poplave s povratno dobo od 10 do 20 let, ter območja pogostih poplav, ki vključujejo poplave s povratno dobo od 2 do 5 let. Uporabljene GIS datoteke in njihovi opisi so dostopni na spletni strani Slovenskega INSPIRE metapodatkovnega sistema, v zavihku Opozorilna karta poplav (2022). Rdeče pike na karti predstavljajo grupirane populacije žlezave nedotike, narejene s pomočjo sistemske funkcije Point Cluster v programu QGIS 3.18.1.

Accompanying flora was similar to the literature reports (Hulme and Bremner 2006, Balogh 2008, Diekmann et al. 2016, Kiełtyk and Delimat 2019), simoultaneously very heterogenous and reflective of Ljubljana Marsh's habitat diversity and its degradation. Even though over half (285) of our localities had at least one other alien plant or other elements of neophytic vegetation, on 263 localities Himalayan balsam was the only one. Those were usually the ditches with native riparian vegetation, most commonly willow thickets and occasional alder communities. Adequate ecological conditions allowed the Himalayan balsam to competitively exclude and supress native riparian forest vegetation, since black poplar, white willow and crack willow appeared to be ecologically most similar species to Himalayan balsam (see Chytrý et al. 2018). Those ecosystems have a crucial role as pionneers in the progressive succession of riparian forests (Garófano-Gómez et al. 2017), but due to their heavy invasion by alien species on entirety of Ljubljana Marsh, those natural processes seem to be significantly disturbed. Our observations and results therefore put focus on disturbance of riparian zones as the potentially biggest negative impact caused by Himalayan balsam on Ljubljana Marsh. Based on the results of robust ecological analysis of indicator values, we suggest that Himalayan balsam should be observed as a species with somewhat broader ecological niche than the one usually ascribed to it. Most surprising variation from its ecological optimum was for moisture, with accompanying flora's average resulting in 6.0. If we look at values of entire Ljubljana Marsh area and compare them with accompanying flora of Himalayan balsam, we see that Himalayan balsam falls into average ecological values of area, with a possibility of realized ecological niche profiling into its alleged nitrophily, since micro-sites where Himalayan balsam grew were by the results of indicator values analysis more suitable for nitrophilous species (averages 6.5 and 5.1, respectively). Those results were probably influenced by large presence of *Urtica dioica*, the second most common species in accompanying flora and only true nitrophilous species, with EV 9. Even though its accompanying flora didn't reflect hidrophily *per se* (with average value of 6.1 and theoretical optimum of 8), there were small differences between its stands and entire area (averages 6.0 and 5.8), hinting that it grows at areas slightly more hydrophilous than the entire area.

Species is probably more tolerant to moderate variations of ecological conditions and capable of surviving in different habitats, co-existing with a range of different species, what Bieberich et al. (2020) list as one of crucial traits which determine the success of invader. As an example, we point out Himalayan balsam's road populations. Even though those stands were usually smaller in size, species is obviously capable of existence in sites which are by default more open, bright and dry than the expected species' ecological optimum (see Chytrý et al. 2018). Accompanying flora at those sites consisted of different ruderal grasses, such as *Lolium perenne* L., *Setaria pumila* (Poir.) Roem. & Schult., *Setaria viridis* (L.) PB., *Panicum capillare* L. and *Digitaria sanguinalis* (L.) Scop., or ruderals like *Cichorium intybus* L. On the other hand, light and/or dryness weren't shown as possible limiting factors when stands were found in an optimum regarding water and/or nutrients, for example along ditches on an open field, where Himalayan balsam might have compensated exposure to open light with sufficient amount of water from ditches. On arable land, Himalayan balsam's stands probably have a dependable source of nutrients, coming from both water and soil. Thus, our results – at least partially - refute findings of Čuda et al. (2014), which indicate that species avoids fully open sites, but agree with the later study (Čuda et al. 2017a), whose results hint that species successfully grows in open areas, when appropriately supplied with water. Optimal conditions for Himalayan balsam (see Chytrý et al. 2018) can therefore be achieved in micro-ecological sites and niches, what is not unusual for Himalayan balsam (see Bieberich et al. 2020 – different approach with a similar final result). In those (relatively) optimal conditions, negative impact of species could come to its peak.

Eradication suggestions

For successful supression of Himalayan balsam's further invasion, we suggest regular and proper eradication, followed by continuous monitoring. Proper eradication methodology is mostly dependent on knowledge of species' phenology, so it is important to choose the right moment for the first summer eradication. In this case, it would be recommended to start in July. If we start priorly, we risk the re-establishment of the population from seeds or by resprouting. If we start later - towards the fall season, we might assist in the seed spreading from ripe capsules whilst handling the plants. Therefore, we suggest one removal in July and one later in the season, to remove potentially regenerated individuals (Hartmann et al. 1995). Skalová et al. (2019) have confirmed the viability of seed bank lasting up to 4 years, so we highly suggest the continuation of proper monitoring and prospective repeated removal in 4-year period at shortest, preferrably longer, since there are also reports on seed viability after 6 years (Schuldes 1995). With regular removal and control, eradication costs (proposed in Results) will probably decrease in the consecutive years. To terminate the invasion process, we must also prevent further importation and re-introduction, which in case of Himalayan balsam would be prohibition of horticultural and beekeeping usage.

Conclusions

This study has confirmed the importance of context-dependence in research field of invasion ecology. Despite great number of studies on Himalayan balsam (*Impatiens glandulifera*), species is still somehow overlooked and trivialized. On Ljubljana Marsh, Himalayan balsam has shown more ecological plasticity than usually considered, surviving in wider range of mesophilic conditions which deviate from its theoretical optimum – moist, fertile and shady places. As long as any of species' critically impactful ecological conditions – light, moisture and nutrients – are at least near its theoretical optimum, deviations of other ecological conditions don't pose as the limiting factor in species' persistance, especially regarding moisture. We have found that species doesn't spread as drastically by water bodies on Ljubljana Marsh in two consecutive seasons as mentioned in other literary sources, meaning that specific hydrology of Ljubljana Marsh cannot contribute to its invasiveness, as other factors – presumably anthropogenic – might. Hydrology of Ljubljana Marsh therefore probably has a role in maintenance of already existing populations and spreading dynamics is not the basis of species' invasiveness in given circumstances. We propose competitive exclusion of native riparian vegetation as the main mechanism of species invasiveness, along with flexibility and adaptability to wide range of ecological conditions. As species of Union concern, Himalayan balsam should be regularly eradicated and monitored at adequate period, dependent on its phenology. At chosen site, in approximately five-year period, species can be successfully controlled with moderate expenses, although prevention of further introductions is crucial.

Povzetek

Biološke invazije so, zaradi negativnega vpliva na biotsko pestrost in ekosistemske storitve eden izmed največjih izzivov, s katerimi se soočajo sodobno naravovarstvo, družba in gospodarstvo. Zaradi velike raznolikosti tujerodnih vrst, povzročajo tudi različne vplive v različnih ekosistemih, kar je odvisno od njihovih bioloških lastnosti, vitalnosti in odpornosti avtohtonih združb. V tem članku so predstavljeni rezultati terenske študije z Ljubljanskega barja iz leta 2019, ko smo primerjali stanje populacij žlezave nedotike (*Impatiens glandulifera*) med letoma 2018 in 2019. Glede na to, da se v literaturi pogosto poudarja sposobnost hitrega širjenja te vrste z vodami, smo predvsem vzdolž vodotokov pričakovali velike razlike stanja populacij med dvema sezonama. Izkazalo

pa se je, da hidrološke in poplavne razmere na Ljubljanskem barju ne omogočajo hitrega širjenja žlezave nedotike, ker med dvema sezonama ni bilo izrazitih razlik. Rezultatov tujih študij razširjanja žlezave nedotike ob hitro tekočih vodah torej ne moremo preprosto uporabiti pri napovedovanju širjenja ob počasi tekočih ali skoraj stoječih voda Barja. Za te se je v študiji pokazalo, da imajo predvsem pomen v vzdrževanju primernih razmer za obstoj populacij nedotike. Ta študija nam je pokazala, da je vrsta ekološko bolj plastična, kot si jo morebiti predstavljamo iz posplošenih navedb iz literaturnih virov, ki jo praviloma obravnavajo kot vlagoljubno, sencoljubno in nitrofilno. Vse dokler je vsaj eden izmed kritičnih okoljskih dejavnikov (svetloba, hranila in vlaga) zunaj pesimuma ali vsaj blizu optimuma, drugi okoljski dejavniki za vrsto očitno niso izrazito omejujoči. Zaradi velikega števila spremljevalnih drugih tujerodnih vrst na rastiščih nedotike in splošnega degradiranega stanja Ljubljanskega barja tudi ni bilo mogoče govoriti o konkretnem vplivu žlezave nedotike na ta ekosistem, vendar naši rezultati kažejo, da vrsta vrhunec svoje invazivnosti kaže v obrežnih gozdovih vrbe in jelše, kjer kompetitivno izključuje avtohtono obrežno floro in moti naravni tok sukcesije. Ker so rezultati naše in drugih študij o žlezavi nedotiki dokaj raznoliki, poudarjamo pomen vsake individualne in primerjalne raziskave. Le tako bomo lažje razumeli biologijo teh vrst v širšem kontekstu, kar je izjemno pomembno pri raziskavah invazivnih tujerodnih vrst v raznolikem in zanje novem okolju, kar nam bo kot biologom omogočilo zaznati ključne momente biologije posamezne invazivne vrste, na katere bomo lahko oprli naravovarstvene ukrepe.

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References

Andrews, M., Maule, H. G., Raven, J. A., Mistry, A., 2005. Extension Growth of *Impatiens glandulifera* at Low Irradiance: Importance of Nitrate and Potassium Accumulation. Annals of Botany, 95 (4), 641-648.

Andrews, M., Maule, H. G., Hodge, S., Cherrill, A., Raven, J. A., 2009. Seed dormancy, nitrogen nutrition and shade acclimation of Impatiens glandulifera: implications for successful invasion of decidious woodland. Plant Ecology and Diversity, 2 (2), 145- 153.

Balogh, L., 2008. Himalayan balsam (*Impatiens glandulifera* Royle). In: Botta-Dukát, Z., Balogh, L. (eds.): The most important invasive plants in Hungary. Hungarian Academy of Sciences, Institute of Ecology and Botany, Vácrátot, pp. 129-137.

Bartelheimer, M., Poschlod, P., 2016. Functional characterizations of Ellenberg indicator values – a review on ecophysiological determinants. Functional Ecology, 30 (4), 506-516.

Beerling, D. J., Perrins, J. M., 1993. Biological flora of the British Isles: Impatiens glandulifera Royle (Impatiens roylei Walp.). Journal of Ecology, 81 (2), 367-382.

Bieberich, J., Feldhaar, H., Lauerer, M., 2020. Micro-habitat and season dependent impact of the invasive *Impatiens glandulifera* on native vegetation. NeoBiota, 57, 109-131.

Bioportal. 2022. http://www.bioportal.si/moj_bp.php

Blackburn, T.M., Essl, F., Evans, T., Hulme, P.E., Jeschke, J.M., Kühn, I., Kumschick, S., Marková, Z., Mrugała, A., Nentwig, W., Pergl, J., Pyšek, P., Rabitsch, W., Ricciardi, A., Richardson, D.M., Sendek, A., Vilà, M., Wilson, J.R.U., Winter, M., Genovesi, P., Bacher, S., 2014. A Unified Classification of Alien Species Based on the Magnitude of their Environmental Impacts. PLoS Biology, 12 (5), e1001850.

Chytrý, M., Tichý, L., Dřevojan, P., Sádlo, J., Zelený, D., 2018. Ellenberg-type indicator values for the Czech flora. Preslia, 90 (2), 83-103.

Clements, D.R., Feenstra, K.R., Jones, K., Staniforth, R., 2008. The Biology of Invasive Alien Plants in Canada. 9. *Impatiens glandulifera* Royle. Canadian Journal of Plant Science, 88 (2), 403-417.

Čuda, J., Skálová, H., Janovský, Z., Pyšek, P., 2014. Habitat requirements, short-term population dynamics and coexistence of native and invasive *Impatiens* species: a field study. Biological Invasions, 16 (1), 177-190.

Čuda, J., Rumlerová, Z., Brůna, J., Skálová, H., Pyšek, P., 2017a. Floods affect the abundance of *Impatiens glandulifera* and its spread from river corridors. Diversity and Distributions, 23 (3-4), 342-354.

Čuda, J., Vítková, M., Albrechtová, M., Guo, W.-Y., Barney, J.N., Pyšek, P., 2017b. Invasive herb *Impatiens glandulifera* has minimal impact on multiple components of temperate forest ecosystem function. Biological Invasions, 19 (10), 3051-3066.

Diekmann, M., Effertz, H., Baranowski, M., Dupré, C., 2016. Weak effects on plant diversity of two invasive *Impatiens* species. Plant Ecology, 217 (12), 1503-1514.

Drescher, A., Prots, B., 2003. Distribution patterns of Himalayan Balsam (*Impatiens glandulifera*) in Austria. Kanitzia, 11, 85-96.

Elst, E.M., Acharya, K.P., Dar, P.A., Reshi, Z.A., Tufto, J., Nijs, I., Graae, B.J., 2016. Pre-adaptation or genetic shift after introduction in the invasive species *Impatiens glandulifera*? Acta Oecologica, 70, 60-66.

Enders, M., Havemann, F., Ruland, F., Bernard-Verdier, M., Catford, J.A., Gómez-Aparicio, L., Haider, S., Heger, T., Kueffer, C., Kühn, I., Meyerson, L.A., Musseau, C., Novoa, A., Ricciardi, A., Sagouis, A., Schittko, C., Strayer, D.L., Vilà, M., Essl, F., Hulme, P.E., van Kleunen, M., Kumschick, S., Lockwood, J.L., Mabey, A.L., McGeoch, M.A., Palma, E., Pyšek, P., Saul, W.-C., Yannelli, F.A., Jeschke, J.M., 2020. A conceptual map of invasion biology: Integrating hypotheses into a consesus network. Global Ecology and Biogeography, 29, 978-991.

European Commission Implementing Regulation (EU) 2017/1263 of 12 July 2017 updating the list of invasive alien species of Union concern established by Implementing Regulation (EU) 2016/1141 pursuant to Regulation (EU) No 1143/2014 of the European Parliament and of the Council, 2017. Official Journal of the European Union, L 182, 37–39.

Flood warning map. 2022. https://eprostor.gov.si/imps/srv/eng/catalog.search#/metadata/854c3f92-48e8-49e6-b9e6-2bb5086e386c

Garófano-Gómez, V., Metz, M., Egger, G., Díaz-Redondo, M., Hortóbagyi, B., Geerling, G., Corenblit, D., Steiger, J., 2017. Vegetation succession processes and fluvial dynamics of a mobile temperate riparian ecosystem: the lower Allier River (France). Géomorphologie : Relief, Processus, Environnement., 23 (3), 187-202.

Geopedia. 2020. http://www.geopedia.si/#T105_x457040_y92728_s12_b2 (6. mar. 2020)

Hartmann, E., Schuldes, R., Kübler, R., Konold, W. 1995. Neophyten. Biologie, Verbreitung und Kontrolle ausgewählter Arten. Ecomed, Landsberg, 302 pp.

Hejda, M., Pyšek, P., 2006. What is the impact of *Impatiens glandulifera* on species diversity of invaded riparian vegetation? Biological Conservation, 132 (2), 143-152.

Hejda, M., Pyšek, P., Varošík, V., 2009. Impact of invasive plants on the species richness, diversity and composition of invaded communities. Journal of Ecology, 97 (3), 393-403.

Hulme, P.E., Bremner, E.T., 2006. Assessing the impact of *Impatiens glandulifera* on riparian habitats: partitioning diversity components following species removal. Journal of Applied Ecology, 43 (1), 43-50.

Jogan, N., Bačič, T., Frajman, B., Leskovar, I., Naglič, D., Podobnik, A., Rozman, B., Strgulc-Krajšek, S., Trčak, B., 2001. GRADIVO za Atlas flore Slovenije. Center za kartografijo flore in favne, Miklavž na Dravskem polju, 443 pp.

Kiełtyk, P., Delimat, A., 2019. Impact of the alien plant *Impatiens glandulifera* on species diversity of invaded vegetation in the northern foothills of the Tatra Mountains, Central Europe. Plant Ecology, 220 (1), 1-12.

Lambdon, P.W., Pyšek, P., Basnou, C., Hejda, M., Arianoutsou, M., Essl, F., Jarošík, V., Pergl, J., Winter, M., Anastasiu, P., Andriopoulos, P., Bazos, I., Brundu, G., Celesti-Grapow, L., Chassot, P., Delipetrou, P., Josefsson, M., Kark, S., Klotz, S., Kokkoris, Y., Kühn, I., Marchante, H., Perglová, I., Pino, J., Vilà, M., Zikos, A., Roy, D., Hulme, P.E., 2008. Alien flora of Europe: species diversity, temporal trends, geographical patterns and research needs. Preslia 80 (2), 101-149.

Lozej, N., 2018. Popis invazivnih tujerodnih vrst – žlezave nedotike in velikega pajesena na območju Krajinskega parka Ljubljansko barje (končno poročilo). Ekološke raziskave in svetovanje, Nina Lozej, 22 pp.

Martinčič, A., Wraber, T., Jogan, N., Podobnik, A., Turk, B., Vreš, B., Ravnik, V., Frajman, B., Strgulc Krajšek, S., Trčak, B., Bačič, T., Fischler, M. A., Eler, K., Surina, B. 2007. Mala flora Slovenije: ključ za določanje praprotnic in semenk. 4. dopolnjena in spremenjena izdaja. Tehniška založba Slovenije, Ljubljana, 967 pp.

Nobis, A., Rola, K., Węgrzyn, M., 2017. Detailed study of a river corridor plant distribution pattern provides implication for river valley conservation. Ecological Indicators, 83, 314-322.

Opozorilna karta poplav. 2022. https://eprostor.gov.si/imps/srv/slv/catalog.search#/metadata/854c3f92-48e8-49e6-b9e6-2bb5086e386c

Perglová, I., Pergl, J., Skálová, H., Moravcová, L., Jarošík, V., Pyšek, P., 2009. Differences in germination and seedling establishment of alien and native *Impatiens* species. Preslia, 81 (4), 357-375.

Perrins, J., Fitter, A., Williamson, M., 1993. Population biology and rates of invasion of three introduced *Impatiens* species in British isles. Journal of Biogeography, 20 (1), 33-44.

Petkovšek, P., 1966. Prispevek k poznavanju vegetacije rečnih obrežij v Sloveniji. Biološki Vestnik, 14: 37–44.

Prekoršek, B., 1964. Po sledovih izgubljene rastline. Proteus, 27, 107-110.

Prowse, A., 1998. Patterns of early growth and mortality in *Impatiens glandulifera*. In: Starfinger, U., Edwards, K., Kowarik, I., Williamson, M. (eds.): Plant Invasions: Ecological Mechanisms and Human Responses. Leiden, Backhuys, pp. 245-252.

Pyšek, P., Jarošík, V., Hulme, P.E., Pergl, J., Hejda, M., Schaffner, U., Vilà, M., 2012. A global assesment of invasive plant impacts on resident species, communities and ecosystems: the interaction of impact measures, invading species' traits and environment. Global Change Biology, 18 (5), 1275-1237.

QGIS.org. 2022. QGIS Geographic Information System. QGIS Association. http://www.qgis.org

Richardson, D.M., Holmes, P.M., Esler, K.J., Galatowitsch, S.M., Stromberg, J.C., Kirkman, S.P., Pyšek, P., Hobbs, R.J., 2007. Riparian vegetation: degradation, alien plant invasions, and restoration prospects. Diversity and Distributions, 13 (1), 126-139.

Richardson, D.M., Pyšek P., 2006. Plant invasions: merging the concepts of species invasiveness and community invasibility. Progress in Physical Geography, 30 (3), 409-431.

Schnitzler, A., Hale, B.W., Alsum, E.M., 2006. Examining native and exotic species diversity in European riparian forests. Biological Conservation, 138 (1-2), 146-156.

Schuldes, H. 1995. Das Indische Springkraut (*Impatiens glandulifera*): Biologie, Verbreitung, Kontrolle. In: Böcker, R., Gebhardt, H., Konold, W., Schmidt-Fischer, S. (eds.): Gebietsfremde Pflanzarten. Auswirkungen auf einheimische Arten, Lebensgemeinschaften und Biotope. Kontrollmöglichkeiten und Management, Ecomed, Landsberg, pp. 83-88.

Skálová, H., Moravcová, L., Čuda, J., Pyšek, P., 2019. Seed-bank dynamics of native and invasive *Impatiens* species during a five-year field experiment under various environmental conditions. NeoBiota, 50, 75-95.

Vilà, M., Basnou, C., Pyšek, P., Josefsson, M., Genovesi, P., Gollasch, S., Nentwig, W., Olenin, S., Roques, A., Roy, D., Hulme, P.E., 2010. How well do we understand the impacts of alien species on ecosystem services? A pan-European, cross-taxa assesment. Frontiers in the Ecology and the Environment, 8 (3), 135-144.

Vilà, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarošík, V., Maron, J.L., Pergl, J., Schaffner, U., Sun, Y., Pyšek, P., 2011. Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. Ecology Letters, 14 (7), 702-708.

Zelnik, I., 2012. The presence of invasive alien plant species in different habitats: case study from Slovenia. Razširjenost tujerodnih in invazivnih vrst rastlin v različnih habitatih: primer iz Slovenije. Acta Biologica Slovenica, 55 (2), 25-38.

Zorn, M., Šmid Hribar, M., 2012. A landscape altered by man as a protected area: A case study of the Ljubljana Marsh (Ljubljansko barje). Ekonomska i Ekohistorija, 8 (1), 45-61.

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Potential of rosemary hydrosol for effective growth inhibition of fungi isolated from buckwheat grains

Potencial rožmarinovega hidrolata za učinkovito zaviranje rasti gliv, izoliranih iz zrnja ajde

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Abstract: Modern botanical fungicides should be non-toxic and readily available. Hydrosols are by-products of essential oil distillation with a large potential market size. They are, therefore, suitable natural candidates for effective fungicide development. Improving grain quality and safety during storage are significant challenges in the contemporary world. We have therefore tested the possible use of rosemary (*Rosmarinus officinalis* L.) hydrosol as an efficient antifungal agent against fungi isolated from buckwheat grain. Fungi from the genus *Fusarium* were the most susceptible to rosemary hydrosol, as growth inhibition was observed in all tested species by 15 % rosemary hydrosol and in *F. graminearum* already by 5 % hydrosol concentration. Since there was no inhibitory effect on the germination of buckwheat grain after exposure to rosemary hydrosol, it could potentially be used as an environmentally friendly alternative for suppressing fungal growth on grains.

Keywords: antimicrobial activity, biopesticides, fungicides, *Fagopyrum esculentum, Fusarium* sp., *Rosmarinus officinalis*

Izvleček: Sodobni rastlinski fungicidi morajo biti nestrupeni in splošno dostopni. Hidrolati so stranski produkti destilacije eteričnih olj, ki imajo velik delež na tržišču, zato predstavljajo primerne naravne mešanice za razvoj novih učinkovitih fungicidov. Velik izziv sodobnega časa predstavljajo izboljšave kakovosti in varnosti zrnja predvsem v času skladiščenja. Zato smo v raziskavi testirali potencialno uporabo hidrolata iz rožmarina (*Rosmarinus officinalis* L.) kot učinkovitega protiglivnega sredstva za zatiranje gliv, izoliranih iz zrn ajde. Glive iz rodu *Fusarium* so se izkazale za najbolj občutljive na rožmarinov hidrolat, saj smo pri 15 % koncentraciji hidrolata opazili zavrtje rasti vseh testiranih vrst, pri *F. graminearum* pa že pri 5 % koncentraciji rožmarinovega hidrolata. Ker nismo opazili nobenega negativnega vpliva na kalitev zrnja ajde po izpostavitvi rožmarinovemu hidrolatu, bi ta lahko bil potencialno uporabljen kot okolju prijazna alternativa za zatiranje glivne rasti na zrnju.

Ključne besede: biopesticidi, fungicidi, *Fagopyrum esculentum, Fusarium* sp., *Rosmarinus officinalis,* protimikrobno delovanje

Introduction

Crops are an essential part of human nutrition, especially cereals and pseudocereals. A major problem in their production is infections with phytopathogenic fungi which can cause seed deterioration (Christensen 1957). They can affect stored seeds' germination and processing quality (Halloin 1983), leading to a considerable financial loss worth millions of dollars (Yoon et al. 2013, Peng et al. 2021).

Several synthetic materials, such as organochlorines, carbamates, organophosphates, pyrethroids, and neonicotinoids (Mahmood et al. 2016) are commercially used against fungi and other harmful microorganisms (Faleiro et al. 1999, Boyraz and Özcan 2005). They are highly effective, but the increased sensitivity of our immune systems against synthetic compounds, environmental pollution, resistance development, and residual toxicity has urged pesticide-producing companies to develop more natural and environmentally friendly products (Faleiro et al. 1999, Boyraz and Özcan 2005, Yoon et al. 2013). Compared to plant metabolites showing insecticidal activity, fungicidal metabolites have little toxicity (Yoon et al. 2013), increasing their importance as potential food preservatives. Essential oil of rosemary, in particular, was recently suggested as an excellent choice for its use as a food preservative, as it is cheap, available, and non-toxic (Nieto et al. 2018, Stojiljkovic et al. 2018).

Plant secondary metabolites, such as essential oils (EOs) and hydrosols, namely the by-products of hydro-distillation of EOs (Taglienti et al. 2022), have been studied for their antifungal, antibacterial, antiviral, insecticidal, and cytotoxic activities. The majority of them focused on the use of EOs, but there are only a few regarding the effects of spice hydrosols. Boyraz and Özcan (2005) have found significant antifungal effects of different spice hydrosols, including savory, cumin, and pickling herb, compared with the lower effect of rosemary and basil hydrosols. However, they also observed that lower doses of hydrosols have even stimulated fungal growth. Politi et al. (2022) have compared the antifungal effect of rosemary, sage, and cypress hydrosols. Among the tested samples, rosemary and sage displayed the greatest effectiveness. They also carried out the biochemical analysis of rosemary hydrosol and found 1,8-cineole, camphor, and camphene as the major compounds that could also be at the basis of its high antifungal activity compared to sage and cypress. They concluded that regarding the dilution and the aqueous nature of the tested hydrosols, the detected activity could give rise to a wide range of their applications.

As described above, hydrosols are a by-product of the production of EOs and are consequently discarded (Rajeswara Rao 2013). In India, one of the most important essential oil-producing countries, the economic value of the lost oil fraction was estimated to be worth about 50–100 million dollars (Rajeswara Rao 2012). Indeed, the value of hydrosols compared to EOs is underrated, especially as the bioactive ingredients are more bioavailable in water solution of hydrosols than in hydrophobic EOs (Di Vito et al. 2019).

In our research, we tested the effectiveness of different concentrations of rosemary hydrosol against some fungi isolated from buckwheat grains. Buckwheat (*Fagopyrum esculentum* Moench.) is an important crop grown mainly in Europe and Asia (Popović et al. 2014). Its production is increasing worldwide because it has an excellent nutritional quality, contains no gluten or gluten-like proteins, and can improve soil fertility (Glamočlija et al. 2011, Popović et al. 2013a,b). Due to its modest growing demands, it is also suitable for organic farming (Popovič et al. 2014). And as for most cereals and pseudocereals, fungal infections are the primary cause of buckwheat diseases at all stages of its production worldwide (Milevoj 1989). Therefore, it is crucial to have effective and environmentally friendly agents for suppressing fungal growth on buckwheat grains.

Materials and methods

Hydrosol preparation

The plant material of rosemary (*Rosmarinus officinalis* L.) was collected in home gardens in Trbovlje and Celje, continental Slovenia, in April 2022. It was hydro-distilled using coppery still Al-Ambiq® with 10 L of volume for plant material. In two rounds of distillation of rosemary, each lasting 20 minutes, we got altogether 1200 mL of rosemary hydrosol (RH) and only a minimal amount of essential oil. They were separated and RH was stored in the refrigerator until further use.

Fungal strains

Seven different species of fungi were tested in our experiment: *Alternaria alternata* (NA007), *Alternaria infectoria* (TA001), *Epicoccum nigrum* (NA020), *Fusarium fujikuroi* SC (NA030), *Fusarium graminearum* (NAX03), *Fusarium oxysporum* (BS2_170) and *Fusarium sporotrichioides* (NA001). They were obtained from the fungal bank of the Plant Physiology Laboratory at the Department of Biology (Biotechnical Faculty, University in Ljubljana, Slovenia) and previously identified based on their morphology and using molecular methods (Kovačec et al. 2016, Mravlje et al. 2021).

Antifungal activity

Antifungal activity was tested in 2% potato dextrose agar (PDA) supplemented with antibiotic chloramphenicol (50 mg L^{-1}). After sterilization and cooling to about 50 °C, an adequate amount of RH was added to the glass bottles to obtain 5%, 10%, and 15% media, and none for the control. The fungi (25 mm^2) were inoculated in Petri dishes, 90 mm in diameter, from the margin of a 7-day-old original fungal culture, in five replicates. Diameter and surface area of fungal mycelia were measured using ImageJ software after a 7-day incubation period using ImageJ software.

The inhibition rate of fungal growth was expressed as the proportion (%) of growth reduction, as calculated according to Equation (1), by Anžlovar et al. (2020), modified by Schoss et al. (2022):

$$\text{Inhibition rate (\%)} = (AC – AT)/AC \times 100 \quad (1),$$

where AC is the area of mycelial growth of the control colonies, and AT is the area of mycelial growth of the treated colonies.

Germination test

The same media were also used for buckwheat grain germination tests (2% PDA with 0, 5, 10, or 15% of RH), to ensure that RH does not suppress the germination of the buckwheat grain. Fifteen buckwheat grains were placed on each Petri dish, evenly separated in-between in five replicates. The plates were incubated in plant growing chambers at 22 °C, 60% humidity, in the dark.

Data analysis

The reported results are expressed as mean ± standard error (SE) of at least five replicates. Our experimental data are presented in Supplementary 1. Statistical significance between groups (treatments) was determined using a one-way analysis of variance (ANOVA) with Duncan's post hoc test (Statistica StatSoft version 7). The significance level was considered at a p-value of less than 0.05, 0.01, or 0.001 as indicated in Fig.1.

Results

The inhibitory effects of rosemary hydrosol, presented as a portion of growth inhibition (%), were confirmed in five of seven fungi tested (Fig. 1). In contrast, *A. alternata*, showed no differences in growth at any RH concentration, whereas in *E. nigrum*, promotion of growth was observed at the highest RH concentration. The highest RH concentration successfully suppressed the growth of *A. infectoria* (by around 20%), and all tested *Fusarium* species, resulting in 35% to almost 80% inhibited growth. *Fusarium graminearum* showed the most susceptible to RH as already 5% RH inhibited its growth by 35%, and 10% RH resulted in almost 50% growth inhibition. 10% RH was also effective towards *F. oxysporum*, while the growth of *F. fujikuroi* SC and *F. sporotrichioides* was only inhibited after the highest (15%) RH.

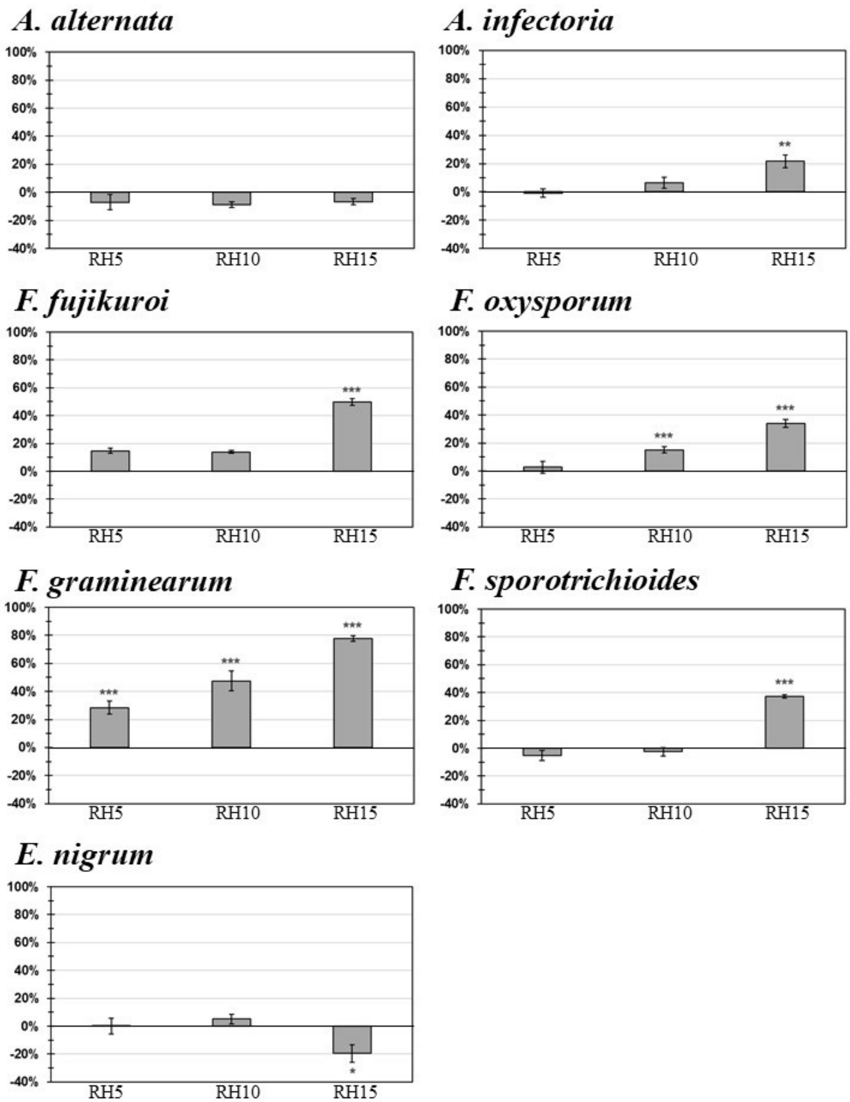

Figure 1: Relative growth inhibition of fungal mycelia (%) by 5%, 10% and 15% rosemary hydrosol (RH) compared to the control group of each fungus after a 7-day inhibition period (N=5). The positive values indicate growth inhibition compared to the control group, and the negative values represent growth stimulation. Asterisks indicate statistical differences from the control at P-value less than 0.05 (*), 0.01 (**) or 0.001 (***).

Slika 1: Relativna inhibicija rasti glivnega micelija (%) pri 5 %, 10 % in 15 % rožmarinovega hidrolata (RH) v primerjavi s kontrolno skupino posamezne glive po 7-dnevni inhibiciji (N = 5). Pozitivne vrednosti predstavljajo inhibicijo rasti v primerjavi s kontrolno skupino, negativne vrednosti pa stimulacijo rasti. Zvezdice ponazarjajo statistične razlike s kontrolo pri P-vrednosti manjši od 0,05 (*), 0,01 (**) ali 0,001 (***).

As expected, no statistically significant difference in buckwheat grain germination was observed after the treatments with selected concentrations of RH (Tab. 1).

Table 1: Germination of buckwheat grain (%) on the control and different rosemary hydrosol (RH) enriched media.
Tabela 1: Kalitev ajdovih zrn (%) na kontrolni plošči in na ploščah z dodanim rožmarinovim hidrolatom (RH).

Treatment	Grain germination (%)
Control	92.0 ± 2.5
5% RH	85.3 ± 2.5
10% RH	89.3 ± 2.7
15% RH	93.3 ± 2.1

Discussion

In the present study, we demonstrate the inhibitory potential of RH against five of the seven fungal strains isolated from grains of buckwheat, namely: *A. alternata*, *A. infectoria*, *E. nigrum*, *F. fujikuroi* SC, *F. graminearum*, *F. oxysporum* and *F. sporotrichioides*.

The only species where no differences in growth at any RH concentration were observed was *A. alternata.* To our best knowledge, there were no studies on RH efficiency against *A. alternata* performed until this date. However, Tabet Zatla et al. (2020) used hydrosol extract of *Marrubium vulgare* at a concentration 0.15 mL L^{-1}, and observed 77.3% of inhibition against *A. alternata*. This indicates that hydrosols can be effective in suppressing the growth of *A. alternata*. In contrast, *A. infectoria* was more susceptible, as around 20% growth inhibition was observed at the highest (15%) RH concentration. As for *A. alternata*, no data for RH against *A. infectoria* was published up to this point. However, some authors report high antifungal activity of EO from *Thymus capitatus,* also from the Lamiaceae family, against different fungi, including *A. infectoria* (Goudjil et al. 2020). In their research, *A. infectoria* was one of the most sensitive fungal species, as no growth was recorded for all concentrations of EO from 0.025% to 0.75%.

Fungi from the genus *Fusarium* are one of the most important mycotoxigenic species found in food and feed, as nearly all species can produce mycotoxins (Thrane 2014). In our research, we observed at least some inhibition rate in all tested *Fusarium* species.

The highest growth inhibition was found for *F. graminearum*, where already at 5% RH concentrations, growth was inhibited by almost 30%. Growth inhibition of *F. graminearum* increased with the increasing RH concentration, reaching around 50% at 10% RH concentration and nearly 80% inhibition of growth at 15% concentration of RH. As for *A. alternata* and *A. infectoria*, no data on RH hydrosols has been published yet. Previously, the antifungal effects of *Thymus vulgaris, Satureja hortensis, Anethum graveolens, Mentha sativa,* and *Capsicum annuum* EOs on the growth of *F. graminearum* and zearalenone production were reported (Hoseiniyeh Faraahani et al. 2012). The highest inhibition was observed by EO of *Thymus vulgaris* with 100% inhibition at 100 µL/100 mL. The antifungal activity of two EOs from *Eryngium triquetrum* and *Smyrnium olusatrum,* from western Algeria, were also tested against different fungal strains, including *F. graminearum* (Merad et al. 2021). Both species showed effective antifungal activity already at low concentrations (1,42*10-1 µL mL^{-1} of air). The antifungal activities of EO from *Eucalyptus camaldulensis* against *F. graminearum* were also reported with a complete mycelial growth arrest at 50 µL 20 mL^{-1} of PDA (Mehani et al. 2014). This indicates that *F. graminearum* can be inhibited with various EOs from different plant species. Our results demonstrate that it can also be effectively controlled with RH, which suggests that the effectiveness of other hydrosols from the above-mentioned species against *F. graminearum* remains to be explored.

In addition, we report quite good antifungal activity of RH against *F. oxysporum*, with nearly 20% inhibition rate at 10% RH, and almost 40% inhibition rate at 15% RH hydrosol, respectively. The antifungal effect of RH on *F. oxysporum* and some other fungal strains has been tested before (Boyraz and Özcan 2005). Interestingly, their results showed a lower inhibitory effect than ours, as they observed only 4% growth inhibition at 10% RH and 18% of inhibition at 15% RH. However, in contrast to our results, they observed some inhibitory effect already at 5% RH, where we found no differences in the growth of *F. oxysporum*. According to their research, where they comparatively tested different Lamiaceae hydrosols, RH has a lower effect than *Satureja hortensis* and a stronger effect than *Ocimum basilicum*. They also found that hydrosols from the Apiaceae family (*Cuminum cyminum, Echinophora tenuifolia*) inhibited the growth of fungi, including *F. oxysporum*, better than RH (Boyraz and Özcan 2005). The effects of some other hydrosols (*Melaleuca alternifolia*, *Ficus carica*, *Zingiber zerumbet*, *Citrus hystrix*) on *F. oxysporum* were also tested (Paramalingam et al. 2021). *M. alternifolia* and *C. hystrix* hydrosols exhibited significant inhibition potential against *F. oxysporum* at all concentrations (50%, 70% and 100%). At 50% concentration of *M. alternifolia* hydrosol the inhibition rate was around 45%. At 15% concentration of RH inhibition against *F. oxysporum* reached 35%. The highest inhibition rate of 70% was achieved at 100% concentration of *M. alternifolia* hydrosol. The growth of *F. fujikuroi* was only affected by the highest (15%) RH concentration. 5% and 10% RH concentrations caused no statistical difference in growth compared to the control group. However, 15% RH suppressed the growth of *F. fujikuroi* by almost 50% in our case. The efficiency of several compounds of EOs against *F. fujikuroi* in *Oryza sativa* was evaluated, and results confirmed the in vitro antifungal activity of tested compounds: specifically, thymol (0.025% vol/vol), terpinen-4-ol (0.1%), and eugenol (0.05%) (Mongiano et al. 2021). Both thymol and terpinen-4-ol are also found in rosemary EO (Angioni et al. 2004).

The same as for *F. fujikuroi*, the growth of *F. sporotrichioides* was also inhibited only in the case of 15% RH, where almost 40% inhibition was observed. EOs of *Bursera morelensis, Lippia graveolens* and *Mentha piperita* had a significant inhibitory effect on *F. sporotrichioides*, too. After 72 h of incubation at 23 °C, *B. morelensis* EO's minimal inhibitory concentration (MIC) was 0.27 $\mu L\ mL^{-1}$, and *L. graveolens* EO's MIC was 0.15 $\mu L\ mL^{-1}$. 4 $\mu L\ mL^{-1}$ of *B. morelensis* EO caused 72% inhibition of growth, while 4 $\mu L\ mL^{-1}$ *L. graveolens* EO inhibited the growth of *F. sporotrichioides* totally. These two EOs had a stronger effect on *F. sporotrichioides* than on two other *Fusarium* species (Rachitha et al. 2017, Medina Romero et al. 2021).

In contrast to *Fusarium* species, where at least some growth inhibition was observed at the highest RH concentration, promotion of growth was observed in the case of *E. nigrum* at the highest RH concentration. Saprophytic fungi, such as *E. nigrum* are found on freshly harvested grains but are usually not significant spoilage species (Hocking 2014). *Epicoccum nigrum* can display an endophytic lifestyle in a variety of plants and can be beneficial to its host plant, as it can increase the root system biomass and control pathogens (Fávaro et al. 2012). Therefore, lack of inhibition or even some level of growth promotion by RH, as in the case of *E. nigrum*, is a positive result. The antifungal activity of rosemary EO against *E. nigrum* and other fungi found on cultural heritage objects has already been tested before (Stupar et al. 2014). In their research, *E. nigrum* was found to be the most sensitive isolate, and the growth of this fungus was completely suppressed at a concentration of 10 $\mu L\ mL^{-1}$. This is contrary to our results obtained using RH, which, in our case, did not suppress the growth but even promoted it at the 15% concentration of RH. This is probably due to the fact that hydrosols contain lower concentrations of active ingredients that have a potential antifungal effect compared to EO, as they are more diluted (Inouye 2008). As *E. nigrum* is a plant endophyte with a possibly beneficial effect on its growth and development, this indicates the advantage of using RH rather than rosemary EO.

Overall, our results show at least some antifungal effect of RH to the majority of tested fungal strains. This is important from both an economic as well as environmental point of view. The majority of the previous studies were performed using plants from the Lamiaceae family, for example, *Lavandula* genus. In the case of *Lavandula × intermedia* (Emeric ex Loisel.), the industry of EOs is highly developed, causing a lot of wasted by-products, such as hydrosols. For example, only around 30 mL of EO can be extracted from 1 kg of *Lavandula × intermedia*, while the quantity of hydrosol from the same amount of plant material is around 830 mL (Politi et al. 2022). Hydrosols usually have similar chemical and sensory properties as EOs, but with a weaker overall effect, as they contain similar active substances but are more diluted due to higher water content (Hay 2015). Chemical compounds from hydrosols can also be partially recovered, the most common technique being re-distillation. With this, the process of extraction of EOs can be made more economically efficient and environmentally friendly (Rajeswara Rao 2012).

Politi et al. (2022) reported lower MIC of RH against some dermatophyte fungal strains than in case of *Salvia officinalis* L. and *Cupressus sempervirens* L. However, they also observed that RH effects more negatively on germination of *Lactuca sativa* seeds than *C. sempervirens* and *S. officinalis* hydrosol. This suppression of germination is caused by a higher content of monoterpenes, like 1,8-cineole, which is a root--growth inhibitor. A higher concentration of basil *Ocimum basilicum* hydrosol also caused a lower germination percentage of both *O. basilicum* and *Chenopodium quinoa* seeds (Çamlica et al. 2017). In contrast, we observed no negative effect of RH on the germination of *Fagopyrum esculentum* grains at any concentration. In our experiment, RH showed inhibitory effects against some phytopathogenic fungi (e.g. *Fusarium* species) but did not suppress germination of *F. esculentum* grains, indicating that it could be utilized as an alternative and environmentally friendly agent for decontamination of *F. esculentum* grains.

Conclusion

Results of our study show that RH inhibits the growth of some fungal pathogens isolated from buckwheat grains. We observed the most significant inhibitory effect in *Fusarium graminearum*, as already the lowest concentration (5%) of RH reduced its growth by around 30% compared to the control group. The highest concentration of RH (15%) inhibited the growth of all fungi from the genus *Fusarium* species by 35-80%, depending on the species. Some inhibitory effect was also observed for *Alternaria infectoria* (20%) at the highest RH concentration, while the growth of *A. alternata* was not affected by the hydrosol. On the contrary, the growth of the endophytic fungus *Epicoccum nigrum* was even stimulated by the highest concentration of RH. There was no inhibitory effect on the germination of buckwheat grain at either hydrosol concentration, which indicates that rosemary hydrosol could potentially be utilized as an alternative agent for suppressing fungal growth on grains.

Povzetek

Hidrolati so stranski produkt proizvodnje eteričnih olj. V primerjavi z eteričnimi olji je njihov izkupiček pri ekstrakciji mnogo večji, imajo vodno osnovo, njihova sestava je podobna sestavi eteričnih olj, le da so bistveno manj koncentrirani. Posledično so tudi njihovi učinki in delovanje do neke mere primerljivi eteričnim oljem. Zaradi vsega tega predstavljajo potencialno alternativo uporabi eteričnih olj, ki imajo znano protimikrobno, insekticidno in citotoksično delovanje. Tako bi lahko hidrolati predstavljali količinsko kot tudi finančno dostopnejšo alternativno obliko pesticidov.

V naši raziskavi smo preverjali potencialno protiglivno delovanje rožmarinovega *(Rosmarinus officinalis* L.) hidrolata na rast izbranih vrst gliv, izoliranih iz ajdovih (*Fagopyrum* sp.) zrn. Testirali smo rast štirih vrst gliv iz rodu *Fusarium* (*F. fujikuroi* SC, *F. graminearum, F. oxysporum* in *F. sporotrichioides*), 2 vrst iz rodu *Alternaria* (*A. alternata* in *A. infectoria*) ter vrsto *Epicoccum nigrum*. Enako velike koščke (5x5 mm) svežih enotedenskih kultur gliv smo nacepili na 2 % gojišča PDA z dodanim antibiotikom kloramfenikolom (50 mg L^{-1}) ter dodali ustrezno količino hidrolata, tako da smo dobili 0 (kontrola), 5, 10 in 15 % koncentracije rožmarinovega hidrolata (RH). Po sedmih dneh smo s pomočjo računalniškega programa izmerili in izračunali obseg glivne rasti ter tega izrazili v deležu inhibicije v primerjavi z rastjo izbrane glive na kontrolnih

ploščah. Izkazalo se je, da RH, vsaj pri najvišji koncentraciji, učinkovito zavira rast gliv vseh gliv iz rodu *Fusarium*, najbolj učinkovito pa vrsto *F. graminearum*, kjer smo že pri najnižji koncentraciji RH (5 %) opazili približno 30 %-inhibicijo rasti, pri 10 % RH pa je bila ta že približno 50 %. Ob izpostavitvi najvišji koncentraciji RH (15 %) smo opazili skoraj 80 %-inhibicijo rasti. Glive rodu *Fusarium* so pomembni fitopatogeni, ki povzročajo mnoge rastlinske bolezni, prav tako pa so praktično vse vrste tega rodu sposobne produkcije mikotoksinov, ki so nevarni za zdravje ljudi in živali. Zato je preprečevanje njihove rasti na semenih in prehranskih izdelkih ključno za zagotavljanje varne in kakovostne rastlinske pridelave. RH je manj zavrl rast vrst iz rodu *Alternaria*, saj smo rahlo inhibicijo rasti (okoli 20 %) opazili le pri 15 % RH pri vrsti *A. infectoria*, na rast glive *A. alternata* pa RH pri nobeni koncentraciji ni imel vpliva. Zanimivo pa je bila rast glive *E. nigrum* na gojišču s 15 % RH celo nekoliko stimulirana, saj je bila opažena približno 20 % boljša rast v primerjavi s kontrolno skupino. Ta vrsta je sicer saprofitska in pogosto opažena kot endofitska gliva pri mnogih rastlinskih vrstah (tudi pri ajdi) ter ima lahko celo pozitivno delovanje na rast rastlin ter jih ščiti pred drugimi patogenimi organizmi.

Testirali smo tudi vpliv različnih koncentracij RH na kalitev zrn navadne ajde. Pri tem nismo opazili statističnih razlik med kontrolno skupino in zrni, ki so kalila pri različnih koncentracijah RH. Rezultati naše raziskave tako nakazujejo na možno uporabo RH kot alternativnega sredstva za zaviranje glivnih okužb na zrnju ajde.

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References

Angioni, A., Barra, A., Cereti, E., Barile, D., Coïsson, J.D., Arlorio, M., Dessi, S., Coroneo, V., Cabras P., 2004. Chemical composition, plant genetic differences, antimicrobial and antifungal activity investigation of the essential oil of *Rosmarinus officinalis* L. Journal of Agricultural and Food Chemistry, 52 (11), 3530–3535.

Anžlovar, S.; Janeš, D.; Koce, J.D. The Effect of Extracts and Essential Oil from Invasive Solidago spp. and Fallopia japonica on Crop-Borne Fungi and Wheat Germination. Food Technology and Biotechnology, 2020, 58, 273.

Boyraz, N., Özcan, M., 2005. Antifungal effect of some spice hydrosols. Fitoterapia, 76 (7), 661–665.

Çamlica, M., Yaldiz, G., Özen, F., 2017. Effects of Different Basil Hydrosol Doses on the Germination and Shoot and Root Lengths of Basil (*Ocimum basilicum*) and Quinoa (*Chenopodium quinoa*) Seeds. Indian Journal of Pharmaceutical Education and Research, 51 (3), S253–S257.

Christensen, C.M., 1957. Deterioration of stored grains by fungi. The Botanical Review, 23 (2), 108–134.

Di Vito, M., Grazia Bellardi, M., Mondello, F., Modesto, M., Michelozzi, M., Bugli, F., Sanguinetti, M., Sclocchi, M.C., Sebastiani, M.L., Biffi, S., Barbanti, L., Mattarelli, P., 2019. *Monarda citriodora* hydrolate vs essential oil comparison in several anti-microbial applications. Industrial Crops and Products, 128, 206–212.

Faleiro, L., Miguel, G.M., Guerrero, C.A.C., Brito, J.M.C., 1999. Antimicrobial activity of essential oils of *Rosmarinus officinalis* L., *Thymus mastichina* (L.) L. ssp. *mastichina* and *Thymus albicans* Hofmanns & Link. Acta Horticulturae, 501, 45–48.

Fávaro, L.C., Sebastianes, F.L., Araújo, W.L., 2012. *Epicoccum nigrum* P16, a sugarcane endophyte,

produces antifungal compounds and induces root growth. PLoS One, 7 (6), e36826.
Glamočlija, Đ., Glamočlija, M., Cvijanović, G., 2011. Heljda, Monografija, Poljoprivredni fakultet, Beograd.
Goudjil. M.B., Zighmi, S., Hamada, D., Mahcene, Z., Bencheikh, S.E., Ladjel S., 2020, Biological activities of essential oils extracted from *Thymus capitatus* (Lamiaceae). South African Journal of Botany, 128, 274–282.
Halloin, J. M. 1983. Deterioration Resistance Mechanisms in Seeds. Phytopathology, 73, 335–339.
Hay, Y.O., Sierra, M.A.A., Tellez, M., Sequeda C, L.G., Tellez A, A.N., Bonnafous, C., Raynaud, C., 2015. Phytochemical, antioxidant and antimicrobial parameters of essential oils and hydrosols of Colombian Thyme and Rosemary obtained using two different steam distillation methods. International Journal of Phytocosmetics and Natural Ingredients, 2 (1), 7–7.
Hocking, A.D., 2014. Spoilage Problems - Problems Caused by Fungi. In: Batt, C., Patel, P. (eds.): Encyclopedia of Food Microbiology, 2nd ed. Academic Press, Amsterdam, pp. 471–481.
Hoseiniyeh Faraahani, S., Mirabolfathy, M., Rezaie Danesh, Y., Karami Osboo, R., 2012. Effect of five essential oils on zearalenon production and growth of *Fusarium graminearum*. Applied Entomology and Phytopathology, 80, 81–94.
Inouye, S., Takahashi, M., Abe, S., 2008. A comparative study on the composition of forty four hydrosols and their essential oils. International Journal of Essential Oil Therapeutics, 3, 89–104.
Kovačec, E., Likar, M., Regvar, M., 2016. Temporal changes in fungal communities from buckwheat seeds and their effects on seed germination and seedling secondary metabolism. Fungal Biology, 120, 666–678.
Mahmood, I., Imadi, S.R., Shazadi, K., Gul, A., Hakeem, K.R., 2016. Effects of Pesticides on Environment. In: K.R. Hakeem et al. (eds.): Plant, Soil and Microbes. Springer International, Switzerland, pp. 253–269.
Medina Romero, Y., Hernandez Hernandez, A., Rodriguez Monroy, M., Canales Martínez, M., 2021. Essential oils of *Bursera morelensis* and *Lippia graveolens* for the development of a new biopesticides in postharvest control. Scientific Reports, 11 (1), 20135.
Mehani, M., Ladjel, S., 2014. Biological Activity of Essential Oil of *Eucalyptus camendulensis* on Some Fungi and Bacteria. Int. Journal of Engineering Research and Applications, 4 (7), 71–73.
Merad, N., Andreu, V., Chaib, S., de Carvalho Augusto, R., Duval, D., Bertrand, C., Boumghar, Y., Pichette, A., Djabou, N., 2021. Essential Oils from Two Apiaceae Species as Potential Agents in Organic Crops Protection. Antibiotics, 10, 636.
Milevoj, L., 1989. Buckwheat diseases. In: Kreft, I. (ed.): Fagopyrum (Buckwheat Newsletter). Biotehniška Fakulteta, Ljubljana, pp. 31–40.
Mongiano, G., Zampieri, E., Morcia, C., Titone, P., Volante, A., Terzi, V., Tamborini, L., Valé, G., Monaco, S. 2021. Application of plant-derived bioactive compounds as seed treatments to manage the rice pathogen *Fusarium fujikuroi*. Crop Protection, 148, 105739.
Mravlje, J., Regvar, M., Starič, P., Mozetič, M., Vogel Mikuš, K., 2021. Cold plasma affects germination and fungal community structure of buckwheat seeds. Plants, 10 (5), 851.
Nieto, G., Ros, G., Castillo, J., 2018. Antioxidant and Antimicrobial Properties of Rosemary (*Rosmarinus officinalis* L.): A Review. Medicines, 5 (3), 98.
Paramalingam, P., Kheiril Anuar, M.S., Akmal Baharum, N., Abdullah, J.O., Abd Aziz, J., Saidi, N.B., 2021. In vitro evaluation of antifungal activity of selected malaysian plants against the wilt pathogen of banana, *Fusarium oxysporum* f.sp. *cubense* tropical race 4. Malaysian Journal of Science, 40 (2), 16–24.
Peng, Y., Li, S.J., Yan, J., Tang, Y., Cheng, J.P., Gao, A.J., Yao, X., Ruan, J.J., Xu, B.L., 2021. Research Progress on Phytopathogenic Fungi and Their Role as Biocontrol Agents. Frontiers in Microbiology, 12, 670135.
Politi, M., Ferrante, C., Menghini, L., Angelini, P., Flores, G.A., Muscatello, B., Braca, A., De Leo,

M., 2022. Hydrosols from *Rosmarinus officinalis*, *Salvia officinalis*, and *Cupressus sempervirens*. Phytochemical Analysis and Bioactivity Evaluation. Plants, 11 (3), 349.
Popović, V., Sikora, V., Berenji, J., Glamočlija, Đ., Marić, V., 2013a. Effect of agroecological factors on buckwheat yield in conventional and organic cropping systems. Zbornik naučnih radova Instituta of PKB Agroeconomik, 19 (1-2), 155–165.
Popović, V., Sikora, V., Ikanovic, J., Rajičič, V., Maksimović, L., Mickovski Stefanovic, V., Katanski, S., 2013b. Production, productivity and quality of buckwheat in organic growing systems with order of environmental protection. In Environment Protection of Urban and Suburban Settlement: Proceedings: 17th International Eco-Conference XVII Eco-Conference, Novi Sad, pp. 395-404.
Popović, V., Sikora, V., Berenji, J., Filipović, V., Dolijanović, Ž., Ikanović, J., Dončić, D., 2014. Analysis of buckwheat production in the world and Serbia. Ekon. Poljopr., 61, 53–62.
Rachitha, P., Krupashree, K., Jayashree, G.V., Gopalan, N., Khanum, F., 2017. Growth Inhibition and Morphological Alteration of *Fusarium sporotrichioides* by *Mentha piperita* Essential Oil. Pharmacognosy Res., 9 (1), 74–79.
Rajeswara Rao, B.R., 2012. Hydrosols and water-soluble essential oils of aromatic plants: Future economic products. Indian Perfumer, 56, 29–33.
Rajeswara Rao, B.R., 2013. Hydrosols and Water-Soluble Essential Oils: Their Medicinal and Biological Properties. In: Govil, J. N., Bhattacharya, S. (eds.): Recent Progress in Medicinal Plants: Essential Oils I, 1st ed. Studium Press LLC, Houston, pp. 119–140.
Schoss, K., Kočevar Glavač, N., Dolenc Koce, J., Anžlovar, S. 2022. Supercritical CO_2 Plant Extracts Show Antifungal Activities against Crop-Borne Fungi. Molecules, 27, 1132.
Stojiljkovic, J., Trajchev, M., Nakov, D., Petrovska, M., 2018. Antibacterial activities of rosemary essential oils and their components against pathogenic bacteria. Advances in Cytology and Pathology, 3, 93–96.
Stupar, M., Grbić, M. L., Džamić, A., Unković, N., Ristić, M., Jelikić, A., Vukojević, J., 2014. Antifungal activity of selected essential oils and biocide benzalkonium chloride against the fungi isolated from cultural heritage objects. South African Journal of Botany, 93, 118–124.
Tabet Zatla, A., Mami, I. , Dib, M.E.A., Sifi, M.E.A., 2020. Efficacy of Essential Oil and Hydrosol Extract of *Marrubium vulgare* on Fungi Responsible for Apples Rot. Anti-Infective Agents, 18, 285–293.
Taglienti, A., Donati, L., Ferretti, L., Tomassoli, L., Sapienza, F., Sabatino, M., Di Massimo, G., Fiorentino, S., Vecchiarelli, V., Nota, P., Ragno, R., 2022. In vivo Antiphytoviral Activity of Essential Oils and Hydrosols From *Origanum vulgare*, *Thymus vulgaris*, and *Rosmarinus officinalis* to Control Zucchini Yellow Mosaic Virus and Tomato Leaf Curl New Delhi Virus in *Cucurbita pepo* L. Frontiers in Microbiology 13, 840893.
Thrane, U., 2014. Fusarium. In: Batt, C. A., Tortorello, M. L. (eds.): Encyclopedia of Food Microbiology, 2nd ed. Academic Press, Amsterdam, pp. 76–81.
Yoon, M.Y., Cha, B., Kim, J.C., 2013. Recent trends in studies on botanical fungicides in agriculture. The Plant Pathology Journal, 29 (1), 1–9.

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Microlitter measurement in fish *Rutilus rutilus* from the Slovenian part of the Mura river basin

Meritve mikroodpadkov v ribah rdečeokah (*Rutilus rutilus*) v porečju reke Mure na območju Slovenije

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Abstract: Knowledge of the impacts of microlitter pollution on the freshwater environment is still less researched when compared to that of marine environments despite rivers being the main pathway for transport of microlitter pollution to the seas and oceans. To better understand the state of pollution with microplastics in Slovenian freshwater fish, we did the first study of microlitter in freshwater fish, in which 50 specimens of common roach (*Rutilus rutilus*) caught in the Slovenian part of the Mura river basin were examined for its presence. The gastrointestinal tract was dissected from each specimen and degraded with 10% KOH. Filtered samples were then checked for microlitter using a stereomicroscope and ATR-FTIR spectroscopy. Microlitter was separated into microplastic particles (0.3 – 5 mm) and textile fibres, which can be of synthetic or seminatural origin. Microlitter was found in 94% of specimens, with an average concentration of 5 ± 3 items/specimen. Colourful fragments and textile fibres were found. Fibres were the predominant form (96%) and indicated households as the main source of microlitter in the Mura river. A strong positive correlation between the number of microlitter and the weight of the fish was found ($R^2 = 0.70$). In the future, simultaneous monitoring of microlitter in sediments, water, and fish would be necessary to assess whether *Rutilus rutilus* is an appropriate species for biomonitoring. Given the growing evidence of the negative effects of microlitter on organisms, it will be important to carry out biomonitoring in terms of assessing environmental status and conditions for human health.

Keywords: fish, microlitter, microplastics, river ecosystem, *Rutilus rutilus*

Izvleček: Vplivi onesnaženja z mikroodpadki v celinskih vodah so še vedno zanemarljivo raziskani v primerjavi s tistimi v morskem okolju, čeprav so reke glavni vir onesnaževanja oceanov z mikroplastičnimi odpadki. Z namenom, da bi bolje razumeli stanje onesnaženosti z mikroodpadki v sladkovodnih ribah v Sloveniji, smo opravili prvo študijo mikroodpadkov v sladkovodnih ribah. Pregledali smo 50 vzorcev rib redečeok (*Rutilus rutilus*) iz slovenskega dela porečja Mure. Vsakemu osebku smo odstranili drobovje in ga razgradili v 10% KOH raztopini. Filtrirane vzorce smo nato pregledali pod stereo-mikroskopom in s pomočjo ATR-FTIR spektrometrije. Mikroodpadke smo ločili na mikroplastične delce (0,3 – 5 mm) in tekstilna vlakna, ki so lahko sintetičnega ali seminaravnega izvora. Mikroodpadke smo našli v kar 94 % vzorcev s povprečno koncentracijo 5 ± 3 delcev/vzorec. Najdeni so bili fragmenti različnih barv in tekstilna vlakna. Vlakna so močno prevladovala (96 %), kar nakazuje, da so glavni vir mikroodpadkov v reki Muri gospodinjstva. Ugotovili smo izrazito korelacijo med številom mikroodpadkov in težo rib (R^2 = 0,70). V prihodnosti bi bilo smiselno sočasno spremljati mikroplastiko v sedimentih, vodi in ribah, da bi ocenili, ali je *Rutilus rutilus* ustrezna ribja vrsta za izvajanje biomonitoringa mikroodpadkov. Glede na naraščajoče število dokazov o negativnih učinkih mikroplastike na žive organizme bo potrebno izvajati biomonitoring tako z vidika ocenjevanja okoljskega stanja kot tudi s stališča varne hrane in s tem vpliva na zdravje ljudi.

Ključne besede: mikroodpadki, mikroplastika, rečni ekosistem, ribe, *Rutilus rutilus*

Introduction

The production of plastic has increased from 1.5 million tons in 1950 to 367 million tons in 2020 (Plastic Europe 2021). It is becoming an ever-increasing environmental threat; however, most people are unaware of the problem hidden from our eyes, microplastic (MP) particles (plastic particles 1 µm – 5 mm in size) that have polluted the entire planet, from its deepest points to its highest summits. They have been found in the Marianas Trench (Peng et al. 2018) as well as on Mount Everest (Napper et al. 2020). In recent years, several studies have been conducted to determine the amount of microplastics in the environment, but primarily in the seas and oceans. As 80% of all plastic litter in the oceans comes from rivers (Jambeck et al. 2015), research in freshwater is equally or even more important.

Before August 2021, only 17 studies about the abundance of microplastics in freshwater fish species had been published from Europe (12 countries) and another 43 from other parts of the world (17 countries). Altogether, MPs were discovered in 199 wild freshwater fish (Galafassi et al. 2021) from 29 countries. The first evidence of microplastics presence in European freshwater fish was published in 2014 by Sanchez et al. in the species *Gobio gobio* from French rivers; MPs were documented in 12% of sampled fish. A relatively low plastic prevalence of 18.8%, with significant differences between rivers (20.6%) and lakes (16.5%) was reported from the federal state of Baden-Württemberg, Germany (Roch et al. 2019). In <10% of studies, the percentage of fish that contained MPs was <20% (Galafassi et al. 2021). In most other subsequent studies, MP abundance in fish samples was even higher; in the river Thames (UK), 33% of fish samples contained microplastics (Horton et al. 2018), in the Chi river, Thailand, 72.9% (Kasamesiri and Thaimuangphol 2020), in the Nile river, Egypt, >75% (Khan et al. 2020), and in the Widawa river, Poland, 54% (Kuśmierek and Popiolek 2020). More than 15% of studies reports >90% of fish guts are contaminated by MPs (Galafassi et al. 2021), with some of them even up to 100%, as in Evergreen Lake and Lake Bloomington, Illinois, USA (Hurt 2020), Lake Taihu, China (Jabeen et al. 2017), and in the Rio de la Plata estuary, Argentina (Pazos et al. 2017). The average MP concentration per fish specimen was similar among studies and ranged between 0–4 items/individual, with a maximum observation from 6–30 items/individual. Most studies (80%; Galafassi et al. 2021) reported that MPs in the form of fibres dominated in >50% per sample (Pazos et al. 2017, Silva-Cavalcanti et al. 2017, Horton et al. 2018, Su et al. 2018, Kasamesiri and Thaimuangphol 2020, Khan et al. 2020, Wang et al. 2020, Yuan et al. 2019). Rare studies reported fragments as the most abundant type of MPs in their fish samples (Garcia et al. 2021, Sun et al. 2021). Overall, polyethylene terephthalate (PET), polyethylene (PE), and polypropylene (PP) were the prevalent types of MPs found in freshwater fish (Horton et al. 2018, Garcia et al. 2021, Khan et al. 2020).

The average MPs concentration in fish is closely related to the state of pollution of habitat (Peters et al. 2016, Horton et al. 2018, Garcia et al. 2021), gastrointestinal tract structure (Jabeen et al. 2017), and species' feeding characteristics (McNeish et al. 2018). Fish consumed more microplastics in the urbanised sections of the river (Silva-Cavalcanti et al. 2017), where domestic sewage is the major source of MPs (Yuan et al. 2019), primarily coming from laundry. In fishing areas, fishing equipment might be the main source of microplastics (Kasamesiri and Thaimuangphol 2020). Omnivorous fish are more exposed to MPs when feeding and the percentage of MP occurrence is higher than in carnivorous species (Kasamesiri and Thaimuangphol 2020, Wang et al. 2020, Zhang et al. 2021), suggesting that particle uptake is more accidental (Roch et al. 2019) and less associated with transmission among trophic levels (Göven et al. 2017). Most particles in fish are found in the stomach and gill tissues, fewer in the edible flesh tissue (Garcia et al. 2021).

The goal of this study was to determine the amount of microlitter (ML) particles in common roach (*Rutilus rutilus*) specimens caught in the Slovenian part of the Mura river basin. This is the first study of ML abundance in freshwater fish in Slovenia. We hypothesized that common roach is common enough in the river systems and have adequate habitat and eating habits to be able to perform microplastic biomonitoring. It is a sentinel species for assessing endocrine disrupting chemicals (PCB and metals) and is widespread and ecologically important in lowland rivers throughout Eurasia (Southam et al. 2011). The results of previous research on the common roach have shown a link between microplastic abundance and anthropogenic pressures. The study was conducted as a preliminary study with a view to developing methods for conducting biomonitoring of ML in the future when ML will be considered also in the European Water Framework Directive (WFD).

Material and methods

Sampling

A sampling of the freshwater fish the common roach (*Rutilus rutilus*) was conducted in the Slovenian part of the Mura river in October 2020. The common roach was chosen because it is not endangered, is easy to catch, and is present in both standing and running inland waters. The Mura river is around 464 kilometres long and it flows through three countries in Central Europe (Austria, Slovenia, and the Croatia/Hungary border). Its basin covers an area of 13,800 km^2 (Ostroški 2015). Fish were caught at five sampling locations, with the same bait (white worm), considering the proximity of urban centres and industrial and municipal outflows. Three locations were on the Mura river (S1, Sladki Vrh; S3, Hrastje–Mota; S5, Krapje) and two were artificial lakes (S2, Lake Stara Jama in Zgornje Konjišče; S4, Lake Gajševci) (Fig. 1). This provides data on the amount of microplastics in fish from running and standing waters. Lakes are still a part of the Mura basin, as the water from Lake Gajševci flows into the Mura and Lake Stara Jama is right next to the river and has the same groundwater.

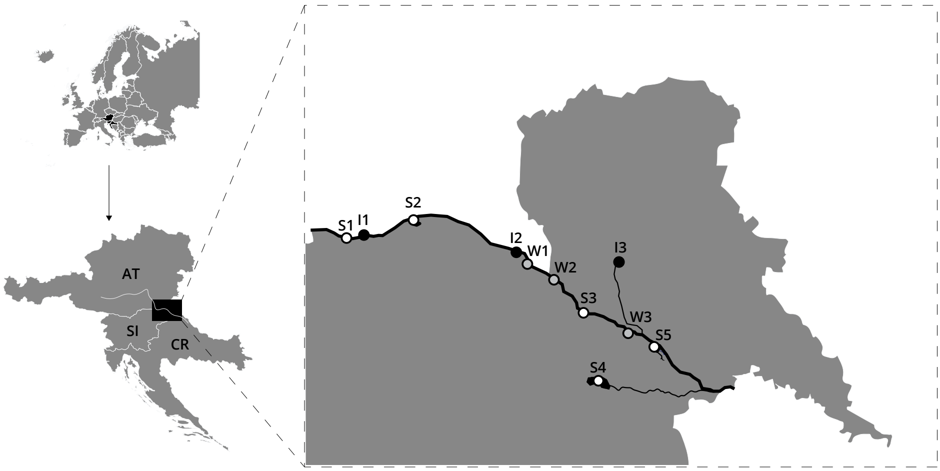


Figure 1: Sampling locations of fish samples, three on the Mura river (S1, Sladki Vrh; S3, Hrastje-Mota; S5, Krapje) and two on the lakes of the Mura basin (S2, Lake Stara Jama in Zgornje Konjišče; S4, Lake Gajševci). The map shows outflows from the WWTPs (W1 – W3) and locations of industrial centres that could have an impact on plastic pollution in the Mura basin (I1 – I3).

Slika 1: Lokacije vzorčenja rib, tri na reki Muri (S1, Sladki Vrh; S3, Hrastje-Mota; S5, Krapje) in dve na jezerih (S2, Jezero Stara Jama v Zgornjem Konjišču; S4, Jezero Gajševci) v porečju reke Mure. Zemljevid prikazuje tudi iztoke čistilnih naprav (W1 – W3) in lokacije industrijskih središč, ki imajo potencialni vpliv na onesnaževanje s plastiko v porečju reke Mure (I1 – I3).

Sample processing

Altogether, 50 specimens were analysed for microplastics (at each location, ten specimens were caught). At first, each fish was measured in length (cm) and weighed (g) with a precision scale (Kern 6W; d = 0.01), then the gastrointestinal tract (GIT) was removed. The fish were cut open along the abdominal region with a scalpel. The GIT was carefully removed from the oesophagus to anus and placed in pre-cleaned glass Petri dishes. GIT was weighed with a precision scale (Kern 6W; d = 0.01) and frozen (-20 °C) immediately after dissection of the fish. When we start with the degradation process, the GIT was thawed, a 10% KOH solution was added at the ratio of 1:1 of the GIT's weight and then incubated at room temperature for 72 hours. After 72 hours, the decomposed contents of the GIT samples were filtered with a Buechner funnel. Cellulose filters (pore size 12 µm) were used. The filters were washed thoroughly with distilled water to rinse off as much KOH as possible. After the filtration was completed, the filters were carefully transferred into clean Petri dishes (checked under a stereomicroscope for MPs before use) with tweezers. The Petri dishes were marked accordingly.

To prevent contamination, work surfaces were thoroughly cleaned. Gloves, face masks, and cotton lab coats were worn throughout the work in the laboratory. All utensils were cleaned and washed with distilled water before use. Other people were forbidden to enter the room during the work, windows were closed, and the air cleaner (Ideal AP30) was turned on. At the same time, procedural blanks were performed, and field blanks were collected. The data was corrected according to the contamination levels found during laboratory analysis.

Petri dishes with filters were examined under a stereomicroscope (Leica ES2; binocular; 10x and 30x magnification) for microlitter 0.3 – 5 mm in size, using a spatula and precision forceps. Microlitter particles were divided into six microplastic categories: fragments, films, foams, fibres, pellets, and granules (Kovač Viršek et al. 2016). Fibres were additionally divided into synthetic textile fibres (polyester and polyamide) and seminatural or natural-based textile fibres (cotton). Synthetic and seminatural fibres were in the first phase distinguished with microscopy (Stanton et al. 2019). In addition, the chemical composition of all fragments and 10% of fibres were identified using the Fourier transform infrared spectrometry method (Spectrum Two, Perkin Elmer), which provides information on the chemical bonds of the particles >0.3 mm in size. Carbon-based polymeric materials are easily analysed using this method, as different chemical bonds have specific vibrational characteristics in the absorption of infrared light, causing them to emit specific spectra that separate plastic from other organic and inorganic particles. By comparing the spectra of the samples with the spectra in the library of polymer spectra (Hummel spectra library), certain types of polymers were identified. To make a positive polymer identification, only matches of >70% similarity to the reference library samples were accepted, according to Frias et al. (2016). Before FTIR, each particle was photographed using a stereomicroscope with a camera (Stereo Discovery V8, software AxioVision SE64 Rel. 4.9.1) and measured lengthwise.

Statistical analysis

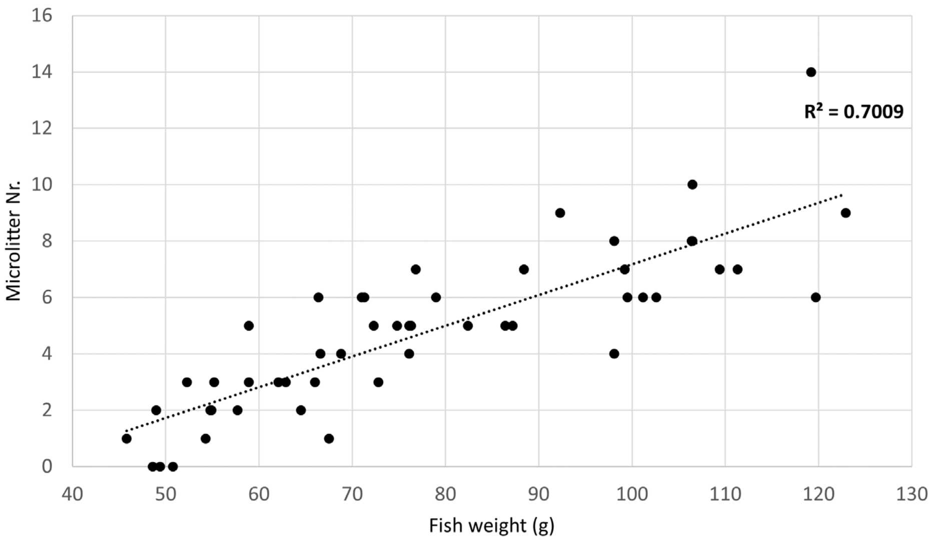
Statistical data analysis was performed using IBM SPSS Statistics 21. Kolmogorov-Smirnov test, Shapiro-Wilk test, Kruskal-Wallis test, Mann-Whitney U test, Pearson correlation and Spearman´s correlation were used.

Results and discussion

Fifty specimens of common roach (*Rutilus rutilus*) from 5 sampling locations on the Mura river were analysed for the presence of ML. The caught fish weighed between 45.8 and 122.9 g and were between 16 and 22 cm in length. ML was found in all sampling locations, in a total of 47 (94%) specimens.

A strong positive correlation was found when ML abundance (in Nr.) was compared with total fish weight ($R^2 = 0.70$; Pearson coefficient: 0.837; Spearman coefficient: 0.874) and moderate positive correlation when ML abundance was compared with fish length ($R^2 = 0.6$; Pearson coefficient: 0.781; Spearman coefficient: 0.816) (Fig. 2). Our study fully supports the results of the study by Horton et al. (2018), where the actual quantity of microplastics in the gut correlated with the size of fish. A positive linear relationship between the number of microplastic particles and the body size was found also in the fish *Neogobius melanostomus* caught in 3 major tributaries of Lake Michigan, USA (McNeish et al. 2018), while this was not found in the marine fish species (Jovanović 2017, Sun et al. 2019). Older fish are, in principle, larger, and a high number of MPs can be associated with a greater frequency of feeding and, as such, a greater chance of ingesting MPs either accidentally directly from the water or indirectly from another contaminated organism. While the whole GIT was degraded, including the wall of the digestive system, the MP particles that accumulated in the GIT tissue should be analysed as well. The particles from our study were large (>300 μm) and, as such, they could not penetrate the other tissue. It is known that fish are even more contaminated with smaller particles, which are hard to find and analyse (Roch et al. 2019).

A



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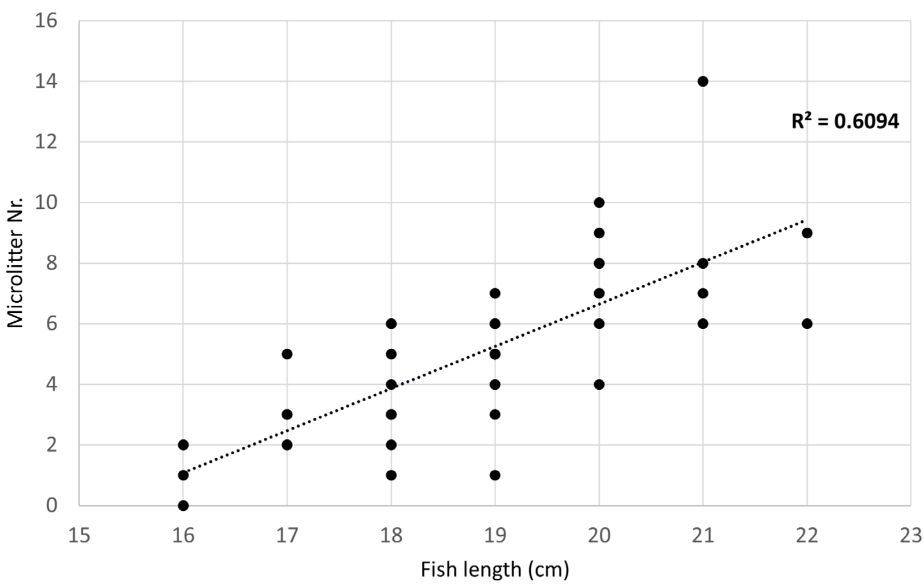

Figure 2: Microlitter correlation with fish weight (**A**) ($R^2 = 0.70$) and fish length (**B**) ($R^2 = 0.61$) for the fish common roach (*Rutilus rutilus*) caught in Mura river basin (Slovenia).

Slika 2: Korelacija mikroodpadkov z maso ribe (**A**) ($R^2 = 0{,}70$) in dolžino ribe (**B**) ($R^2 = 0{,}61$) za ribo rdečeoko (*Rutilus rutilus*), ulovljeno v porečju reke Mure (Slovenija).

The mean ML concentration among all sampling locations was 5 ± 3 items/specimen. The average ML concentration was the lowest at the first sampling location (S1: 3.8 ± 2.2 items/specimen) and the highest at the last sampling location (S5: 5.8 ± 3.5 items/specimen) (Fig. 3). The results from the riverine sampling sites show an increase in ML presence in fish downstream (total MPs S1: 38, S3: 43, S5: 58), but with no significant difference among all sampling locations (Kruskal - Wallis test, $p = 0.54$) and also among the first (S1) and the last (S5) sampling location (Mann - Whitney test, $p = 0.28$). The average ML concentration in the fish samples from the riverine system was 4 ± 2.5 items/specimen, while the average ML concentration from the two lakes (S2 and S4) was higher (5 ± 3.3 items/specimen), but with no statistically significant difference (Mann - Whitney test, $P = 0.479$). Statistical significance in our study was not reached most probably due to too short distances among sampling sites and small differences in land use. Otherwise, it is known that the maximum number of ingested microplastic particles for individual fish can strongly correlate to exposure (Horton et al. 2018), which is probably high in the lower reaches of the river due to agricultural and urban activities.

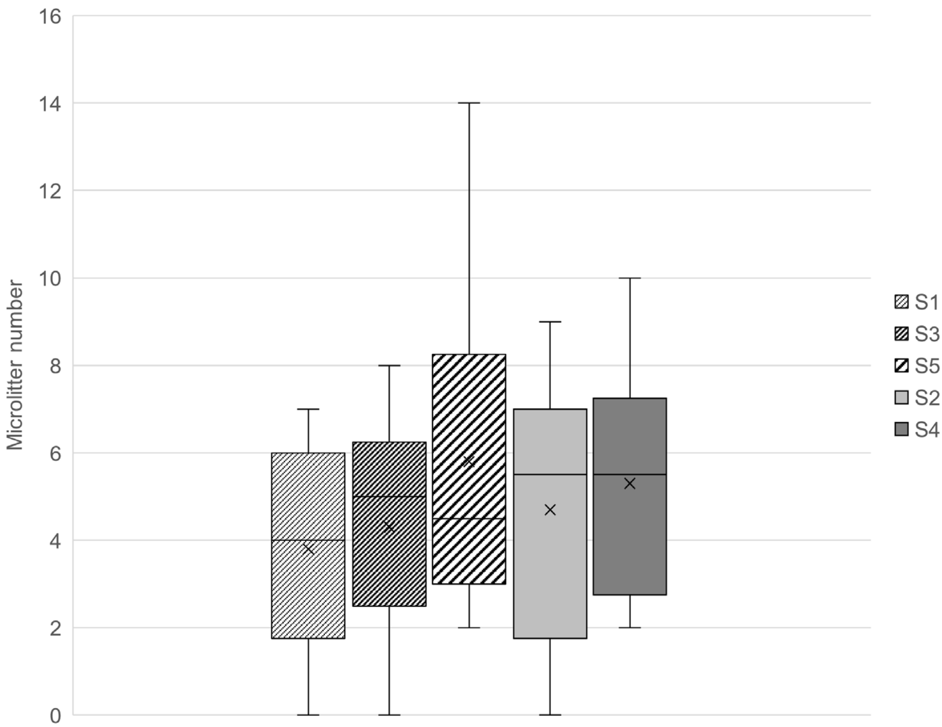

Figure 3: Boxplot for microlitter (ML) found in the fish gastrointestinal tract of common roach (*Rutilus rutilus*) from each sampling location (riverine sampling sites: S1, S3, S5; lake sampling sites: S2 and S4) in Mura river basin (Slovenia). Marks: error bars, the min and max number of ML particles found in fish; x, the average concentration of ML in fish.

Slika 3: Boxplot diagram za mikroodpadke, ki smo jih našli v prebavnem traktu rdečeok (*Rutilus rutilus*) z vsake vzorčne lokacije (rečna vzorčna mesta: S1, S3, S5; jezerska vzorčna mesta: S2 in S4) v porečju reke Mure (Slovenija). Oznake: odkloni, najmanjše in največje število delcev mikroodpadkov, ki smo jih našli v ribah; x, povprečna koncentracija mikroodpadkov v ribah.

Microplastic concentration in the fish species *Rutilus rutilus* was also investigated in the river Thames (UK) (Horton et al. 2018), rivers and lakes in the federal state of Baden-Württemberg (Germany) (Roch et al. 2019), and Widawa river, Poland (Kuśmierek and Popiolek 2020). The mean ML concentration found in *Rutilus rutilus* in our study was 4.2 and 7.2 times higher than the mean concentration from the Widawa and Thames, respectively. As the methodology for sample analysis differs among studies, this comparison does not necessarily reflect the true state. Detection of ML under a stereomicroscope is subjective and dependent on the experience of the expert. The most numerous mistakes are made in the determination of fibres, which are not addressed in all studies and where it is hard to distinguish whether they are of natural or synthetic origin. To some extent, it is possible to distinguish between them with a stereomicroscope, however, chemical analysis by FTIR is crucial to get proper results. Altogether around 50% (Galafassi et al. 2021) of studies did not use any chemical analysis of microparticles, which can lead to over- or underestimation of the concentration of ML.

So far, no study on the presence of ML/MPs in the Mura river (water or sediments) has been reported. Thus, the obtained results cannot be directly correlated to ML pollution in the river. A few studies reported that MP concentrations in river water/sediment and fish seemed not to be strictly dependent (McNeish et al. 2018, Galafassi et al. 2021), nevertheless, our results indicate high ML pollution of the Mura river at the time of sampling. The incidence of ML in the water rises with resuspension of sediments and in them accumulated ML, thus pelagic fish are more exposed to incidentally ingest ML. *Rutilus rutilus* is a pelagic omnivorous fish and the incidence of MPs in pelagic fish should be higher in more polluted rivers and near sewage discharges (Pazos et al. 2017). The concentration of MPs in fish is also closely related to feeding habits. Omnivorous fish are more susceptible to MP ingestion than carnivorous ones (Wang et al. 2020). But regardless of the eating habits of individual fish species and their habitat, the uptake of microplastics by fish is inadvertent rather than intentional (Li et al. 2021).

Only two categories of microparticles were found: fibres and fragments, of which most were fibres (96%), as in many previous studies that addressed microplastic quantity in freshwater fish (Pazos et al. 2017, Silva-Cavalcanti et al. 2017, Horton et al. 2018, Su et al. 2018, Yuan et al. 2019, Kasamesiri and Thaimuangphol 2020, Khan et al. 2020, Wang et al. 2020). Most of them do not distinguish between synthetic and seminatural textile fibres with any method (microscopy or spectroscopy). It follows that, in such cases, the term microplastic can be misleading, as it is not necessary that all the fibres were of synthetic polymer origin. Therefore, in our study, the term ML was used as in the document Guidance of Monitoring of Marine Litter in European Seas (MSFD Technical Subgroup on Marine Litter 2013).

In this study, altogether 230 fibre particles were isolated from the 50 specimens of the common roach. The fibres were different in colour, shape, length, and thickness. Based on these characteristics and ATR-FTIR analysis (10% of fibres), we distinguished between anthropogenic fibres of seminatural (cellulose-cotton) and artificial origin (polymer material). Fibres of natural origin are flat, twisted, and uneven in diameter, while fibres of artificial origin are equal in diameter along their length (Stanton et al. 2019). Based on ATR-FTIR analysis, 86% of all fibres were of anthropogenic seminatural origin, similar to the studies of Garcia et al. 2021 and Jabeen et al. 2017. While these kinds of fibres are of anthropogenic origin with potential risk to nature due to their dye content and other chemical used in processing material (Sharma et al. 2007, Khan and Malik 2018), is crucial to include them into microlitter studies, although they are not of polymer origin.

Among fibres of artificial origin, PET (1x), PE (1x), and ethylene-vinyl acetate (EVA) (1x) were also detected (Fig. 4). PET is most widely used (60%; Li-Na, 2013) in the production of synthetic fibres (polyester fibres). Polyester fibres are often spun together with natural fibres such as cotton and wool to produce a fabric with blended properties. For this reason, both contribute to ML environmental pollution. Fish are exposed to fibres via feeding and breathing, but not all the fibres accumulate in the fish gills or GIT since they can be spontaneously excreted with mucus (Li et al. 2021). The abundance of fibres in the GIT increases in the presence of food (Li et al. 2021). Fibres were most likely to be intertwined in other organic matter (natural cellulose fibres) in water environments, thus, with feeding, omnivorous fish also accidentally eat textile fibres.

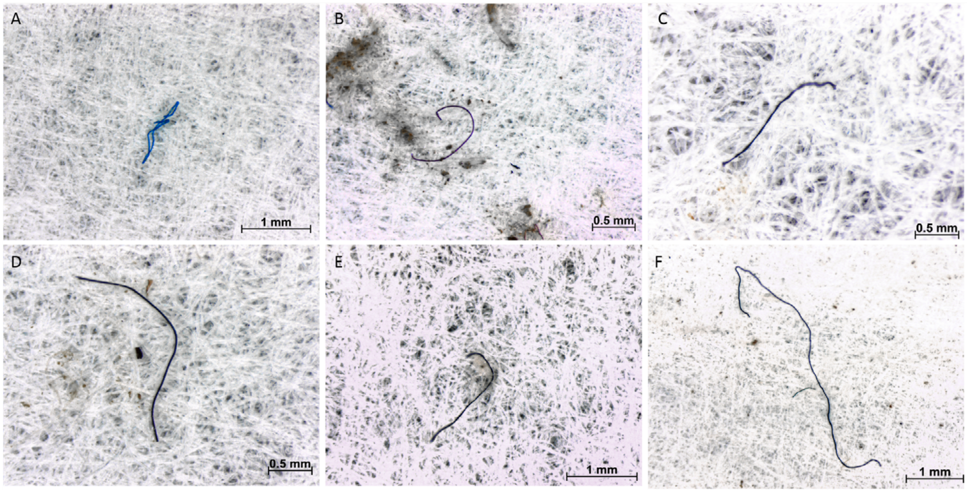

Figure 4: Examples of fibres of anthropogenic origin from the fish samples (*Rutilus rutilus*) caught in the Mura river basin. Figures **A, B, C** show cellulose fibres of various colours, **D** was identified as ethylene vinyl acetate, **E** as polyethylene, and **F** as polyethylene terephthalate.

Slika 4: Primeri vlaken antropogenega izvora iz vzorcev rib (*Rutilus rutilus*), ujetih v porečju reke Mure. Slike **A, B, C** prikazujejo celulozna vlakna različnih barv, **D** je vlakno etilen vinil acetata, **E** polietilen in **F** polietilen tereftalat.

The finding that most of the ML were fibres suggests that the main sources of pollution in the Mura river are wastewater treatment plants. Fibres enter the freshwater systems mainly via wastewater treatment plant outflows (Cesa et al. 2017). Just one load of polyester laundry can release hundreds to thousands of fibres per gram of fabric and the values depend on the kind of fabric tested and on the washing conditions/laundry products (de Falco et al. 2018). The majority accumulate in wastewater sludge and the percentage that is released from WWTPs depends on the WWTP technology. Secondary treatment plants have been found to retain approximately 92% of microplastics, tertiary treatment plants up to 96%, and membrane filtration plants more than 99% of microplastics (Blair et al. 2019). The presence of fibres rises with levels of urbanisation (Huang et al. 2020). Fibres can also be transported into the atmosphere and enter freshwater systems via precipitation. Atmospheric transport allows them to reach even very distant areas. Rain and storm events are key for microplastic contamination and microplastic cycling in the environment. One storm event can multiply microplastic contamination in rivers over 40-fold (Hitchcock 2020).

Altogether nine colourful fragments were found (white, transparent, pink, green, and blue) (Fig. 5), 0.37 mm - 0.89 mm in length (S1, 1 fr.; S2, 1 fr.; S3, 2 fr.; S4, 2 fr.; S5, 3 fr.). ATR-FTIR analysis proved diverse chemical compositions – PE (3x), PP (2x), PET (2x), polystyrene (PS) (1x), and styrene copolymer (1x). Similar chemical composition of MPs from the freshwater fish was also reported by Horton et al. (2018), Khan et al. (2020) and Yuan et al. (2019). The polymer composition of plastic fragments was expected, while the largest groups in total non-fibre plastic production are PE (36%), PP (21%), and polyvinyl chloride (PVC) (12%), followed by PET, polyurethane (PUR), and PS (<10% each). The majority (42%) of the PE, PP, and PVC is used only for packaging (Geyer et al. 2017). This relationship is reflected in environmental studies on MPs. The main polymer constituents of microplastics found in freshwaters, identified as PE, PP, PS, and PET, account for nearly three-quarters of the pollution in freshwater systems (Li et al. 2020).

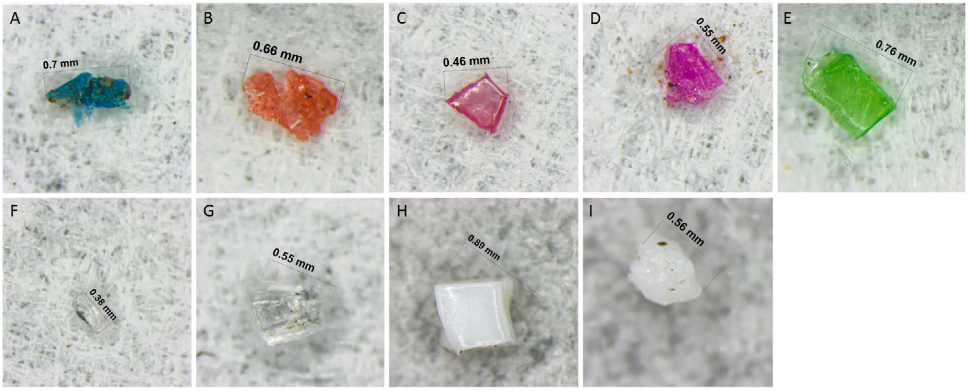

Figure 5: Microplastic fragments from the fish samples (*Rutilus rutilus*) caught in the Mura river and its tributaries: **A**, styrene copolymer; **B**, polyethylene; **C**, polystyrene; **D**, polypropylene; **E**, polyethylene terephthalate; **F**, polyethylene terephthalate; **G**, polyethylene; **H**, polyethylene; **I**, polypropylene.

Slika 5: Mikroplastični fragmenti iz vzorcev rib (*Rutilus rutilus*), ujetih v porečju reke Mure; **A**, stiren kopolimer; **B**, polietilen; **C**, polistiren; **D**, polipropilen; **E**, polietilen tereftalat; **F**, polietilen tereftalat; **G**, polietilen; **H**, polietilen; **I**, polipropilen.

Conclusions

This is the first study on the presence of microlitter in freshwater fish in Slovenia, which will contribute to a better understanding of ML in fish. We demonstrated relatively high concentrations of ML in the fish species *Rutilus rutilus* from the Mura river basin in comparison with other European studies. Fibres as the main ML type indicate households and WWTPs effluents as the main source of ML pollution. The study also clearly demonstrates a positive correlation between the amount of microplastics and the weight/length of the fish. These results indicate the potential suitability of fish *Rutilus rutilus* for the microlitter biomonitoring.

A significant amount of research is being carried out on microplastics in the marine environment, which in Europe is directly linked to the implementation of the Marine Strategy Framework Directive that treats marine litter as one of the key indicators of the health status of marine environments. The fact that rivers are the main route of microplastics to the sea, makes the research on freshwater ecosystems even more important. As a start, identification of sources of pollution through regular monitoring in freshwater ecosystems should be necessary in the frame of the European Water Framework Directive. Only by taking measures to reduce pollution in freshwater, will the measures to improve ecological status regarding litter in the marine environment be effective.

Povzetek

Študije mikroodpadkov v prebavilih sladkovodnih rib so še dokaj redke, čeprav so reke zelo izpostavljene onesnaževanju s plastičnimi delci in glavna transportna pot odpadkov do morja. Opisana študija predstavlja prve rezultate o prisotnosti mikroodpadkov v prebavilih rib rdečeok (*Rutilus rutilus*), ki so bile ulovljene v reki Muri jeseni 2020 na petih lokacijah (S1, Sladki Vrh; S2, jezero Stara Jama / Zgornje Konjišče; S3, Hrastje–Mota; S4, jezero Gajševci; S5, Krapje). Iz vsake lokacije so bila analizirana prebavila desetih rib. Vzorci prebavil so bili razgrajeni v 10 % KOH in filtrirani. Tako pripravljeni vzorci so bili pregledani s streomikroskopom za prisotnost mikroodpadkov, katerim je bila določena tudi kemijska sestava s ATR-FTIR spektrofotometrom. Mikroodpadke smo ločili na mikroplastične delce, kamor uvrščamo fragmente, pene, filme, pelete in granule ter na tekstilna vlakna, katere ločimo glede na izvor na sintetična in naravna (bombaž).

Izmed 50 pregledanih rib, jih je 94 % vsebovalo mikroodpadke v povprečni koncentraciji 5 ± 3 delcev/osebek, med katerimi so prevladovala tekstilna vlakna (96 %). Dokazana je bila korelacija med koncentracijo mikroodpadkov in težo rib (R^2 = 0,70). Skupno je bilo najdenih 9 mikroplastičnih fragmentov različnih barv in kemijskih struktur (polietilen - 3x, polipropilen - 2x, polietilentereftalat - 2x, polistiren - 1x, stiren kopolimer - 1x). Med tekstilnimi vlakni so prevladovala vlakna naravnega izvora (barvan bombaž; 86 %), med sintetičnimi vlakni so bili identificirani primerki iz etilen vinil acetata, polietilena in polietilentereftalata. Rezultati, s prevladujočimi mikroodpadki v obliki tekstilnih vlaken, kažejo na gospodinjstva in z njimi povezane odpadne komunalne vode, kot največji vir onesnaževanja reke Mure z mikroodpadki. Rezultati se po strukturi mikroodpadkov ne razlikujejo od študij opravljenih v morskem okolju v Sloveniji. Okvirna direktiva o morski strategiji že obravnava morske odpadke in predvideva izvajanje monitoringa morskih odpadkov. Poleg tega se znotraj izvajanja direktive sprejemajo ukrepi za zmanjševanje onesnaženosti morskega okolja s plastičnimi odpadki in doseganje dobrega stanja morskega okolja, medtem ko Okvirna vodna direktiva te problematike še ne obravnava, čeprav se glavna pot odpadkov do morskega okolja prične na rekah. Tako so študije mikroodpadkov v celinskih vodah izrednega pomena, tako z vidika ugotavljanja stanja okolja in vpliva na organizme kot tudi vpliva na zdravje ljudi.

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References

Blair, R.M., Waldron, S., Phoenix, V.R., Gauchotte-Lindsay, C., 2019. Microscopy and elemental analysis characterisation of microplastics in sediment of a freshwater urban river in Scotland, UK. Environmental Science and Pollution Research, 12491–12504.

Cesa, F.S., Turra, A., Baruque-Ramos, J., 2017. Synthetic fibers as microplastics in the marine environment: a review from textile perspective with a focus on domestic washings. Science of the Total Environment, 598, 1116-1129.

De Falco, F., Gullo, M. P., Gentile, G., Di Pace, E., Cocca, M., Gelabert, L., Brouta-Agnésa, M., Rovira, A., Escudero, R., Villalba, R., Mossotti, R., Montarsolo, A., Gavignano, S., Tonin, C., Avella, M., 2018. Evaluation of microplastic release caused by textile washing processes of synthetic fabrics. Environmental Pollution, 236, 916-925.

Frias, J. P., Gago, J., Otero, V., Sobral, P., 2016. Microplastics in coastal sediments from Southern Portuguese shelf waters. Marine Environmental Research, 114, 24–30.

Galafassi, S., Campanale, C., Massarelli, C., Uricchio, V.F., Volta, P., 2021. Do freshwater fish eat microplastics? A review with a focus on effects on fish health and predictive traits of mps ingestion. Water, 13(16), 2214.

Garcia, A.G., Suárez, D.C., Li, J., Rotchell, J.M., 2021. A comparison of microplastic contamination in freshwater fish from natural and farmed sources. Environmental Science and Pollution Research, 28, 14488–14497.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made. Science Advances, 3, 25–29.

Güven, O., Gökdağ, K., Jovanović, B., Kıdeyş A.E., 2017. Microplastic litter composition of the Turkish territorial waters of the Mediterranean Sea, and its occurrence in the gastrointestinal tract of fish. Environmental Pollution, 223, 286–294.

Hitchcock, J.N., 2020. Storm events as key moments of microplastic contamination in aquatic ecosystems. Science of the Total Environment, 734, 139436.

Horton, A.A., Jürgens, M.D., Lahive, E., van Bodegom, P.M., Vijver, M.G., 2018. The influence of exposure and physiology on microplastic ingestion by the freshwater fish Rutilus rutilus (roach) in the River Thames, UK. Environmental Pollution, 236, 188–194.

Huang, Y., Tian, M., Jin, F., Chen, M., Liu, Z., He, S., Li, F., Yang, L., Fang, C., Mu, J., 2020. Coupled effects of urbanization level and dam on microplastics in surface waters in a coastal watershed of Southeast China. Marine Pollution Bulletin, 154, 111089.

Hurt, R., O'Reilly, C.M., Perry, W.L., 2020. Microplastic prevalence in two fish species in two U.S. reservoirs. Limnol. Oceanographic Letters, 5, 147–153.

Jabeen, K., Su, L., Li, J., Yang, D., Tong, C., Mu, J., Shi, H., 2017. Microplastics and mesoplastics in fish from coastal and fresh waters of China. Environmental Pollution, 221, 141–149.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347. 6223, 768–771.

Jovanović, B., 2017. Ingestion of microplastics by fish and its potential consequences from a physical perspective. Integrated Environmental Assessment and Management, 13(3), 510-515.

Kasamesiri, P., Thaimuangpho, W., 2020. Microplastics ingestion by freshwater fish in the Chi River, Thailand. International Journal of GEOMATE, 18, 114–119.

Khan, S., Malik, A., 2018. Toxicity evaluation of textile effluents and role of native soil bacterium in biodegradation of a textile dye. Environmental Science and Pollution Research, 25(5), 4446-4458.

Khan, F.R., Shashoua, Y., Crawford, A., Drury, A., Sheppard, K., Stewart, K., Sculthorp, T., 2020. "The plastic nile": First evidence of microplastic contamination in fish from the Nile river (Cairo, Egypt). Toxics, 8(2), 22.

Kovač Viršek, M., Palatinus, A., Koren, Š., Peterlin, M., Horvat, P., Kržan, A., 2016. Protocol for Microplastics Sampling on the Sea Surface and Sample Analysis. Journal of Visualized Experiments 1–9.

Kuśmierek, N., Popiołek, M., 2020. Microplastics in freshwater fish from Central European lowland river (Widawa R., SW Poland). Environmental Science Pollution Research,. 27, 11438–11442.

Li, C., Busquets, R., Campos, L.C., 2020. Assessment of microplastics in freshwater systems: A review. Science of the Total Environment, 707, 135578.

Li, B., Liang, W., Liu, Q. X., Fu, S., Ma, C., Chen, Q., Su, L., Craig, N. J., Shi, H., 2021. Fish Ingest Microplastics Unintentionally. Environmental Science & Technology, 55(15), 10471-10479.

Li-Na, J., 2013. Study on preparation process and properties of polyethylene terephthalate (PET). Applied Mechanics and Materials, 312, 406–410.

McNeish, R.E., Kim, L.H., Barrett, H.A., Mason, S.A., Kelly, J.J., Hoellein, T.J., 2018. Microplastic in riverine fish is connected to species traits. Scientific Reports, 8, 1–12.

MSFD Technical Group on Marine Litter, 2013. Guidance on monitoring of marine litter in European seas. Publications Office of the European Union.

Napper, I.E., Davies, B.F.R., Clifford, H., Elvin, S., Koldewey, H.J., Mayewski, P.A., Miner, K.R., Potocki, M., Elmore, A.C., Gajurel, A.P., Thompson, R.C., 2020. Reaching New Heights in Plastic Pollution—Preliminary Findings of Microplastics on Mount Everest. One Earth 3, 621–630.

Ostroški, L., December 2015. Statistical Yearbook of the Republic of Croatia 2015. Zagreb: Croatian Bureau of Statistics. p. 49. ISSN 1333-3305. Retrieved 27 December 2015.

Pazos, R.S., Maiztegui, T., Colautti, D.C., Paracampo, A.H., Gómez, N., 2017. Microplastics in gut contents of coastal freshwater fish from Río de la Plata estuary. Marine Pollution Bulletin, 122, 85–90.

Peters, C.A., Bratton, S.P., 2016. Urbanization is a major influence on microplastic ingestion by sunfish in the Brazos River Basin, Central Texas, USA. Environmental Pollution, 210, 380–387.

Peng, X., Chen, M., Chen, S., Dasgupta, S., Xu, H., Ta, K., Du, M., Li, J., Guo, Z., Bai, S., 2018. Microplastics contaminate the deepest part of the world's ocean. Geochemical Perspectives Letters, 9, 1–5.

Plastic Europe-Association of Plastics Manufactures, 2020. Plastics – the Facts 2020. PlasticEurope 1–64.

Roch, S., Walter, T., Ittner, L.D., Friedrich, C., Brinker, A., 2019. A systematic study of the microplastic burden in freshwater fishes of south-western Germany – Are we searching at the right scale? Science of the Total Environment, 689, 1001–1011.

Sanchez, W., Bender, C., Porcher, J.M., 2014. Wild gudgeons (Gobio gobio) from French rivers are contaminated by microplastics: Preliminary study and first evidence. Environmental Research, 128, 98–100.

Sharma, S., Suryavathi, V., Singh, P.K., Sharma, K.P., 2007. Toxicity assessment of textile dye wastewater using swiss albino rats. Australasian Journal of Ecotoxicology, 13(2), 35-39.

Silva-Cavalcanti, J.S., Silva, J.D.B., França, E.J. de, Araújo, M.C.B. de, Gusmão, F., 2017. Microplastics ingestion by a common tropical freshwater fishing resource. Environmental Pollution, 221, 218–226.

Southam, A.D., Lange, A., Hines, A., Hill, E.M., Katsu, Y., Iguchi, T., Tyler, C.R., Viant, M.R., 2011. Metabolomics reveals target and off-target toxicities of a model organophosphate pesticide to roach (Rutilus rutilus): implications for biomonitoring. Environmental Science and Technology, 45(8), 3759-3767.

Stanton, T., Johnson, M., Nathanail, P., MacNaughtan, W., Gomes, R.L., 2019. Freshwater and airborne textile fiber populations are dominated by 'natural', not microplastic, fibers. Science of the Total Environment, 666, 377–389.

Su, L., Cai, H., Kolandhasamy, P., Wu, C., Rochman, C.M., Shi, H., 2018. Using the Asian clam as an indicator of microplastic pollution in freshwater ecosystems. Environmental Pollution, 234, 347–355.

Sun, X., Li, Q., Shi, Y., Zhao, Y., Zheng, S., Liang, J., Liu, T., Tian, Z., 2019. Characteristics and retention of microplastics in the digestive tracts of fish from the Yellow Sea. Environmental Pollution, 249, 878-885.

Sun, D., Wang, J., Xie, S., Tang, H., Zhang, C., Xu, G., Zou, J., Zhou, A., 2021. Characterization and spatial distribution of microplastics in two wild captured economic freshwater fish from north and west rivers of Guangdong province. Ecotoxicology and Environmental Safety, 207, 111555.

Wang, S., Zhang, C., Pan, Z., Sun, D., Zhou, A., Xie, S., Wang, J., Zou, J., 2020. Microplastics in wild freshwater fish of different feeding habits from Beijiang and Pearl River Delta regions, south China. Chemosphere 258, 127345.

Yuan, W., Liu, X., Wang, W., Di, M., Wang, J., 2019. Microplastic abundance, distribution and composition in water, sediments, and wild fish from Poyang Lake, China. Ecotoxicolology and Environmental Safety, 170, 180–187.

Zhang, C., Wang, J., Zhou, A., Ye, Q., Feng, Y., Wang, Z., Wang, S., Xu, G., Zou, J., 2021. Species-specific effect of microplastics on fish embryos and observation of toxicity kinetics in larvae. Journal of Hazardous Materials, 403, 123948.

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70 let revije Acta Biologica Slovenica

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Znanstvena revija Acta Biologica Slovenica (ABS) je glasilo stanovskega združenja biologov Društva biologov Slovenije. V letošnjem letu obeležujemo 70 let, kar je začel izhajati Biološki vestnik, predhodnik revije ABS. Novo ime Acta Biologica Slovenica je revija dobila leta 1997. Biološki vestnik je sprva objavljal izsledke domačih raziskovalcev z različnih področij biologije v slovenskem jeziku. Kasneje so se izdajatelji odločili je objavljanje pretežno v angleškem jeziku. To je povečalo krog bralcev in omogočilo posredovanje izsledkov slovenskih znanstvenikov tudi v mednarodni prostor. Širša dostopnost revije se je še povečala z izmenjavo z več kot 200 domačimi in tujimi inštitucijami. Poleg tega pa so v Biološko knjižnico prihajale številne tuje publikacije s področja biologije, ki so bile na razpolago slovenskim raziskovalcem.

Uredništvo revije je bilo vseskozi prostovoljno in je zahtevalo obilo srčnosti in predanosti. Sprva so uredniški odbor sestavljali slovenski znanstveniki, od leta 1997 dalje ima uredniški odbor mednarodno sestavo. Do danes se je zvrstilo več odgovornih in tehničnih urednikov. V obdobju od leta 1962-69 je bil odgovorni urednik entomolog Štefan Michieli, med leti 1970 in letom 1992 je urednikoval zoolog, predavatelj in akademik Janez Matjašič, od leta 1995 do 2008 predavatelj in limnolog Mihael J. Toman, z letom 2009 sem uredništvo prevzela Alenka Gaberščik in ga lansko leto predala predavateljici in botaničarki Jasni Dolenc Koce.

Revija je z nekaj krajšimi prekinitvami redno izhajala 2-krat letno. Do sedaj je izšlo 64 zvezkov. Do leta 2009 je revija izhajala le v tiskani obliki, s tem, da so bili angleški in slovenski izvlečki dostopni tudi na spletu (http://bijh.zrc-sazu.si/ABS/SI/ABS/index.asp). Po letu 2009 smo začeli izdajati tudi spletno izdajo, z letom 2021 pa smo tiskano izdajo ukinili in prešli le na spletno izdajo, ki je v odprtem dostopu dostopna tako na portalu ZRC-SAZU, kot tudi na portalu digitalne knjižnice Slovenije http://www.dlib.si/details/URN:NBN:SI:spr-ETYMF1IG). ABS je vključena tudi v svetovne baze, kot so CABI in Clarivate.

Revijo sofinancirajo člani društva s svojimi članarinami in Javna agencija za raziskovalno dejavnost Republike Slovenije (ARRS), v preteklosti pa Javna agencija za knjigo (JAK), kar člani društva cenimo.

Revija objavlja raziskave lokalnega in širšega pomena z vseh osnovnih področij biologije, od ekologije, botanike, zoologije, antropologije, molekularne biologije, biokemije, genetike, mikrobiologije, kot tudi prispevke na področju aplikativne biologije. Objavlja izvirne, pregledne znanstvene in kratke članke, novice o različnih znanstvenih dogodkih, recenzije znanstvenih knjig ter prispevke ob obletnicah znanstvenikov in znanstvenih ustanov. Občasno izidejo tudi posebne tematske številke (npr. o risu, ob različnih obletnicah). V reviji svoje izsledke objavljajo uveljavljeni domači in tuji raziskovalci (v razmerju 80 % / 20 %), k objavljanju pa vabimo tudi mlade raziskovalce in znanstvenike na začetku njihove raziskovalne poti. Revija objavlja tudi slovenske znanstvene članke, kar je posebej pomembno z vidika razvoja slovenskega strokovnega izrazja. K temu doprinesejo tudi slovenski izvlečki, povzetki ter napisi k slikam in tabelam v angleških člankih.

Splet je precej olajšal in pospešil uredniško delo, pa tudi povečal dostopnost revije, obenem pa se vsebina lahko kaj hitro utopi v množici informacij, ki so danes na voljo uporabnikom spleta. Zato je treba vseskozi vzdrževati visoko kakovost in izvirnost znanstvenih prispevkov in si tako zagotoviti "vidnost".

INSTRUCTIONS FOR AUTHORS

1. Types of Articles

SCIENTIFIC ARTICLES are comprehensive descriptions of original research and include a theoretical survey of the topic, a detailed presentation of results with discussion and conclusion, and a bibliography according to the IMRAD outline (Introduction, Methods, Results, and Discussion). In this category ABS also publishes methodological articles, in so far as they present an original method, which was not previously published elsewhere, or they present a new and original usage of an established method. The originality is judged by the editorial board if necessary after a consultation with the referees. The recommended length of an article including tables, graphs, and illustrations is up to fifteen (15) pages; lines must be double-spaced. Scientific articles shall be subject to peer review by two experts in the field.

REVIEW ARTICLES will be published in the journal after consultation between the editorial board and the author. Review articles may be longer than fifteen (15) pages.

BRIEF NOTES are original articles from various biological fields (systematics, biochemistry, genetics, physiology, microbiology, ecology, etc.) that do not include a detailed theoretical discussion. Their aim is to acquaint readers with preliminary or partial results of research. They should not be longer than five (5) pages. Brief note articles shall be subject to peer review by one expert in the field.

CONGRESS NEWS acquaints readers with the content and conclusions of important congresses and seminars at home and abroad.

ASSOCIATION NEWS reports on the work of Slovene biology associations.

2. Originality of Articles

Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

3. Language

Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

4. Titles of Articles

Title must be short, informative, and understandable. It must be written in English and in Slovene language. The title should be followed by the name and full address of the authors (and if possible, fax number and/or e-mail address). The affiliation and address of each author should be clearly marked as well as who is the corresponding author.

5. Abstract

The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is up to 250 words, and for brief note articles, 100 words. Article must have an abstract in both English and Slovene.

6. Keywords

There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

7. Running title

This is a shorter version of the title that should contain no more than 60 characters with spaces.

8. Introduction
The introduction must refer only to topics presented in the article or brief note.

9. Illustrations and Tables
Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided in electronic form. Tables should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1). A full name is used in the legend title (e.g. Figure 1, Table 2 etc.), written bold, followed by a short title of the figure or table, also in bold. Subpanels of a figure have to be unambiguously indicated with capital letters (A, B, …). Explanations associated with subpanels are given alphabetically, each starting with bold capital letter, a hyphen and followed by the text (A - text...).

10. The quality of graphic material
All the figures have to be submitted in the electronic form. The ABS publishes figures either in pure black and white or in halftones. Authors are kindly asked to prepare their figures in the correct form to avoid unnecessary delays in preparation for print, especially due to problems with insufficient contrast and resolution. Clarity and resolution of the information presented in graphical form is the responsibility of the author. Editors reserve the right to reject unclear and poorly readable pictures and graphical depictions. The resolution should be 300 d.p.i. minimum for halftones and 600 d.p.i. for pure black and white. The smallest numbers and lettering on the figure should not be smaller than 8 points (2 mm height). The thickness of lines should not be smaller than 0.5 points. The permitted font families are Times, Times New Roman, Helvetica and Arial, whereby all figures in the same article should have the same font type. The figures should be prepared in TIFF, EPS or PDF format, whereby TIFF (ending *.tif) is the preferred type. When saving figures in TIFF format we recommend the use of LZW or ZIP compression in order to reduce the file sizes. The photographs can be submitted in JPEG format (ending *.jpg) with low compression ratio. Editors reserve the right to reject the photos of poor quality. Before submitting a figure in EPS format make sure first, that all the characters are rendered correctly (e.g. by opening the file first in the programs Ghostview or GSview – depending on the operation system or in Adobe Photoshop). With PDF format make sure that lossless compression (LZW or ZIP) was used in the creation of the *.pdf file (JPEG, the default setting, is not suitable). Figures created in Microsoft Word, Excel, PowerPoint etc. will not be accepted without the conversion into one of the before mentioned formats. The same goes for graphics from other graphical programs (CorelDraw, Adobe Illustrator, etc.). The figures should be prepared in final size, published in the magazine. The dimensions are 12.5 cm maximum width and 19 cm maximum height (width and height of the text on a page).

11. Conclusions
Articles shall end with a summary of the main findings which may be written in point form.

12. Summary
Articles written in Slovene must contain a more extensive English summary. The reverse also applies.

13. Literature
References shall be cited in the text. If a reference work by one author is cited, we write Allan (1995) or (Allan 1995); if a work by two authors is cited, (Trinajstić and Franjić 1994); if a work by three or more authors is cited, (Pullin et al. 1995); and if the reference appears in several works, (Honsig-Erlenburg et al. 1992, Ward 1994a, Allan 1995, Pullin et al. 1995). If several works by the same author

published in the same year are cited, the individual works are indicated with the added letters a, b, c, etc.: (Ward 1994a,b). If direct quotations are used, the page numbers should be included: Toman (1992: 5) or (Toman 1992: 5–6).The bibliography shall be arranged in alphabetical order beginning with the surname of the first author, comma, the initials of the name(s) and continued in the same way with the rest of the authors, separated by commas. The names are followed by the year of publication, the title of the article, the full name of the journal (periodical), the volume, the number in parenthesis (optional), and the pages. Example:

Mielke, M.S., Almeida, A.A.F., Gomes, F.P., Aguilar, M.A.G., Mangabeira, P.A.O., 2003. Leaf gas exchange, chlorophyll fluorescence and growth responses of Genipa americana seedlings to soil flooding. Experimental Botany, 50(1), 221–231.

Books, chapters from books, reports, and congress anthologies use the following forms:

Allan, J.D., 1995. Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

Pullin, A.S., McLean, I.F.G., Webb, M.R., 1995. Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: Pullin A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150-164.

Toman, M.J., 1992. Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 1-7.

14. Format and Form of Articles

The manuscripts should be sent exclusively in electronic form. The format should be Microsoft Word (*.doc) or Rich text format (*.rtf) using Times New Roman 12 font with double spacing, align left only and margins of 3 cm on all sides on A4 pages. Paragraphs should be separated by an empty line. The title and chapters should be written bold in font size 14, also Times New Roman. Possible sub-chapter titles should be written in italic. All scientific names must be properly italicized. Used nomenclature source should be cited in the Methods section. The text and graphic material should be sent to the editor-in-chief as an e-mail attachment. For the purpose of review the main *.doc or *.rtf file should contain figures and tables included (each on its own page). However, when submitting the manuscript the figures also have to be sent as separate attached files in the form described under paragraph 10. All the pages (including tables and figures) have to be numbered. All articles must be proofread for professional and language errors before submission.

A manuscript element checklist (For a manuscript in Slovene language the same checklist is appropriately applied with a mirroring sequence of Slovene and English parts):
English title – (Times New Roman 14, bold)
Slovene title – (Times New Roman 14, bold)

Names of authors with clearly indicated addresses, affiliations and the name of the corresponding author – (Times New Roman 12)
Author(s) address(es) / institutional addresses – (Times New Roman 12)
Fax and/or e-mail of the corresponding author – (Times New Roman 12)
Keywords in English – (Times New Roman 12)
Keywords in Slovene – (Times New Roman 12)
Running title – (Times New Roman 12)

Abstract in English (Times New Roman 12, title – Times New Roman 14 bold)
Abstract in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Introduction – (Times New Roman 12, title – Times New Roman 14 bold)
Material and methods – (Times New Roman 12, title – Times New Roman 14 bold)
Results – (Times New Roman 12, title – Times New Roman 14 bold)
Discussion – (Times New Roman 12, title – Times New Roman 14 bold)
Summary in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Figure legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, figure designation and figure title – Times New Roman 12 bold)
Table legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, table designation and table title – Times New Roman 12 bold)
Acknowledgements – (Times New Roman 12, title – Times New Roman 14 bold)
Literature – (Times New Roman 12, title – Times New Roman 14 bold)
Figures, one per page; figure designation indicated top left – (Times New Roman 12 bold)
Tables, one per page; table designation indicated top left – (Times New Roman 12 bold)
Page numbering – bottom right – (Times New Roman 12)

15. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. With articles written in Slovene and dealing with a very local topic, both reviewers will be Slovene. In the compulsory accompanying letter to the editor the authors must nominate one foreign and one Slovene reviewer. However, the final choice of referees is at the discretion of the Editorial Board. The referees will remain anonymous to the author. The possible outcomes of the review are: 1. Fully acceptable in its present form, 2. Basically acceptable, but requires minor revision, 3. Basically acceptable, but requires important revision, 4. May be acceptable, but only after major revision, 5. Unacceptable in anything like its present form. In the case of marks 3 and 4 the reviewers that have requested revisions have to accept the suitability of the corrections made. In case of rejection the corresponding author will receive a written negative decision of the editor-in-chief. The original material will be erased from the ABS archives and can be returned to the submitting author on special request. After publication the corresponding author will receive the *.pdf version of the paper.

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PREGLEDNI ČLANEK – REVIEW PAPER:

ZNANSTVENI ČLANKI – SCIENTIFIC ARTICLES:

KRATKA NOTICA / BRIEF NOTE: