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PRIRODOSLOVNO-MATEMATIČKI FAKULTET

BIOLOŠKI ODSJEK

Milena Milaković

UTJECAJ OTPADNIH VODA FARMACEUTSKIH INDUSTRIJA NA RAZVOJ I ŠIRENJE OTPORNOSTI NA ANTIBIOTIKE

DOKTORSKI RAD

Zagreb, 2020.

University of Zagreb

FACULTY OF SCIENCE

DEPARTMENT OF BIOLOGY

Milena Milaković

IMPACT OF PHARMACEUTICAL WASTEWATERS ON SELECTION AND DISSEMINATION OF ANTIBIOTIC RESISTANCE

DOCTORAL THESIS

Zagreb, 2020

Ovaj je doktorski rad izrađen u Laboratoriju za okolišnu mikrobiologiju i biotehnologiju Instituta Ruđer Bošković u Zagrebu pod vodstvom dr. sc. Nikoline Udiković Kolić, više znanstvene suradnice, u sklopu Sveučilišnog poslijediplomskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Rad je financiran u okviru dva znanstveno-istraživačka projekta koje je vodila dr. sc. N. Udiković Kolić, Uspostavnog projekta Hrvatske zaklade za znanost „Istraživanje utjecaja otpadnih voda iz farmaceutskih industrija na sastav i profil antibiotičke rezistencije izloženih mikrobnih zajednica u slatkovodnim sedimentima“ (WINAR; broj projekta: UIP-2014-09-9350) i projekta Zaklade ADRIS „Proučavanje povezanosti zagađenja okoliša antibioticima i razvoja rezistencije na antibiotike u ljudskih patogena“ te stipendijom COST akcije NEREUS (ES1403).

INFORMACIJE O MENTORU

Dr. sc. Nikolina Udiković Kolić rođena je 20. ožujka 1978. godine u Ogulinu. Diplomirala je 2001. godine na studiju Biotehnologije, smjeru biokemijsko-mikrobiološkom na Prehrambeno-biotehnološkom fakultetu Sveučilišta u Zagrebu. Na istom fakultetu obranila je doktorsku disertaciju 2008. godine pod naslovom „Katabolički potencijal i genetička struktura bakterijskih zajednica porijeklom iz okoliša onečišćenog atrazinom i drugim *s*-triazinskim spojevima“. Zaposlena je na mjestu više znanstvene suradnice i voditeljice Laboratorija za okolišnu mikrobiologiju i biotehnologiju na Institutu Ruđer Bošković u Zagrebu, gdje radi od 2001. godine. U više navrata usavršavala se na međunarodno renomiranim institucijama u inozemstvu uključujući 11-mjesečni boravak na Sveučilištu Yale u SAD-u kao stipendistica prestižnog programa Fulbright. Održala je niz predavanja na međunarodnim i na domaćim znanstvenim skupovima i/ili institucijama te dobila nekoliko domaćih i međunarodnih nagrada za svoj znanstveni rad. Pridruženi je urednik međunarodnog časopisa *Journal of Environmental Quality* te članica uredništva još dva međunarodna časopisa. Sudjeluje i u Sveučilišnoj nastavi, gdje je nositeljica/sunositeljica kolegija na doktorskim studijima na Prehrambeno-biotehnološkom fakultetu Sveučilišta u Zagrebu i Sveučilištu u Osijeku. Autorica je i suautorica brojnih znanstvenih radova te voditeljica nekoliko znanstveno-istraživačkih projekata.

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„Believe you can and you're halfway there.“

Theodore Roosvelt

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorski rad

UTJECAJ OTPADNIH VODA FARMACEUTSKIH INDUSTRIJA NA RAZVOJ I ŠIRENJE OTPORNOSTI NA ANTIBIOTIKE

MILENA MILAKOVIĆ

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Cilj rada bio je istražiti utjecaj ispusta otpadnih voda iz dviju lokalnih farmaceutskih industrija na recipijentni vodeni okoliš, s naglaskom na razvoj i širenje otpornosti na antibiotike te sastav bakterijskih zajednica. Kemijskom analizom potvrđene su relativno visoke koncentracije antibiotika u otpadnim vodama tih industrija te znatno više koncentracije antibiotika u sedimentima recipijentnih voda nizvodno od ispusta u odnosu na referentnu lokaciju uzvodno od ispusta. Funkcionalnom metagenomikom identificirani su poznati i potencijalno novi geni za otpornost na ciljane antibiotike u otpadnim vodama i sedimentima. Kvantifikacijom odabranih gena otpornosti utvrđene su značajno više količine u sedimentima na lokaciji ispusta i nizvodno od ispusta u odnosu na referentnu uzvodnu lokaciju. Egzogenom izolacijom plazmida potvrđeno je povećano horizontalno širenje gena otpornosti na makrolide i/ili tetracikline u sedimentima onečišćenima otpadnim vodama farmaceutskih industrija. Filogenetskom analizom nukleotidnih sekvenci gena *erm*B potvrđena je njihova visoka sličnost između okolišnih bakterijskih izolata porijeklom iz riječnog sedimenta izrazito onečišćenog makrolidima i kliničkih izolata streptokoka, što ukazuje na mogući prijenos ovog gena između bakterija kliničkog i okolišnog podrijetla. Sekvenciranjem amplikona 16S rRNA gena utvrđene su značajne prostorne i sezonske promjene u sastavu bakterijskih zajednica izloženih sedimenata. Rezultati ovog istraživanja ukazali su na problem onečišćenja okoliša otpadnim vodama farmaceutskih industrija, što može predstavljati potencijalnu opasnost za zdravlje ljudi i okoliša.

(253 stranice, 34 slike, 15 tablica, 297 literaturnih navoda, jezik izvornika hrvatski)

Ključne riječi: otpornost na antibiotike, otpadne vode farmaceutskih industrija, sedimenti, bakterijska zajednica, plazmidi

Mentor: dr. sc. Nikolina Udiković Kolić, viša znanstvena suradnica

Ocjenjivači: dr. sc. Ines Sviličić Petrić, viša znanstvena suradnica
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doc. dr. sc. Ana Bielen

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University of Zagreb
Faculty of Science
Department of Biology

Doctoral thesis

IMPACT OF PHARMACEUTICAL WASTEWATERS ON SELECTION AND DISSEMINATION OF ANTIBIOTIC RESISTANCE

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The aim of this work was to explore the impact of the wastewater discharge from two local pharmaceutical industries on the recipient freshwater environments, particularly regarding the development and spread of antibiotic resistance and bacterial communities composition. Chemical analyses confirmed relatively high antibiotic concentrations in the wastewater from those industries and considerably higher antibiotic concentrations in recipient sediments downstream from the discharge site when compared with upstream reference site. Functional metagenomics revealed already known and potentially novel genes for resistance to targeted antibiotics in wastewater and sediments. Significantly higher concentrations of selected resistance genes were found in sediments at the disharge site and downstream from the pharmaceutical industries discharge site relative to the upstream reference site. Exogenous plasmid isolation revealed the increased horizontal transfer of macrolide and/or tetracycline resistance genes in antibiotic-polluted sediments. Phylogenetic analysis confirmed high similarity of the nucletiode sequences of the *erm*B gene between environmental bacterial isolates that originated from highly macrolide-polluted river sediments and clinical streptococcal isolates indicating potential transfer of this gene between bacteria of environmental and clinical origin. Sequencing of 16S rRNA gene amplicons revealed significant spatial and seasonal changes in bacterial communities composition of wastewater-exposed sediments. The results of this study highlight the problem of environmental pollution with pharmaceutical wastewater, which may pose a potential threat to both human and environmental health.

(253 pages, 34 figures, 15 tables, 297 references, original in Croatian)

Keywords: antibiotic resistance, pharmaceutical wastewater, sediments, bacterial community, plasmids

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UVOD

1. UVOD

Otpornost bakterija na antibiotike predstavlja jedan od vodećih problema današnje medicine i gorući javnozdravstveni problem svjetskih razmjera. Otkriće prvog antibiotika, penicilina, 1928. godine bilo je jedno od najvećih znanstvenih postignuća zahvaljujući kojem su spašeni mnogi životi u to vrijeme i mnoge zarazne bolesti stavljene pod kontrolu. Obećavajuća je bila i brzina otkrivanja novih i sve potentnijih antibiotika u razdoblju do 70-tih godina prošlog stoljeća te se vjerovalo da zarazne bolesti više neće biti problem (Kraemer i sur., 2019.; Peterson i Kaur, 2018.). Međutim, učinkovitost antibiotika dovedena je u pitanje, ponajviše zbog prekomjerne i nerazumne primjene u medicini i veterinarstvu, kao i njihovog kontinuiranog ispuštanja u okoliš. Do danas su bakterije razvile mehanizme otpornosti na gotovo sve skupine antibiotika koji se koriste u medicini. Takva moć bakterijske evolucije, zajedno s ograničenim investiranjem farmaceutske industrije u razvoj novih antibiotika, učinili su otpornost na antibiotike jednom od najozbiljnijih prijetnji modernom zdravlju u 21. stoljeću (Tambić Andrašević, 2011.). Dužnost nam je stoga što je moguće više usporiti razvoj otpornosti na antibiotike među bakterijama kako bi sačuvali djelotvornost postojećih antibiotika za buduće naraštaje. Toga su postala svjesna ne samo stručna društva već i vlade mnogih zemalja pa je stoga problem otpornosti na antibiotike istaknut kao jedan od prioriteta Svjetske zdravstvene organizacije (SZO) (WHO, 2017.).

Neke su bakterije prirodno (urođeno) otporne na određene antibiotike; međutim, zabrinjavajući je problem kad bakterije koje su obično osjetljive na antibiotike steknu otpornost kao rezultat genetičkih promjena, bilo novim mutacijama u genomu ili horizontalnim prijenosom gena iz drugih, otpornih bakterija (von Wintersdorff i sur., 2016.). Te procese stjecanja otpornosti pospješuje ne samo prekomjerna primjena antibiotika u liječenju ljudi i životinja nego i sve veće onečišćenje okoliša antibioticima. Antibiotici dospijevaju u okoliš najčešće putem otpadnih voda iz različitih izvora (kućanstva, bolnice, farmaceutska industrija, akvakultura), ali i upotrebom stajskog gnojiva ili obrađenog mulja s uređaja za pročišćavanje komunalnih otpadnih voda na poljoprivrednim zemljištima (Gothwal i Shashidhar, 2015.; Singh i sur., 2019.). Pod stalnim selektivnim pritiskom antibiotika u okolišu ubrzava se razvoj otpornosti na antibiotike u okolišnih bakterija (Buschmann i sur., 2012.; Heuer i Smalla, 2007.; Jechalke i sur., 2013.; Kristiansson i sur., 2011.; Li i sur., 2010.; Martinez, 2009.). Pokazalo se da čak i niske, subinhibitorne koncentracije antibiotika, slične onima nađenim u različitim vodenim i kopnenim sustavima (Kümmerer, 2009.) mogu promicati nastanak otpornih bakterija (Gullberg i sur., 2011.). Nadalje, prisutnost metala, čak i u niskim koncentracijama, potiče

selekciju otpornosti na antibiotike zbog zajedničkih genetičkih ili fizioloških mehanizama otpornosti na antibiotike i metale (Selier i Berendonk, 2012.). S obzirom na to da čovjek stalno izmjenjuje svoju mikrobiotu s okolišnom, razvoj otpornosti na antibiotike u okolišu povećava rizik prijenosa otpornih bakterija na ljude. Tome u prilog idu i studije koje su pokazale identične gene za otpornost na antibiotike u okolišnih (većinom bezopasnih) bakterija i patogenih bakterija (Surette i Wright, 2017.). Putevi kojima ljudi mogu doći u kontakt s okolišnim bakterijama otpornim na antibiotike su brojni. Oni uključuju pijenje vode za piće porijeklom iz onečišćene podzemne ili površinske vode, sudjelovanje u rekreativnim aktivnostima u onečišćenoj površinskoj vodi ili inhalaciju aerosola (Pruden i sur., 2013.). Osim utjecaja na razvoj i širenje otpornosti, onečišćenje okoliša antibioticima može utjecati i na sastav i/ili funkcionalna svojstva prirodnih bakterijskih zajednica te na taj način narušiti zdravlje cijelog ekosustava (Grenni i sur., 2018.).

Do danas se relativno malo zna o važnosti vodenog okoliša kao mjesta nakupljanja, ali i izvora antibiotika i determinanti za otpornost na antibiotike. Na primjeru Indije i Kine se pokazalo da bi vodeni okoliš koji služi kao recipijent otpadnih voda iz postrojenja za proizvodnju antibiotika mogao predstavljati značajan izvor budući da takve otpadne vode mogu sadržavati smjese antibiotika u visokim koncentracijama (mg L^{-1}), ali i druga onečišćivala kao što su metali te bakterije otporne na antibiotike (Larsson, 2014a.; Li i sur., 2009., 2010.; Lübbert i sur., 2017.; Marathe i sur., 2013.). Zbog toga se smatra da okoliš koji je pod utjecajem industrijskih otpadnih voda, naročito sedimenti, predstavlja jedinstvene "vruće točke" u kojima može, uslijed intenzivnog selekcijskog pritiska, doći do obogaćivanja postojećih, ali i razvoja novih gena otpornosti na antibiotike. Zbog sposobnosti bakterija da prenose genetički materijal na pokretnim genetičkim elementima, geni za otpornost na antibiotike mogu se prenijeti iz bezopasnih bakterija u štetne, patogene bakterije unutar onečišćenog okoliša ili pak unutar ljudi i životinja, što može imati štetne zdravstvene posljedice za sve, bez obzira na to gdje se onečišćenje dogodilo.

Zbog svega navedenog, bitno je razumjeti moguće posljedice kontinuiranog ispuštanja antibiotika u vodeni okoliš, posebice na razvoj i širenje bakterijske otpornosti na antibiotike. Za postizanje inovativnog proboja na ovoj temi koja je od globalne važnosti za zdravlje ljudi i okoliša, postoji potreba za provođenjem interdisciplinarnog pristupa kojim će se istražiti odgovor bakterijskih zajednica, a ne pojedinačnih bakterijskih vrsta, na kemijsko i mikrobiološko onečišćenje te pronaći poveznice s izvorom onečišćenja.

1.1. Ciljevi i svrha rada

Posljedice unosa razmjerno visokih koncentracija antibiotika u okoliš putem otpadnih voda farmaceutskih industrija još su uvijek nedovoljno istražene, iako su nužne za procjenu rizika za zdravlje ljudi i okoliša. Zato je svrha ove doktorske disertacije pridonijeti boljem razumijevanju utjecaja otpadnih voda dviju lokalnih farmaceutskih industrija na recipijentni vodeni okoliš, s naglaskom na razvoj i širenje otpornosti na antibiotike te sastav bakterijskih zajednica. Sukladno navedenom, specifični ciljevi istraživanja bili su:

- procijeniti kemijsko onečišćenje otpadnih voda iz sinteze ili formulacije antibiotika te vodenog okoliša koji služi kao recipijent tih otpadnih voda mjerenjem koncentracija antibiotika vezanim sustavom tekućinska kromatografija − tandemna spektrometrija masa (LC-MS/MS) i metala masenom spektrometrijom s induktivno spregnutom plazmom (ICP-MS) te određivanjem fizikalno-kemijskih pokazatelja za ispitivanje stanja površinskih voda, sedimenata i otpadnih voda pomoću međunarodno validiranih metoda (ISO norme);
- identificirati i karakterizirati gene odgovorne za otpornost na antibiotike iz mikroorganizama porijeklom iz otpadnih voda i slatkovodnih sedimenata inovativnim pristupom funkcionalne metagenomike;
- procijeniti širenje otpornosti na antibiotike u sedimentima rijeke Save i potoka Kalinovica na lokacijama industrijskih ispusta i nizvodno od ispusta kvantifikacijom gena za otpornost pomoću kvantitativne lančane reakcije polimerazom (qPCR), analizom horizontalnog prijenosa gena pomoću egzogene izolacije plazmida te analizom gena otpornosti na makrolidne antibiotike u okolišnih i kliničkih bakterijskih izolata pomoću lančane reakcije polimerazom (PCR) i sekvenciranja;
- odrediti prostorne i sezonske promjene u sastavu bakterijskih zajednica iz otpadnih voda i izloženih sedimenata sekvenciranjem amplikona 16S rRNA gena tehnologijom Illumina MiSeq i bioinformatičkom analizom dobivenih podataka u programu QIIME2.

1.2. Hipoteze rada

Istraživanjem provedenim u ovom radu testirali smo sljedeće hipoteze:

H1. Otpadne vode iz lokalnih farmaceutskih industrija onečišćuju recipijentni vodeni okoliš antibioticima, metalima i bakterijama otpornima na antibiotike;

H2. Otpadne vode i sedimenti recipijentnih voda su spremnici poznatih i potencijalno novih gena za otpornost na antibiotike;

H3. Industrijski ispusti potiču širenje otpornosti na antibiotike među bakterijama u izloženim sedimentima;

H4. Industrijski ispusti mijenjaju strukturu i sastav izloženih prirodnih bakterijskih zajednica.

LITERATURNI PREGLED

2. LITERATURNI PREGLED

2.1. Antibiotici i otpornost bakterija na antibiotike

Otkriće antibiotika smatra se jednim od najvećih znanstvenih postignuća 20. stoljeća kojim su brojne zarazne bolesti stavljene pod kontrolu uz bitno smanjenje smrtnosti, a mnoga područja medicine su napredovala zahvaljujući profilaktičkoj primjeni antibiotika (Carvalho i Santos, 2016.). Glavno obilježje tih dragocjenih lijekova je to što su selektivno toksični, odnosno toksični su za bakterije, dok su manje toksični za ljudski organizam. Iako je primjena antibiotika smanjila smrtnost i znatno povećala životni vijek, nažalost, sve više slabi njihov utjecaj na bakterije, jer je nerazumna i prekomjerna potrošnja te prekomjerno ispuštanje antibiotika u okoliš dovelo do razvoja otpornosti na njih među bakterijama (Larsson, 2014b.; Ventola, 2015.). Otpornost na antibiotike je urođeno ili stečeno svojstvo neke bakterijske vrste ili soja da se obrani od djelovanja antibiotika u svojem okolišu zahvaljujući mehanizmima strukturne ili fiziološke te genetičke prilagodbe (Martínez i sur., 2015.). Zbog učestalog izlaganja antibioticima i sposobnosti brze prilagodbe okolišu u kojem žive, bakterije su do danas razvile mehanizme otpornosti na gotovo sve skupine antibiotika, a do novih antibiotika se sve teže dolazi, jer farmaceutska industrija ne ulaže u njihov razvoj zbog slabije isplativosti u odnosu na druge lijekove (Ventola, 2015.). Grube procjene pokazuju da su infekcije izazvane bakterijama otpornim na antibiotike već do sada u svijetu uzrokovale nekoliko stotina tisuća smrtnih slučajeva godišnje, a predviđa se da će do 2050. godine te brojke narasti i do 10 milijuna smrtnih slučajeva (O'Neill, 2016.). Stoga se otpornost bakterija na antibiotike ističe kao jedan od najvećih javnozdravstvenih problema današnjice te se borba protiv otpornosti ubraja u prioritete SZO (WHO, 2017.), a predstavlja i jedan od zahtjeva Vijeća Europske Unije postavljen svim zemljama članicama (EFSA, 2017.; Tambić Andrašević, 2011.).

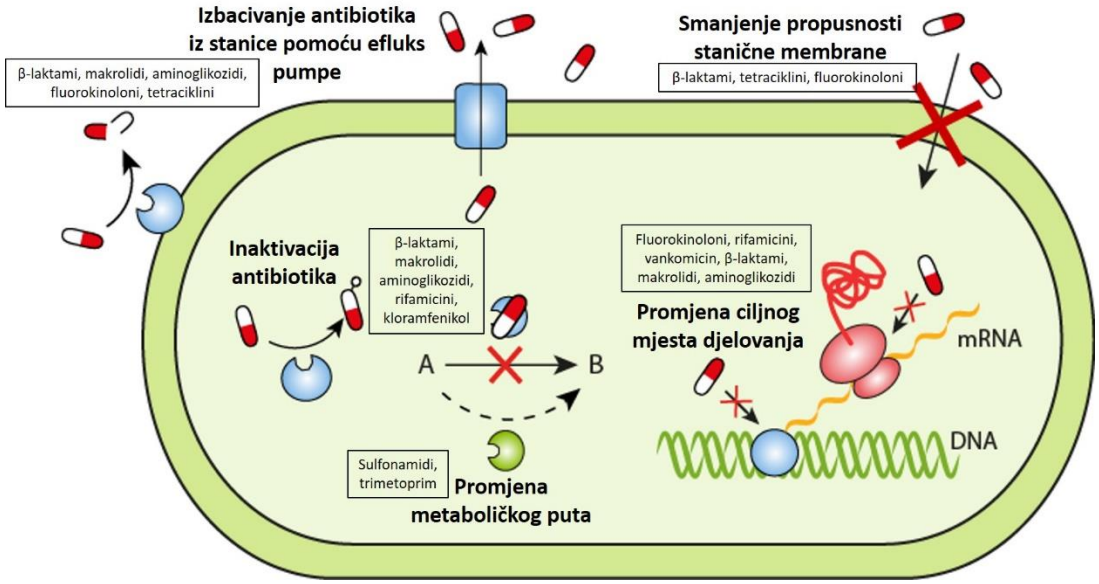
2.1.1. Urođena i stečena otpornost

Otpornost bakterija na antibiotike može biti urođena ili primarna (intrinzična) i stečena ili sekundarna. Urođena otpornost je strukturalno ili funkcionalno svojstvo bakterija koje im omogućuje preživljavanje u prisutnosti antibiotika, a prenosi se staničnom diobom na bakterije iste vrste, što zovemo vertikalnim prijenosom. Bakterije su najčešće urođeno otporne na antibiotike zbog smanjene propusnosti stanične stijenke i posljedično, nemogućnosti ulaska antibiotika u bakterijsku stanicu ili pojačanog izbacivanja antibiotika iz stanice pomoću efluks pumpi (Reygaert, 2018.). Primjeri urođene otpornosti su otpornost Gram-negativnih bakterija na glikopeptide ili Gram-pozitivnih bakterija na polimiksine zbog nemogućnosti ili smanjenog

prolaza antibiotika kroz staničnu stijenku (Grenni i sur., 2018.; Trimble i sur., 2016.). Stečena otpornost nastaje pod selektivnim pritiskom antibiotika kao rezultat novih mutacija u bakterijskom kromosomu ili izmjenom genetičkog materijala među bakterijama, što je posebno olakšano za gene koji kodiraju otpornost, a nalaze se na plazmidima (Gothwal i Shashidhar, 2015.). Tako primjerice, točkasta mutacija u genu koji kodira ciljno mjesto djelovanja antibiotika dovodi do nemogućnosti njegovog vezanja te posljedično djelovanja na stanicu. Ako bakterije s novostečenom otpornošću nisu izložene tom antibiotiku, one ostaju rijetke unutar osjetljive populacije bakterija. Međutim, nakon izlaganja antibiotiku, bakterijski mutanti sa stečenom otpornošću su u selektivnoj prednosti, preživljavaju i razmnožavaju se te s vremenom prevladaju u toj bakterijskoj populaciji (Davies i Davies, 2010.). Ipak, kromosomske mutacije su rijetke i za većinu bakterija nemaju klinički značaj. Puno češće se otpornost na antibiotike stječe horizontalnim prijenosom gena među bakterijama (engl. *Horizontal Gene Transfer,* HGT) mehanizmima transformacije, transdukcije ili konjugacije (Poglavlje 2.1.7.).

Mehanizmi stečene otpornosti bakterija na antibiotike, prikazani na Slici 1. (Munita i Arias, 2016.), uključuju:

i. **inaktivaciju antibiotika** putem kemijske promjene ili razgradnje molekule antibiotika pomoću enzima;
ii. **promjenu ili zaštitu ciljnog mjesta djelovanja antibiotika** čime se antibiotiku onemogućuje vezanje. Promjene ciljnog mjesta djelovanja uključuju enzimske i kemijske promjene te točkaste mutacije gena za ciljno mjesto djelovanja antibiotika;
iii. **promjene metaboličkog puta** čime se zaobilazi metabolički put na koji djeluje antibiotik te se stvara novi, alternativni metabolički put koji nije inhibiran djelovanjem antibiotika;
iv. **smanjenje propusnosti stanične membrane ili aktivno izbacivanje antibiotika iz stanice** čime se sprječava akumulacija antibiotika unutar bakterijske stanice. Ovaj je mehanizam posredovan smanjenjem ekspresije i broja porina, proteina stanične membrane, te njihovom selektivnošću za određenu skupinu antibiotika ili povećanom ekspresijom gena koji kodiraju efluks pumpe (Reygaert, 2018.).



Slika 1. Mehanizmi otpornosti bakterija na antibiotike. Preuzeto i prilagođeno iz Gullberg (2014.).

2.1.2. β-laktamski antibiotici

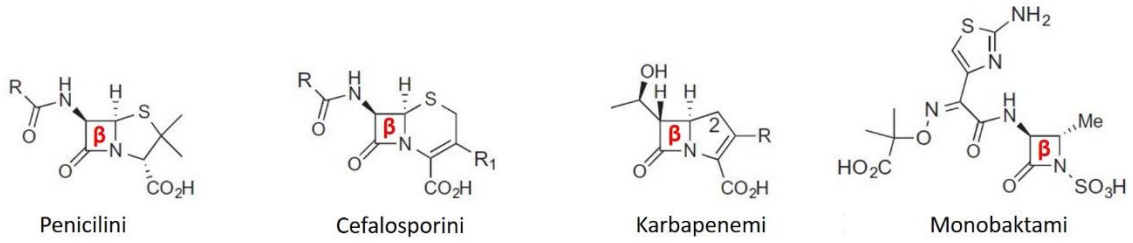
β-laktamski antibiotici su najraširenija skupina antibiotika koja se zbog vrlo snažnog antimikrobnog djelovanja i vrlo niske toksičnosti najčešće koristi u medicini širom svijeta pa tako i u Republici Hrvatskoj (Bhattacharjee, 2016.; Bush i Bradford, 2016.; Tambić Andrašević i sur., 2018.). Zbog širokog spektra djelovanja, β-laktamski antibiotici se primjenjuju u liječenju bakterijskih infekcija izazvanih Gram-pozitivnim bakterijama iz rodova *Staphylococcus*, *Streptococcus*, *Enterococcus* te Gram-negativnim bakterijama iz rodova kao što su *Escherichia*, *Klebsiella* i *Proteus* (Fernandes i sur., 2013.; Singh i sur., 2017.). Pored toga, β-laktamski antibiotici su druga najčešće korištena skupina antibiotika u veterinarstvu, pri čemu se najčešće primjenjuju penicilini (Cháfer-Pericás i sur., 2010.; ESVAC, 2016.).

2.1.2.1. Kemijska struktura i mehanizam djelovanja

Osnovna kemijska struktura β-laktamskih antibiotika sastoji se od jedinstvenog četveročlanog β-laktamskog prstena (Slika 2.) koji je ključan za antibakterijsku aktivnost i po kojem je cijela skupina dobila ime (Singh i sur., 2017.). S obzirom na dodatne kemijske skupine vezane na prsten, β-laktamski antibiotici se mogu podijeliti u četiri skupine: peniciline, cefalosporine, karbapeneme i monobaktame (Slika 2.) (Singh i sur., 2017.). Neki od tih β-laktamskih antibiotika poput treće i četvrte generacije cefalosporina te karbapenema nalaze se

na listi kritički značajnih antibiotika u medicini koju je nedavno objavila SZO (WHO, 2017.), i to zato što su za neka po život opasna stanja ili jedini antibiotici koji su još preostali u terapiji ili su jedni od samo nekoliko antibiotika koji se mogu koristiti u liječenju specifičnih infekcija.

Osim sličnosti u kemijskoj građi, β-laktamski antibiotici imaju isti mehanizam djelovanja koji se temelji na inhibiciji sinteze stanične stijenke vezanjem na proteine koji vežu penicilin (engl. *Penicilin Binding Proteins,* PBP), nakon čega dolazi do inhibicije povezivanja peptidoglikanskih lanaca i do prestanka sinteze peptidoglikana. To dovodi do smrti bakterijske stanice zbog osmotske nestabilnosti ili autolize (Kohanski i sur., 2010.; Singh i sur., 2017.).



Slika 2. Kemijske strukture predstavnika β-laktamskih antibiotika. Slika je preuzeta i prilagođena iz rada Singh i sur. (2017.).

2.1.2.2. Mehanizmi bakterijske otpornosti

U kliničkoj praksi važna su četiri mehanizma bakterijske otpornosti na β-laktamske antibiotike: enzimska inaktivacija molekule antibiotika, nemogućnost ili smanjeni prolaz antibiotika kroz vanjsku membranu stanice, aktivno izbacivanje antibiotika iz stanice i promjena ciljnog mjesta djelovanja (smanjen afinitet PBP-a za vezanje β-laktamskih antibiotika) (Fernandes i sur., 2013.; Reygaert, 2018.). Među navedenim mehanizmima najučestaliji je inaktivacija antibiotika putem bakterijskih enzima β-laktamaza koje cijepaju β-laktamski prsten te dovode do gubitka strukturnog integriteta molekule antibiotika, a time i njegove antibakterijske aktivnosti (Reygaert, 2018.; Sultan i sur., 2018.; van Hoek i sur., 2011.). Stoga se danas, uz β-laktamske antibiotike, primjenjuju i inhibitori β-laktamaza kao što su primjerice klavulanska kiselina, sulbaktam ili tazobaktam (Tooke i sur., 2019.). Prema specifičnosti za inaktivaciju pojedinih vrsta β-laktamskih antibiotika, razlikujemo tri vrste β-laktamaza - penicilinaze, cefalosporinaze i karbapenemaze, a prema molekularnoj strukturi (tzv. Amblerova klasifikacija), postoje četiri vrste β-laktamaza - skupine A, B, C i D (Fernandes i sur., 2013.; Munita i Arias, 2016.). β-laktamaze skupina A, C i D su enzimi čiji se hidrolitički

mehanizam bazira na serinu u aktivnom mjestu. Skupinu B, tzv. metalo-β-laktamaze, čine enzimi u čijem se aktivnom mjestu nalazi jedan ili dva cinkova iona. Klinički je najznačajnija skupina A β-laktamaza koja uključuje penicilinaze (TEM-1 i SHV-1) koje hidroliziraju samo penicilin, a pripadaju joj i β-laktamaze sa spektrom proširenim prema cefalosporinima (engl. *Extended Spectrum β-Lactamase*, ESBL) te karbapenemaze koje dodatno hidroliziraju i karbapeneme i od kojih su najučestalije KPC i GES (Munita i Arias, 2016.). Međutim, svi spomenuti enzimi podložni su djelovanju inhibitora klavulanske kiseline i tazobaktama. Metalo-β-laktamaze su enzimi s najširim spektrom koji mogu hidrolizirati sve β-laktamske antibiotike osim monobaktama (aztreonam), a otporne su na klasične inhibitore β-laktamaza poput klavulanske kiseline (Munita i Arias, 2016.). U ovu skupinu spadaju neki klinički važni enzimi kao što su IMP, VIM i NDM. Skupina C β-laktamaza obuhvaća enzime na koje inhibitori β-laktamaza ne djeluju te ispoljavaju otpornost na sve peniciline i cefalosporine, a klinički najznačajniji enzim je AmpC. Skupina D β-laktamaza obuhvaća enzime koji hidroliziraju oksacilin te se stoga ova skupina naziva i β-laktamaze OXA (Evans i Amyes, 2014.). Tu skupinu čini više od 400 različitih varijanti enzima šarolike hidrolitičke aktivnosti. Neke varijante su β-laktamaze uskog spektra, neke posjeduju ESBL karakteristike, a samo manji udio ovih enzima ispoljava karbapenemaznu aktivnost (Evans i Amyes, 2014.). Do danas je poznato preko 1150 različitih β-laktamaza koje mogu biti kodirane genima smještenim na kromosomu ili na pokretnim genetičkim elementima poput plazmida koji predstavljaju veliki problem zbog brzog širenja među bakterijama (van Hoek i sur., 2011.; Sultan i sur., 2018.).

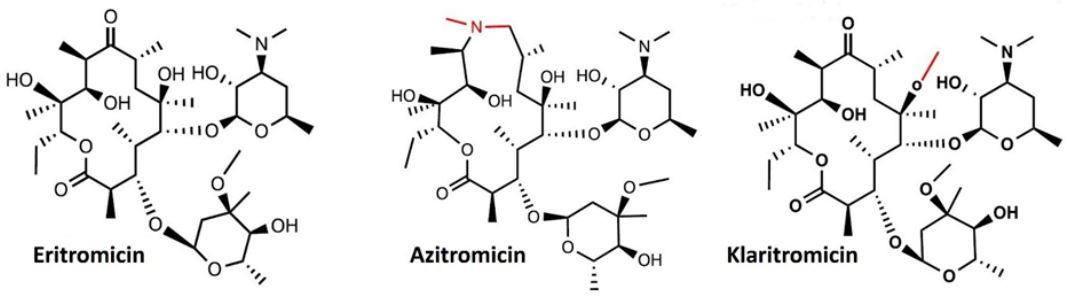
2.1.3. Makrolidni antibiotici

Antibiotici iz skupine makrolida druga su najčešće korištena skupina antibiotika u liječenju različitih infekcija izazvanih Gram-pozitivnim i Gram-negativnim bakterijama (Bhattacharjee, 2016.; Tambić Andrašević i sur., 2018.). Ovi su antibiotici, zajedno s penicilinima, osnova prve linije antibiotske terapije pri infekcijama dišnih puteva, najčešće izazvanim pneumokokima (*Streptococcus pneumoniae*) i β-hemolitičkim streptokokom A (Tambić Andrašević, 2008.), a primjenjuju se i za liječenje nekompliciranih infekcija kože i mekih tkiva uzrokovanih vrstama iz rodova *Streptococcus* i *Staphylococcus* (Eraković Haber, 2011.; Kuzman, 2018.). Osim Gram-pozitivnih bakterija, makrolidi su djelotvorni i u liječenju Gram-negativnih uzročnika poput *Haemophilus influenze*, *Bordetella* spp., *Legionella* spp., *Campylobacter* spp., *Helicobacter pylori* te klamidija i mikoplazmi (Eraković Haber, 2011.). Međutim, većina Gram-negativnih bakterija pokazuje urođenu otpornost na makrolide zbog nepropusnosti vanjske stanične membrane za ove antibiotike te zbog prisutnosti efluks pumpi

(Tambić Andrašević, 2008.). Važno je također naglasiti da su makrolidni antibiotici, pored nekih β-laktamskih antibiotika, uvršteni na listu kritički značajnih antibiotika u medicini objavljenu od strane SZO (WHO, 2017.), što znači da je važno čim više usporiti širenje otpornosti na ove dragocjene lijekove kako bi se njihova djelotvornost očuvala za buduće naraštaje.

2.1.3.1. Kemijska struktura i mehanizam djelovanja

Svi makrolidni antibiotici u svojoj kemijskoj strukturi sadrže veliki 14-, 15- ili 16-eročlani makrociklički laktonski prsten na koji su vezani različiti amino i deoksi šećeri, obično kladinoza i dezozamin (Slika 3.) (Aminov, 2017.; Dinos, 2017.; Fyfe i sur., 2016.). Prvi makrolidni antibiotik u kliničkoj primjeni bio je eritromicin (Slika 3.), prirodni 14-eročlani antibiotik izoliran iz vrste *Saccharopolyspora erythraea*, koji se upotrebljava i danas kako u medicini i tako i u veterinarstvu (Oliynyk i sur., 2007.). Kemijskim modifikacijama prirodnog eritromicina razvijena je druga generacija makrolida, 14-eročlani klaritromicin i 15-eročlani azitromicin (Slika 3.), koji je danas jedan od najprodavanijih antibiotika u svijetu zbog jednostavne farmakokinetike, dobre djelotvornosti, jednostavnog doziranja te mogućnosti primjene kod trudnica i djece (Aminov, 2017.; Dinos, 2017.; Kuzman, 2018.). Razvoj novih generacija makrolidnih antibiotika nadalje je bio usmjeren ka poboljšanju antibakterijske aktivnosti što je dovelo do razvoja novih generacija, ketolida i fluoroketolida.



Slika 3. Kemijske strukture najčešće korištenih makrolidnih antibiotika u medicini. Preuzeto i prilagođeno iz Dinos i sur. (2017.).

Makrolidni antibiotici selektivno djeluju tako da se reverzibilno vežu na 23S rRNA regiju koja se nalazi na velikoj (50S) podjedinici bakterijskog ribosoma i sprječavaju sintezu proteina inhibirajući aktivnost peptidil-transferaznog centra odnosno mjesta gdje se odvija kataliza stvaranja peptidnih veza (Cattoir i Leclercq, 2017.; Vázquez-Laslop i Mankin, 2018.).

2.1.3.2. Mehanizmi bakterijske otpornosti

Otpornost na makrolidne antibiotike pojavila se nedugo nakon kliničke primjene eritromicina te je prvi put opisana kod stafilokoka (MacCabe i Gould, 1956.; Weisblum, 1995.). Do danas su bakterije razvile različite mehanizme otpornosti na makrolidne antibiotike koji uključuju promjene ciljnog mjesta djelovanja na ribosomu, kemijske modifikacije makrolida posredovane enzimima, aktivno izbacivanje antibiotika iz stanice te zaštitu mjesta vezanja antibiotika na ribosomu (Fyfe i sur., 2016.).

Promjene ciljnog mjesta djelovanja makrolida posredovane su enzimima metiltransferazama (Erm) koji metiliraju 23S rRNA što rezultira smanjenim afinitetom vezanja makrolida za njihovo vezno mjesto na ribosomu zbog nastalih steričkih smetnji (Dinos, 2017.). Ove enzime kodiraju geni *erm* (engl. *erythromycin resistance methylase*) koji su rasprostranjeni u okolišu, ali i u kliničkim patogenim bakterijama, posebice stafilokokima i streptokokima (Berglund, 2015.; Fyfe i sur., 2016.). U okolišnih bakterija i kliničkih patogena najučestaliji *erm* geni su *erm*B, *erm*C i *erm*F (Berglund, 2015.; Fyfe i sur., 2016.). Osim posredstvom enzima metiltransferaza do promjene ciljnog mjesta može doći i zbog mutacija koje dovode do strukturalnih promjena ciljnog mjesta djelovanja na 23S rRNA ili na ribosomskim proteinima.

Kemijske modifikacije makrolida temelje se na djelovanju enzima makrolidnih fosfotransferaza i esteraza. Makrolidne fosfotransferaze (Mph) fosforiliraju specifične hidroksilne skupine makrocikličkog laktonskog prstena, a najčešće su kodirane genima *mph* (engl. *macrolide phosphotransferase*) uključujući *mph*A, *mph*C, *mph*D i *mph*E te relativno nedavno otkriveni gen *mph*G (Fyfe i sur., 2016.; Nonaka i sur., 2015.). Makrolidne esteraze (Ere) hidroliziraju estersku vezu makrocikličkog laktonskog prstena pri čemu lineariziraju molekulu makrolida koja se tada više ne može vezati na bakterijski ribosom. Ti enzimi su najčešće kodirani genima *ere*A i *ere*B koji su široko rasprostranjeni među kliničkim patogenima i okolišnim bakterijama (Dinos, 2017.).

Aktivno izbacivanje makrolida iz bakterijske stanice odvija se pomoću efluks pumpi, proteinskih transportera koji izbacuju 14-eročlane i 15-eročlane makrolide iz stanice u zamjenu za proton (Fyfe i sur., 2016.). Ove su pumpe najčešće kodirane genima *mef* (engl. *macrolide efflux pump*) koji se većinom nalaze u Gram-pozitivnih bakterija te manjim dijelom i u Gram-negativnih bakterija (Ojo i sur., 2004.). Najučestaliji geni su *mef*A, *mef*E, *mef*B i *mef*C koji se nalaze na različitim pokretnim genetičkim elementima, najčešće transpozonima i plazmidima (Dinos, 2017.; Fyfe i sur., 2016.).

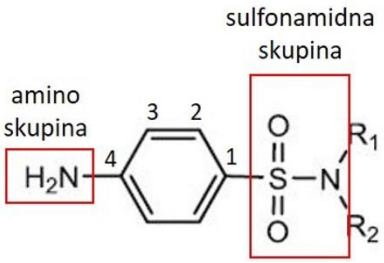
Zaštita veznog mjesta makrolida na ribosomu posredovana je proteinima iz porodice Msr koji svojim vezanjem za ribosom onemogućavaju vezanje makrolidnih antibiotika (Dinos, 2017.). Ti proteini kodirani su genima *msr*A, *msr*C, *msr*D i *msr*E koji omogućuju otpornost na 14-eročlane i 15-eročlane makrolide (Dinos, 2017.). Osim toga, Duval i sur. (2018.) opisali su novi mehanizam otpornosti posredovan djelovanjem specifičnih proteina koji vežu GTPaze, a kodira ih gen *hfl*X. Smatra se da navedeni proteini reagiraju s molekulom makrolida vezanom na ribosomu čime ju izbacuju iz veznog mjesta dopuštajući da se ribosom ponovno uključi u sintezu staničnih proteina.

2.1.4. Sulfonamidni antibiotici

Sulfonamidi pripadaju skupini sintetskih antibiotika koji se osim u medicini široko primjenjuju i u liječenju mnogih domaćih životinja (Prescott, 2013.). Prvi komercijalno dostupan sulfonamidni antibiotik bio je *Prontosil rubrum*, azo-boja koja se u organizmu raspadala na sulfanilamid koji ima antibakterijsku aktivnost (Aminov, 2017.; Sköld, 2000.). Antibiotici ove skupine imaju široki spektar djelovanja prema Gram-pozitivnim bakterijama iz rodova kao što su primjerice *Streptococcus* i *Staphylococcus* te Gram-negativnim bakterijama iz rodova kao što su *Escherichia*, *Shigella*, *Salmonella* i dr. (Prescott, 2013.).

2.1.4.1. Kemijska struktura i mehanizam djelovanja

Sulfonamidni antibiotici su derivati sulfanilamida koji imaju sulfonamidnu i amino skupinu vezanu za benzenski prsten u *para*-položaju (Slika 4.) (Prescott, 2013.). Najpoznatiji predstavnici ove skupine antibiotika su sulfametoksazol koji se u medicini najčešće primjenjuje za liječenje infekcija urinarnog trakta, bronhitisa i prostatitisa, dok se sulfadiazin i sulfametazin primjenuju za liječenje infekcija izazvanih bakterijama te eukariotskim parazitima poput *Pneumocystis jirovecii* i *Toxoplasmosa gondii* u ljudi i životinja (Prescott, 2013.; Tačić i sur., 2017.). Djelovanje svih sulfonamidnih antibiotika temelji se na kompeticiji s *p*-aminobenzojevom kiselinom (PABA) čime se inhibira sinteza folne kiseline koja je nužna za sintezu molekule DNA. Naime, zbog velike strukturne sličnosti sulfonamidi se mogu vezati na aktivno mjesto enzima dihidropteroat sintaze (DHPS) umjesto PABA čime nastaju defektni spojevi koji ne dovode do sinteze folne kiseline (Bhattacharjee, 2016.).



Slika 4. Osnovna kemijska struktura sulfonamidnih antibiotika. Preuzeto i prilagođeno iz Tačić i sur. (2014.).

2.1.4.2. Mehanizmi bakterijske otpornosti

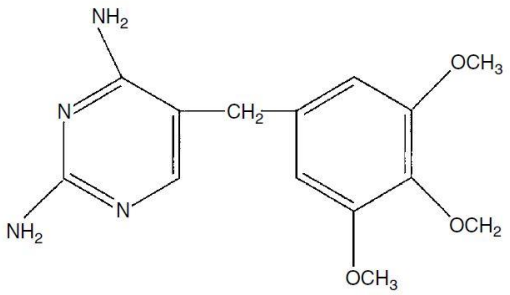
Otpornost bakterija na sulfonamidne antibiotike posljedica je mutacija ili rekombinacija u bakterijskom kromosomu, pretjerane proizvodnje PABA molekula ili horizontalnog prijenosa gena za otpornost na sulfonamide među bakterijama (Sköld, 2000., 2001.). Točkaste mutacije gena *fol*P koji je smješten na kromosomu i kodira za enzim DHPS dovode do supstitucije aminokiseline fenilalanina izoleucinom, što rezultira smanjenim afinitetom vezanja sulfonamida na DHPS (Sköld, 2001.). Ovaj mehanizam prvotno je opisan kod bakterije *E. coli*, a potom i kod različitih bakterijskih vrsta iz roda *Streptococcus* te vrsta *Campylobacter jejuni* i *Haemophilus influenzae* (Buwembo i sur., 2013.), čestih uzročnika bolesti u ljudi. Osim toga, otpornost na sulfonamide može se steći horizontalnom izmjenom te potom rekombinacijom gena čime nastaju nove varijante gena *fol*P kao što je prethodno opisano u vrste *Neisseria meningitidis* (Sköld, 2000., 2001.; Sköld i Swedberg, 2017.). Nadalje, pretjerana proizvodnja PABA molekula u bakterijskoj stanici može rezultirati smanjenom antibakterijskom aktivnošću sulfonamida, a zamijećena je u bakterijskih rodova *Neisseria* i *Streptococcus* (Prescott, 2013.; Then, 1982.). Međutim, najčešće je otpornost bakterija na sulfonamide posljedica postojanja varijanti enzima DHPS koji su otporni na djelovanje antibiotika što je posredovano genima *sul*1 i *sul*2 te, u manjoj mjeri, genom *sul*3 (Sköld, 2001.). Budući da su ovi geni često smješteni na pokretnim genetičkim elementima kao što su plazmidi, široko su rasprostranjeni među bakterijskim populacijama pa otpornost bakterija na sulfonamide danas predstavlja veliki problem u liječenju ljudi i životinja.

2.1.5. 2,4-diaminopirimidinski antibiotici

2,4-diaminopirimidinski antibiotici su sintetska skupina antibiotika koji djeluju kao inhibitori enzima dihidrofolat reduktaze (DHFR) (Prescott, 2013.). Najvažniji predstavnik ove skupine je trimetoprim, diaminopirimidinski derivat koji se vrlo često upotrebljava u kombinaciji sa sulfonamidima (najčešće sulfametoksazolom) zbog njihova sinergističkog djelovanja (Prescott, 2013.). Trimetoprim ima širok spektar djelovanja, a najčešće se primjenjuje za liječenje infekcija urinarnog trakta izazvanih Gram-pozitivnim i Gram-negativnim bakterijama (Prescott, 2013.).

2.1.5.1. Kemijska struktura i mehanizam djelovanja

Osnovna kemijska struktura trimetoprima sastoji se od dvije amino skupine vezane na 2 i 4 položaju pirimidinskog prstena, a prikazana je na Slici 5.



Slika 5. Kemijska struktura trimetoprima. Preuzeto iz Prescott i sur. (2013.).

Mehanizam djelovanja trimetoprima zasniva se na blokiranju sinteze folne kiseline inhibicijom enzima DHFR čime ometa pretvorbu dihidrofolne kiseline u tetrahidrofolnu kiselinu (Prescott, 2013.). Ovaj antibiotik primijenjen pojedinačno djeluje na bakterijsku stanicu bakteriostatski (inhibira rast i razvoj bakterijske stanice), dok u kombinaciji sa sulfonamidima ima baktericidno djelovanje (ubija stanicu) koje ostvaruje blokiranjem dviju različitih faza u sintezi tetrahidrofolne kiseline (Bhattacharjee, 2016.).

2.1.5.2. Mehanizmi bakterijske otpornosti

Budući da je trimetoprim, kao i sulfonamidi, sintetski antibiotik, mala je vjerojatnost postojanja prirodnih enzima koji modificiraju ili inaktiviraju antibiotik. Stoga je otpornost bakterija na trimetoprim uzrokovana različitim promjenama na kromosomu kao što su mutacije u promotorskoj regiji što uzrokuje prekomjerno stvaranje enzima DHFR te točkaste mutacije

gena *dfr*, što rezultira stvaranjem izmijenjenog enzima DHFR i smanjenim afinitetom vezanja trimetoprima za taj enzim (Prescott, 2013.; Sköld, 2001.). Ovaj posljednji mehanizam dovodi do visokog stupnja otpornosti na trimetoprim, a najčešće je posredovan genima *dfr*A i *dfr*B koji su često smješteni na plazmidima pa se olakšano šire među bakterijama (van Hoek i sur., 2011.; Sköld, 2001.). Pored toga, otpornost na trimetoprim može biti i posljedica promjena u strukturi porina te, posljedično, smanjene stanične propusnosti zbog čega antibiotik ne može ući u bakterijsku stanicu što je zamijećeno kod nekih Gram-negativnih bakterija iz rodova *Klebsiella, Enterobacter* i *Serratia*. Uz to, niska razina otpornosti na trimetoprim može biti posredovana i mutacijom gena *thy* koji kodira za enzim timidilat sintazu (TYMS) (Sköld, 2001.; Sköld i Swedberg, 2017.). To dovodi do inaktivacije enzima TYMS zbog čega stanica postaje ovisna o vanjskoj zalihi timina, dušične pirimidinske baze potrebne za sintezu molekule DNA. Time se zaobilazi put sinteze folne kiseline, neophodne za sintezu timina, zbog čega se sinteza bakterijske DNA nastavlja neovisno o djelovanju antibiotika.

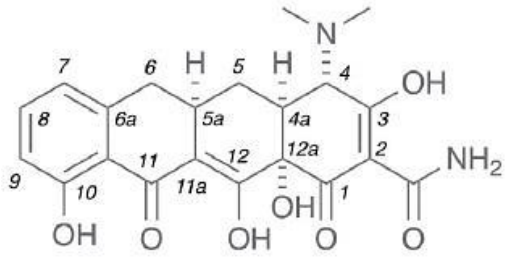
2.1.6. Tetraciklinski antibiotici

Antibiotici iz skupine tetraciklina prirodni su antibiotici nastali kao proizvod sekundarnog metabolizma bakterija roda *Streptomyces* (Aminov, 2017.). Ovi se antibiotici širokog spektra djelovanja primjenjuju u liječenju brojnih bakterijskih infekcija u ljudi kao što su infekcije probavnog, mokraćnog i dišnog sustava te infekcije kože i očiju (Aminov, 2017.). Osim u medicini, tetraciklini se učestalo primjenjuju i u veterinarstvu te u akvakulturi kod uzgoja vodenih organizama poput riba i mekušaca (Rocha Guidi i sur., 2018.; Quesada i sur., 2013.). Uz to, ovi se antibiotici u nekim državama primjenjuju i na životinjskim farmama kao promotori rasta, što je u Europskoj Uniji zabranjeno od 2006. godine (Aminov, 2017.).

2.1.6.1. Kemijska struktura i mehanizam djelovanja

Tetraciklini pripadaju skupini poliketidnih antibiotika koji u osnovnoj kemijskoj strukturi imaju oktahidronaftacen kojeg čine četiri linearno povezana kondenzirana šesteročlana ugljikova prstena („tetraciklinska jezgra“) (Slika 6.). Na tu jezgru mogu biti vezane različite funkcionalne skupine od kojih su dimetilamino- i hidroksilna skupina najvažnije za antibakterijsku aktivnost (Aminov, 2017.; Chopra i Roberts, 2001.). Najpoznatiji predstavnici tetraciklinske skupine antibiotika su klortetraciklin, oksitetraciklin, tetraciklin i doksiciklin (Daghrir i Drogui, 2013.; Markley i Wencewicz, 2018.). Mehanizam njihovog bakteriostatskog djelovanja temelji se na inhibiciji sinteze proteina vezanjem na 30S podjedinicu ribosoma, što

onemogućava vezanje aminoacilirane tRNA na vezno mjesto ribosoma i produljenje peptidnog lanca (Bhattacharjee, 2016.; Chopra i Roberts, 2001.).



Slika 6. Osnovna kemijska struktura tetraciklinskih antibiotika. Preuzeto iz Markley i Wencewicz (2018.).

2.1.6.2. Mehanizmi bakterijske otpornosti

Nekoliko je mehanizama odgovorno za otpornost na tetracikline, a to su izbacivanje antibiotika iz stanice, promjena i zaštita ciljnog mjesta djelovanja antibiotika, enzimska inaktivacija antibiotika te smanjenje propusnosti stanične stijenke (Markley i Wencewicz, 2018.; Roberts, 2005.). Najčešće je otpornost na tetracikline posredovana aktivnim izbacivanjem antibiotika iz stanice pomoću efluks pumpi pri čemu dolazi do zamjene kompleksa tetraciklin-kation za proton suprotno koncentracijskom gradijentu (Roberts, 2005.). Geni koji kodiraju za efluks pumpe, primjerice *tet*A, *tet*C i *tet*39, su uglavnom pronađeni u Gram-negativnim bakterijama (Roberts, 2002., 2005.), dok su neki, kao što su *tet*K i *tet*L, pronađeni kod Gram-pozitivnih bakterija. Zaštita ciljnog mjesta djelovanja tetraciklina je posredovana proteinima odgovornim za zaštitu ribosoma koji vezanjem na ribosom uzrokuju alosteričke promjene uslijed čega dolazi do otpuštanja tetraciklina, vraćanja ribosoma u početnu konformaciju i daljnje neometane sinteze proteina (Roberts, 2005.). Ovaj mehanizam posredovan je genima kao što su *tet*M, *tet*O i *tet*Q koji su podjednako zastupljeni među Gram-pozitivnim i Gram-negativnim bakterijama (Roberts, 2002.). Pored toga, promjena ciljnog mjesta djelovanja može biti i posljedica mutacija u genima koji kodiraju za vezno mjesto što rezultira smanjenim afinitetom vezanja tetraciklina za ribosom (Markley i Wencewicz, 2018.). Nadalje, inaktivacija tetraciklina temelji se na selektivnoj oksidaciji pomoću enzima oksidoreduktaza, što dovodi do razgradnje molekule antibiotika u prisutnosti kisika i nikotinamida adenin dinukleotid fosfata (NADPH) (Markley i Wencewicz, 2018.). Suprotno prethodno spomenutim mehanizmima, na ovaj način dolazi do potpunog nestanka tetraciklina

i smanjenja njegove koncentracije unutar i izvan bakterijske stanice. Najučestalija oksidoreduktaza je Tet(X) oksidoreduktaza ovisna o NADPH-u posredovana genom *tet*X koji je široko rasprostranjen među okolišnim bakterijama te kliničkim patogenim bakterijama (Deng i sur., 2014; Leski i sur., 2013.; Ming i sur., 2017.). Naposljetku, otpornost na tetracikline posredovana smanjenom propusnošću stanične stijenke posljedica je morfoloških promjena ili smanjene ekspresije porina zbog čega je smanjen ili u potpunosti onemogućen unos tetraciklina u bakterijsku stanicu (Markley i Wencewicz, 2018.).

2.1.7. Mehanizmi širenja otpornosti na antibiotike među bakterijama

Geni za otpornost na antibiotike mogu biti smješteni na bakterijskom kromosomu i/ili na pokretnim genetičkim elementima kao što su plazmidi, integroni i transpozoni, a među bakterijama se mogu prenositi vertikalno ili horizontalno (Scott i sur., 2016.). Vertikalan prijenos je prijenos gena za otpornost, smještenih najčešće na kromosomu, na nove generacije jedne bakterijske vrste razmnožavanjem tj. staničnom diobom (Kalenić, 2000.). Horizontalni prijenos gena je izmjena genetičkog materijala među filogenetski bliskim ili udaljenim bakterijskim vrstama, rodovima ili čak koljenima (Peterson i Kaur, 2018.). Ovaj prijenos je stoga važan izvor genetičke varijabilnosti u bakterija i često je odgovoran za stjecanje otpornosti na antibiotike. Na ovaj način se šire geni otpornosti koji su smješteni na pokretnim genetičkim elementima kao što su plazmidi (Partridge i sur., 2018.; Sultan i sur., 2018.). Osim toga, na ovakav način mogu se širiti i geni smješteni na bakterijskom kromosomu ako se radi o transpozonu koji se može prenijeti s kromosoma na plazmid.

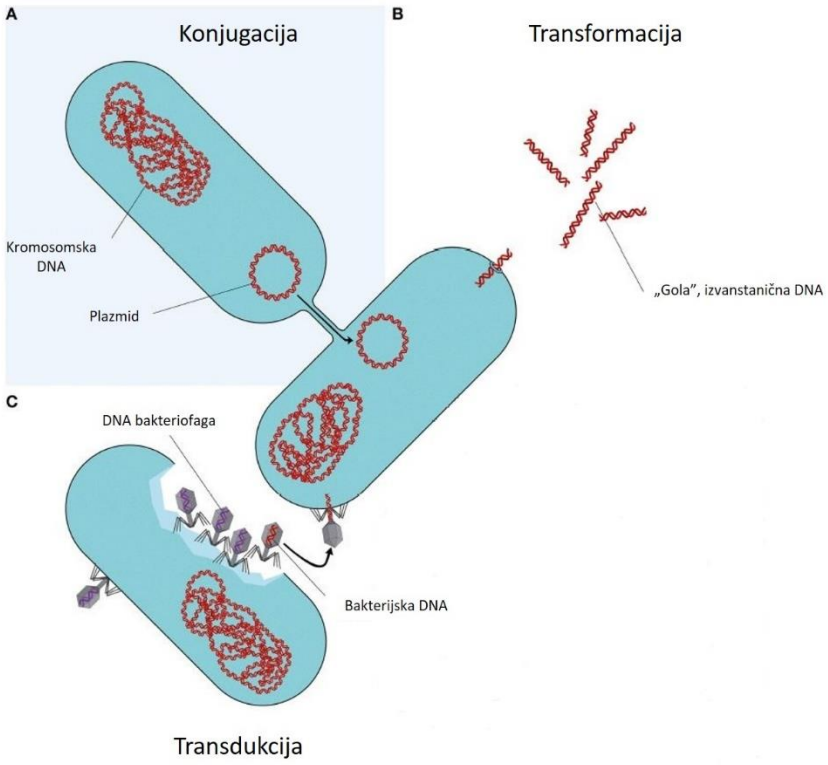
Mehanizmi horizontalnog prijenosa gena kojima bakterije mogu steći otpornost su konjugacija, transformacija i transdukcija, a prikazani su na Slici 7.

Konjugacija je prijenos fragmenata kromosoma ili plazmida iz stanice davatelja (donor) u stanicu primatelja (recipijent) koji zahtijeva direktni kontakt između dvije bakterijske stanice preko pilusa (konjugacijskog mostića) (von Wintersdorff i sur., 2016.). Neki su plazmidi vrlo specifični pa se konjugacija odvija samo među određenim vrstama bakterija, dok drugi imaju manju specifičnost i mogu se prenositi između bakterija različitih vrsta, rodova ili porodica (Volkova i sur., 2014.; von Wintersdorff i sur., 2016.). Prijenos bakterijskih plazmida konjugacijom smatra se najčešćim HGT mehanizmom za širenje gena otpornosti na antibiotike, dok se transformacija i transdukcija smatraju manje važnima. Razlog tome je to što konjugacija omogućava bolju zaštitu prijenosa DNA od utjecaja okoline i djelotvornije načine unošenja DNA u stanicu recipijenta od transformacije te postoji širi raspon stanica koje mogu konjugirati

u odnosu na one u koje se DNA može prenijeti putem transdukcije bakteriofagom (Norman i sur., 2009.).

Transdukcija je prijenos DNA iz stanice donora u stanicu recipijenta pomoću bakterijskog virusa, bakteriofaga (Modi i sur., 2013.). Iako se transdukcijom ne ostvaruje fizički kontakt između stanice donora DNA i stanice recipijenta, bakteriofag se mora replicirati unutar stanice donora litičkim ili lizogenim ciklusom. U lizogenom ciklusu, bakteriofag ugrađuje svoj genom u genom stanice domaćina (profag) te se replicira kao dio domaćina (Balcazar, 2014.). U nepovoljnim uvjetima može doći do indukcije profaga, odnosno izlaska fagne DNA iz bakterijskog kromosoma pri čemu profag zahvaća i dio bakterijskog kromosoma i potom ulazi u litički ciklus. Tijekom litičkog ciklusa u stanici domaćina dolazi do replikacije bakteriofaga pri čemu se mali dio DNA stanice domaćina može upakirati u novosintentizirani omotač bakteriofaga (Norman i sur., 2009). Tako novonastale virusne čestice se iz stanice domaćina lizom oslobađaju u okoliš u kojem mogu zaraziti novu stanicu domaćina. Transdukcijom je došlo do širenja otpornosti na različite skupine antibiotika među kliničkim patogenima iz rodova *Enterococcus*, *Streptococcus* i *Salmonella* (von Wintersdorff i sur., 2016.). To ukazuje da bi bakteriofagi mogli biti važan spremnik gena za otpornost na antibiotike, a novija istraživanja sugeriraju da bi njihova uloga u širenju otpornosti na antibiotike mogla biti veća nego što se prethodno smatralo (Balcazar, 2014.; von Wintersdorff i sur., 2016).

Transformacija je prijenos „golih“ fragmenata DNA iz okoline u stanicu recipijenta koji se potom ugrađuju u kromosom i/ili plazmid te funkcionalno eksprimiraju u bakterijskoj stanici (Gothwal i Shashidhar, 2015.; von Wintersdorff i sur., 2016.). Često su ovi „goli“ fragmenti DNA oslobođeni iz stanice staničnom smrću ili aktivnim transportom (Chen i Dubnau, 2004.). Ovaj je proces kod bakterija prirodan, a zahtijeva prisutnost fragmenta DNA u izvanstaničnom prostoru, kompetentnost bakterije recipijenta te da se DNA koja se prenosi stabilizira procesom rekombinacije u genom recipijenta ili ponovnom cirkularizacijom, u slučaju plazmidne DNA (Thomas i Nielsen, 2005.). Premda se konjugacija smatra najčešćim HGT mehanizmom, pokazalo se da i transformacija ima značajnu ulogu u širenju otpornosti na antibiotike (Domingues i sur., 2012a.; Mao i sur., 2014.; von Wintersdorff i sur., 2016.).

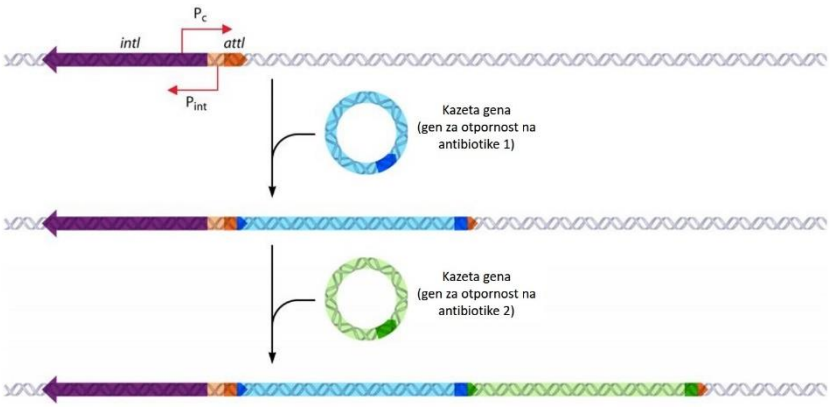


Slika 7. Mehanizmi horizontalnog prijenosa gena i stjecanja otpornosti na antibiotike u bakterija. Slika je preuzeta i prilagođena iz von Wintersdorff i sur. (2016.).

2.1.7.1. Integroni

Integroni su pokretni genetički elementi koji imaju sposobnost prihvaćanja i uključivanja novih gena mjesno-specifičnom rekombinacijom u već postojeći genetički materijal te mogu uspješno provoditi ekspresiju tih gena kao dio kazeta gena (Domingues i sur., 2012b.). Oni su naširoko poznati po svojoj ulozi u širenju otpornosti na antibiotike, posebice u Gram-negativnih bakterija. Osim toga, pokazalo se da imaju važan utjecaj na adaptaciju bakterija i evoluciju genoma. Iako sami po sebi nisu pokretni, među bakterijama se prenose kao dijelovi konjugacijskih plazmida ili transpozona (Deng i sur., 2015.). Kao što je prikazano na Slici 8., svaki integron sastoji se od: 1) gena *int*I koji kodira protein integron-integrazu i promotora P_{int} odgovornog za ekspresiju tog gena, 2) gena *attI* koji čini rekombinacijsko mjesto i 3) promotora P_c potrebnog za uspješnu transkripciju i ekspresiju genskih kazeta prisutnih u sklopu integrona (Davies i Davies, 2010.; Jové i sur., 2017.). Uloga integraze je da katalizira rekombinaciju između ulaznih kazeta gena i *attI* mjesta na integronu u rekombinacijsko mjesto *attC* (Slika 8.) (Partridge i sur., 2018.). Ovaj proces je reverzibilan tj. kazete gena se mogu i

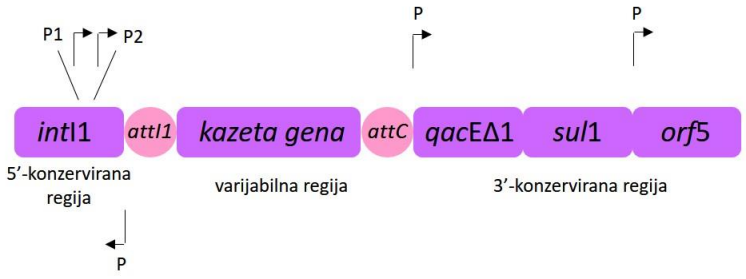
izrezati u obliku slobodnih, cirkularnih molekula DNA (Collis i Hall, 1992.). Nakon što se kazeta gena rekombinacijom ugradi u integron provodi se ekspresija gena preko promotora P_c (Slika 8.) (Deng i sur., 2015.; Domingues i sur., 2012b.; Partridge i sur., 2018.). To omogućuje stjecanje novih gena za otpornost kao dio kazeta gena. Kazete gena su mali mobilni elementi koji se od sastoje nešto više od jednog gena, otvorenog okvira čitanja i rekombinacijskog mjesta *attC* koje se originalno nazivalo element 59 nukleinskih baza. Do danas je identificirano više od 130 genskih kazeta za otpornost na antibiotike u sklopu integrona (Cambray i sur., 2010.). Na temelju razlike u sekvenci integron-integraze, integroni se mogu podijeliti u nekoliko skupina od kojih skupine 1, 2 i 3 imaju glavnu ulogu u širenju gena za otpornost na antibiotike (Deng i sur., 2015.; Sultan i sur., 2018.). Pogotovo su integroni koji pripadaju skupinama 1 i 2 često detektirani u okolišu onečišćenom antibioticima noseći različite tipove gena otpornosti (Kristiansson i sur., 2011.; Marathe i sur., 2013.; Moura i sur., 2012.).



Slika 8. Struktura integrona i stjecanje genskih kazeta unutar integrona. Slika je preuzeta i prilagođena iz rada Davies i Davies (2010.).

Integroni skupine 1 smatraju se glavnim vektorima za širenje otpornosti na antibiotike posljednjih 50 godina. Prvo su otkriveni u kliničkim bakterijskim sojevima gdje su često bili integrirani u transpozone iz porodice Tn*402* (Cambray i sur., 2010.; Gillings, 2014.). Istraživanja su potom pokazala da ti integroni nisu ograničeni samo na bolničke sredine nego su također rasprostranjeni i u različitim dijelovima okoliša uključujući otpadne vode, površinske vode i sediment noseći različite tipove gena otpornosti na antibiotike (Huang i sur., 2019.; Kristiansson i sur., 2011.; Li i sur., 2010.). Osim strukture karakteristične za ostale integrone opisane u gornjem odjeljku, integroni skupine 1 imaju tri vrste rekombinacijskih

mjesta (*attI1*, *attC* i sekundarna mjesta), integron-integrazu kodiranu genom *int*I1, dok se nizvodno od toga nalazi 3'-konzervirana regija u kojoj su smješteni geni odgovorni za otpornost na sulfonamide (*sul*1) i antiseptike (kvaterne amonijeve spojeve, *qac*EΔ1) (Slika 9.) (Cambray, i sur., 2010.; Deng i sur., 2015.). Spomenuti integroni široko su rasprostranjeni među Gram-negativnim bakterijama, uključujući rodove *Acinetobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas* i *Salmonella* te Gram-pozitivnim bakterijama uključujući rodove *Streptococcus*, *Staphylococcus*, *Enterococcus* i *Aerococcus* (Deng i sur., 2015.), uzročnike brojnih bolesti u ljudi. Gen *int*I1 predložen je kao biomarker za procjenu antropogenog onečišćenja okoliša (Gillings, 2017., 2018.; Gillings i sur., 2015.), a pronađen je u otpadnim vodama i obrađenom aktivnom mulju uređaja za pročišćavanje otpadnih voda, stajskom gnojivu kao i u različitim sastavnicama okoliša poput rijeka, jezera i sedimenata (Gillings, 2018.). Tako je povećani sadržaj navedenog gena nađen i u vodenom okolišu onečišćenom antibioticima putem ispusta otpadnih voda iz farmaceutskih industrija (Kristiansson i sur., 2011.; Li i sur., 2009., 2010.).



Slika 9. Struktura integrona skupine 1. Preuzeto i prilagođeno iz Deng i sur. (2015.).

Integroni skupine 2 imaju sličnu funkciju kao integroni skupine 1, ali posjeduju samo mali dio gena za otpornost koji se nalaze u sastavu integrona skupine 1. Najčešće se povezuju s otpornošću na aminoglikozide i trimetoprim, a pronađeni su kod bakterija iz rodova kao što su *Salmonella*, *Pseudomonas*, *Acinetobacter* te kod porodice *Enterobacteriaceae* (Deng i sur., 2015.; Sultan i sur., 2018.). Suprotno tome, integroni skupine 3 najčešće su povezani s otpornošću na β-laktamske antibiotike noseći gene od kliničkog značaja kao što su bla_{GES-1} i $bla_{IMP-1,}$ a pronađeni su u brojnim patogenim bakterijama iz rodova kao što su *Klebsiella*, *Escherichia*, *Acinetobacter*, *Serratia* i *Pseudomonas* (Deng i sur., 2015.; Sultan i sur., 2018.).

2.1.7.2. Plazmidi

Najistaknutiju ulogu u horizontalnom prijenosu gena za otpornost na antibiotike zasigurno imaju plazmidi. Plazmidi su male, kružne, samoreplicirajuće molekule DNA koje u bakterijskoj stanici mogu bili slobodne u citoplazmi ili se mogu integrirati u kromosomsku DNA. Svaki plazmid ima određeni broj gena koji nisu od esencijalnog značenja za bakterijsku stanicu, ali mogu biti vrlo korisni za brzu prilagodbu i preživljavanje bakterije u novostvorenim uvjetima okoliša (Grenni i sur., 2018.; Smalla i sur., 2015.). Pretpostavlja se da ovi pokretni genetički elementi potiču adaptaciju bakterija na stresne i promjenjive uvjete u okolišu (Heuer i Smalla, 2012.). Razlikujemo konjugacijske plazmide koji posjeduju faktore fertilnosti (F-faktore) pomoću kojih se uspostavlja kontakt između dvije bakterijske stanice nužan za prijenos plazmida iz stanice donora u stanicu recipijenta i plazmide koji ne posjeduju F-faktore te se među bakterijskim stanicama prenose uz pomoć konjugacijskih plazmida (Partridge i sur., 2018.; Sultan i sur., 2018.). Smatra se da su, uz integrone, plazmidi ključni vektori u globalnom širenju gena za otpornost na antibiotike i promotori višestruke otpornosti jer često nose gene za otpornost na više skupina antibiotika (Blau i sur., 2018.; Flach i sur., 2015.; Heuer i sur., 2009.; Heuer i Smalla, 2012.; Jechalke i sur., 2013.).

Klasifikacija plazmida je moguća na nekoliko načina, a najčešća je ona na temelju analize replikona. Replikoni su konzervirane regije plazmidne molekule DNA koje su ključne za proces replikacije. Zaduženi su za kontrolu broja kopija plazmida u bakterijskoj stanici pri čemu definiraju i spektar bakterijskih vrsta u kojima plazmidi mogu opstati (Shintani i sur., 2015.). Plazmidi koji imaju isti replikon su inkompatibilni i svrstavaju se u istu skupinu inkompatibilnosti, Inc (Shintani i sur., 2015.), dok su plazmidi s različitim replikonom kompatibilni (Partridge i sur., 2018.). Prema potencijalu širenja u različite bakterijske vrste, plazmidi se dijele na one koji su vezani uz uzak spektar bakterijskih domaćina (npr. IncF) i na one koji se mogu naći u velikom broju različitih bakterijskih vrsta (npr. IncP-1, IncN, IncA/C, IncQ, IncL/M) (Klümper i sur., 2015.; Shintani i sur., 2010., 2014.; Suzuki i sur., 2010.). Pritom se posebno ističu plazmidi skupine IncP-1 koji su važni u kliničkom i okolišnom kontekstu, a vrlo su „promiskuitetni“ i često nose gene za otpornost na više skupina antibiotika (Heuer i sur., 2012.; Popowska i Krawczyk-Balska, 2013.; Wolters i sur., 2015.). Spomenuti plazmidi su detektirani u različitim okolišnim sredinama kao što su tlo (Klümper i sur., 2015.), riječni sedimenti onečišćeni metalima (Cyriaque i sur., 2020.) te komunalne otpadne vode (Rahube i sur., 2014.). Plazmidi IncP-1 često nose gene otpornosti na aminoglikozide, β-laktame, sulfonamide, tetracikline i trimetoprim te tako doprinose širenju višestruke otpornosti

(Popowska i Krawczyk-Balska, 2013.). Uočena je također povezanost gena za otpornost na antiseptike (*qac*EΔ1) s ovim plazmidima (Popowska i Krawczyk-Balska, 2013.). Pored plazmida IncP-1, u širenju otpornosti na antibiotike u okolišu važnu ulogu imaju i plazmidi IncA/C i IncN skupina inkompatibilnosti. Navedeni plazmidi su konjugacijski i povezuju se s onečišćenjem okoliša antibioticima putem ispusta iz farmaceutskih industrija u Indiji (Flach i sur., 2015.). Pokazalo se da plazmidi IncA/C iz tako onečišćenog okoliša nose velik broj gena odgovornih za otpornost na različite skupine antibiotika koji su uglavnom lokalizirani u sklopu integrona skupine 1, insercijskih sekvenci (IS*CR*) ili transpozona (Tn*21*). Nasuprot tome, plazmidi IncN skupine inkompatibilnosti porijeklom iz okoliša onečišćenog antibioticima obično nose gene odgovorne za otpornost na fluorokinolone i trimetoprim u sastavu integrona skupine 1 (Flach i sur., 2015.).

2.2. Otpornost bakterija na antibiotike u okolišu

Iako se bakterijska otpornost na antibiotike tradicionalno veže uz bolničke sredine, istraživanja provedena zadnjih desetak godina ukazala su na okoliš kao važan spremnik i izvor otpornih mikroorganizama koji posjeduju veliku zalihu gena otpornosti na antibiotike koja se naziva rezistom (D'Costa i sur., 2011.; Martínez, 2008.). To je dijelom posljedica prisutnosti mikroorganizama koji proizvode antibiotike (neke gljive i bakterije) pa su razvili otpornost kao obranu od samouništenja (Surette i Wright, 2017.). S druge strane, kontinuiranim onečišćenjem okoliša antibioticima narušila se ravnoteža prirodnog ekosustava te je došlo do selekcije bakterijskih populacija otpornih na antibiotike. Pokazalo se da čak i niske, subinhibitorne koncentracije antibiotika, slične onima nađenim u različitim vodenim i kopnenim sustavima (Kümmerer, 2009.), mogu potaknuti selekciju otpornih bakterija putem horizontalnog prijenosa plazmida s genima za otpornost (Andersson i Hughes, 2014.; Gullberg i sur., 2011.; Sandegren, 2014.). Pored antibiotika, istraživanja su pokazala da onečišćenje okoliša metalima, čak i u relativno niskim koncentracijama, također potiče selekciju otpornosti na antibiotike zbog zajedničkih genetičkih ili fizioloških mehanizama otpornosti na antibiotike i metale (Di Cesare i sur., 2016.; Pal i sur., 2017.). Naime, geni za otpornost na antibiotike i metale često su smješteni na istim pokretnim genetičkim elementima poput plazmida ili se nalaze u istoj bakterijskoj stanici pa uslijed izloženosti metalima dolazi do indirektne selekcije otpornosti na antibiotike (tzv. ko-otpornost) (Baker-Austin i sur., 2006.; Pal i sur., 2017.). Druga mogućnost uključuje tzv. unakrsnu otpornost kada jedan gen ili mehanizam otpornosti (npr. prekomjerna ekspresija gena, efluks pumpa) dovodi do otpornosti bakterija na obje skupine spojeva, antibiotike i metale (Baker-Austin i sur., 2006.; Pal i sur., 2017.). Osim toga, sve više

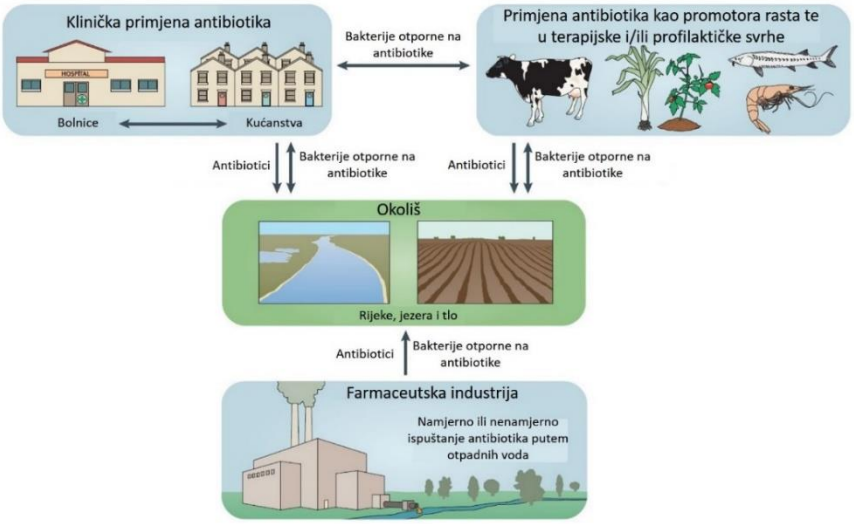
istraživanja ukazuje na povezanost povećanog razvoja otpornosti na antibiotike u okolišu i pojave otpornosti na antibiotike među patogenima čovjeka. Tako je primjerice zapaženo da geni za otpornost na β-laktame (*bla*$_{\text{CTX-M}}$ i *bla*$_{\text{OXA-48}}$) i kinolone (*qnr*A) koji su široko rasprostranjeni među kliničkim patogenima iz porodice *Enterobacteriaceae* imaju vjerojatno porijeklo u okolišnim bakterijama iz roda *Kluvyera* i *Shewanella* te bakterijama iz porodice *Vibrionaceae* (Cantón i Coque, 2006.; Poirel i sur., 2005a., 2005b., 2012.). Pored toga, Forsberg i sur. (2012.) pokazali su da mikroorganizmi iz tla posjeduju gene za otpornost na β-laktame (*bla*$_{\text{P-1}}$), tetracikline (*tet*A), aminoglikozide (*aad*B i *aac*A4), sulfonamide (*sul*1) i amfenikol koji su 100 % identični onima iz kliničkih patogena poput *A. baumanii, K. pneumoniae, S. typhimurium, P. aeruginosa, E. coli, En. cloacae* i dr. Takve spoznaje ukazuju na važnost okoliša kao izvora i spremnika gena za otpornost na antibiotike koji su raspoloživi za razmjenu s ljudskim i životinjskim patogenim bakterijama. Putevi kojima ljudi mogu doći u kontakt s tim otpornim bakterijama i/ili njihovim genima uključuju konzumaciju onečišćene hrane ili vode, rekreativne aktivnosti u onečišćenoj površinskoj vodi te inhalaciju aerosola (O'Flaherty i Cummins, 2017.; Pruden i sur., 2013.). Stoga onečišćenje okoliša antibioticima predstavlja ne samo direktnu opasnost za zdravlje ekosustava izazivanjem potencijalno toksičnih učinaka na žive organizme nego i indirektnu opasnost za zdravlje ljudi i životinja putem razvoja i širenja otpornosti na antibiotike. Čovjek stalno izmjenjuje svoju mikrobiotu s okolinom te se stoga ograničavanju širenja otpornosti na antibiotike mora pristupiti zajednički s obje strane, bolničke i izvanbolničke. Pritom se uspjeh pojedinih intervencija usmjerenih prema kontroli širenja otpornosti može mjeriti jedino ako postoje podaci o razini otpornosti u obje sredine prije i poslije intervencije. Međutim, za razliku od bolničkih sredina u kojima se otpornost kontinuirano prati na nacionalnoj i međunarodnoj razini, programi za praćenje otpornosti u okolišu još uvijek ne postoje. Takvi podaci su pak prijeko potrebni za procjenu rizika te provođenje mjera za zaštitu zdravlja ljudi i okoliša.

2.2.1. Unošenje antibiotika i bakterija otpornih na antibiotike u okoliš

Premda su antibiotici spojevi koji su prirodno prisutni u okolišu, istraživanja provedena posljednjih desetak godina ukazala su da se ovi spojevi kontinuirano unose u okoliš kroz razne antropogene aktivnosti. Stoga su antibiotici tek u novije vrijeme privukli pozornost kao potencijalna prijetnja za okoliš te se ubrajaju u tzv. „nova zagađivala" (engl. *Emerging Contaminants*). Za razliku od klasičnih prioritetnih zagađivala, poput primjerice pesticida čije je unošenje u okoliš odavno ograničeno propisima, koncentracije antibiotika u okolišu još uvijek nisu regulirane zakonom, iako se ovi spojevi odlikuju visokom biološkom aktivnošću pa

mogu izazvati nepovoljne učinke na okolišne organizme. Naime, kontinuiranim unosom antibiotika u okoliš vodeni organizmi mogu biti izloženi neprekidnom djelovanju takvih spojeva (Scott i sur., 2016.). To djelovanje se može, s jedne strane, manifestirati toksičnim učincima na alge, biljke i životinje te bakterijske zajednice inhibirajući ili uništavajući pojedine skupine koje su važne za normalno funkcioniranje ekosustava, čime se direktno ugrožava zdravlje okoliša (Grenni i sur., 2018.; Carvalho i Santos, 2016.). S druge strane, onečišćenje okoliša antibioticima može imati nepovoljan utjecaj na zdravlje ljudi zbog opasnosti od nastanka bakterija otpornih na antibiotike koje se mogu prenijeti izvan okoliša (Forsberg i sur., 2012.; Grenni i sur., 2018.; Huijbers i sur., 2015.).

Putevi unošenja antibiotika u okoliš prikazani su na Slici 10. Oni uključuju ispuste komunalnih i bolničkih otpadnih voda koje sadrže antibiotike izlučene iz ljudskog ili životinjskog organizma nakon terapijske primjene te ispuste otpadnih voda iz proizvodnih pogona farmaceutske industrije (Andersson i Hughes, 2014.; Larsson, 2014b.). Osim toga, antibiotici dospijevaju u okoliš i gnojenjem obradivih površina stajskim gnojivom s farmi domaćih životinja na kojima se antibiotici koriste kao promotori rasta te radi liječenja ili prevencije bolesti (Hölzel i sur., 2010.; Kemper, 2008.), ali i primjenom obrađenog aktivnog mulja s komunalnih uređaja za obradu otpadnih voda (Gothwal i Shashidar, 2015.). Izvor onečišćenja okoliša također predstavlja primjena antibiotika u uzgoju voća i povrća (McManus i sur., 2002.) te u akvakulturi kod uzgoja vodenih organizama poput riba i mekušaca (Cabello i sur., 2016.). Pritom treba naglasiti da bakterije otporne na antibiotike uglavnom slijede iste puteve unosa u okoliš kao i antibiotici.



Slika 10. Glavni putevi unošenja antibiotika i bakterija otpornih na antibiotike u okoliš. Preuzeto i prilagođeno iz Andersson i Hughes (2014.).

Uređaji za pročišćavanje otpadnih voda prepoznati su kao jedni od glavnih izvora onečišćenja vodenog okoliša antibioticima i bakterijama otpornim na antibiotike (Manaia i sur., 2018.; Michael i sur., 2013.; Rizzo i sur., 2013.). Budući da se dio antibiotika nakon terapijske primjene (od 30 do 90 %) izlučuje iz ljudskog organizma u nepromijenjenom obliku, ti spojevi putem komunalnih otpadnih voda dospijevaju do uređaja za pročišćavanje otpadnih voda u kojima se slabo ili nepotpuno uklanjaju (Carvalho i Santos, 2016.; O'Flaherty i Cummins, 2017.). Bakterije otporne na antibiotike se također izlučuju iz ljudskog organizma fekalijama te tako dospijevaju u komunalne otpadne vode i putem njih u uređaje za obradu otpadnih voda (Cai i sur., 2014.). Zbog nepotpunog uklanjanja u uređajima za pročišćavanje, obrađene otpadne vode koje se ispuštaju u prirodne vode (najčešće rijeke) često sadržavaju antibiotike u koncentraciji tipično do 10 μg L^{-1} te otporne bakterije u koncentraciji od 10^5 do 10^7 u 100 mL otpadne vode (Manaia i sur., 2016.; Michael i sur., 2013.; Zhou i sur., 2019.). Što se tiče antibiotika, pokazalo se da su te koncentracije u obrađenim komunalnim otpadnim vodama minijaturne u usporedbi s koncentracijama u otpadnim vodama iz pogona za proizvodnju antibiotika. Naime, koncentracije antibiotika u industrijskim otpadnim vodama u nekim azijskim zemljama poput Indije i Kine bile su reda veličine nekoliko desetaka mg L^{-1}, što je oko 1.000 puta više nego u komunalnim otpadnim vodama pa se farmaceutski pogoni smatraju značajnim točkastim izvorima onečišćenja okoliša antibioticima (Larsson, 2014a.; Šimatović i Udiković-Kolić, 2019.). Ispusti otpadnih voda iz takvih pogona su stoga rezultirali onečišćenjem površinskih voda i njihovih sedimenata, a zatim i podzemnih voda te voda za piće antibioticima (Fick i sur., 2009.; Kristiansson i sur., 2011.; Li i sur., 2009., 2010.; Rutgersson i sur., 2014.). Posljedično, pokazalo se da je takvo onečišćenje pridonijelo razvoju i širenju bakterija otpornih na antibiotike, uključujući i nastanak patogena otpornih na više skupina antibiotika (Li i sur., 2009., 2010.; Lübbert i sur., 2017.; Marathe i sur., 2013.; Sidrach-Cardona i sur., 2014.). Pritom se također pokazalo da su sedimenti važni spremnici ne samo antibiotika nego i otpornih bakterija te njihovih gena, jer zadržavaju hranjive tvari koje pogoduju rastu bakterija te štite bakterijske stanice/gene od svjetla i predatorskih praživotinja (Sidrach-Cardona i sur., 2014.; Singh i sur., 2019.; Rutgersson i sur., 2014.).

2.3. Otpadne vode farmaceutskih industrija

Otpadne vode iz farmaceutskih industrija nastaju kao nusprodukt tehnoloških procesa kemijske sinteze antibiotika ili formulacije lijekova s antibioticima kao aktivnim tvarima, a mogu nastati i pranjem međuprodukata i konačnog produkta te kao rezultat pranja opreme i površina tijekom određenih procesa proizvodnje antibiotika. Njihov sastav je često promjenjiv

i vrlo kompleksan, a ovisi o polaznoj sirovini, tehnološkim postupcima i procesima te otpadnim produktima (Gadipelly i sur., 2014.; Rana i sur., 2017.). U pravilu te otpadne vode sadrže smjesu različitih toksičnih tvari kao što su neizreagirani reaktanti, međuprodukti, ostaci produkata tj. aktivnih tvari antibiotika, katalizatori te aditivi u organskom otapalu. Također mogu sadržavati kiseline, baze, halide, nitrate, sulfate, cijanide te različite metale (Cardoso i sur., 2014.; Šimatović i Udiković-Kolić, 2019.). Te otpadne vode karakteriziraju i povišene vrijednosti različitih pokazatelja kakvoće otpadne vode, uključujući pokazatelje kemijske (KPK) i biokemijske potrošnje kisika (BPK_5), ukupnog organskog ugljika (TOC) te ukupnih suspendiranih tvari (TSS) s pH od 3 do 11 (Gadipelly i sur., 2014.). Stoga je pročišćavanje takvih otpadnih voda vrlo specifično i složeno te redovito zahtijeva kombinaciju različitih naprednih postupaka obrade kako bi se zadovoljili zakonom propisani uvjeti i granične vrijednosti emisija prije ispusta u okoliš. U ekonomski bolje razvijenim zemljama provodi se predobrada industrijskih otpadnih voda u krugu farmaceutske industrije te se potom tako obrađene otpadne vode ispuštaju u sustav javne odvodnje gdje se dodatno pročišćavaju na gradskim uređajima za pročišćavanje komunalnih otpadnih voda (Graham i sur., 2013.). Suprotno tome, u slabije razvijenim zemljama, posebice u Indiji i Kini, situacija je puno lošija, jer se velik dio industrijskih otpadnih voda često izravno ispušta u prirodne vode bez ikakve prethodne obrade ili nakon samo djelomične obrade (Larsson, 2014a.), što predstavlja značajan rizik za zdravlje okoliša i ljudi. Iako u Hrvatskoj nisu dostupni javni podaci o postupcima obrade otpadnih voda lokalnih farmaceutskih industrija, poznato je da se dio otpadnih voda iz proizvodnje azitromicina nakon predobrade u membranskom bioreaktoru obrađuje u uređaju za pročišćavanje komunalnih otpadnih voda grada Zaprešića, dok se dio ispušta u rijeku Savu (Tehničko-tehnološko rješenje 25-12-387/44). Također je poznato da se otpadne vode iz farmaceutske industrije koja se bavi formulacijom veterinarskih lijekova ispuštaju u obližnji plitki potok (oko 0,3-0,5m) nakon samo mehaničke predobrade (V. Tomić, osobno priopćenje). Pokazatelji kakvoće navedenih otpadnih voda te njihove granične vrijednosti uređeni su Pravilnikom o graničnim vrijednostima emisija otpadnih voda NN 80/2013 (43/14, 27/15, 3/16), ali antibiotici nisu obuhvaćeni tim propisima.

2.3.1. Unošenje antibiotika u vodeni okoliš putem industrijskih otpadnih voda

U današnje se vrijeme većina svjetskih antibiotika proizvodi u azijskim zemljama, posebice u Indiji i Kini, vjerojatno zbog jeftinije radne snage i labavijih zakona za zaštitu okoliša (Šimatović i Udiković-Kolić, 2019.). Istražujući kemijski sastav otpadnih voda iz 90-tak farmaceutskih industrija u industrijskoj zoni Patancheru u Indiji, švedski znanstvenici

su po prvi put pronašli visoke koncentracije različitih farmaceutskih spojeva uključujući antibiotike, pri čemu je koncentracija fluorokinolonskog antibiotika ciprofloksacina dosezala čak 31 mg L^{-1} (Larsson i sur., 2007.). Procijenjeno je da se na taj način u okoliš dnevno ispusti oko 44 kg ciprofloksacina, što bi bilo dovoljno za liječenje čak 45.000 ljudi (Larsson, 2014a.). Slična je situacija zapažena i u Kini. Tako je u obrađenoj otpadnoj vodi farmaceutske industrije koja proizvodi oksitetraciklin izmjerena koncentracija oksitetraciklina od 19,5 mg L^{-1} (Li i sur., 2008a.), dok je u otpadnoj vodi industrije koja proizvodi penicilin G nađena penilo kiselina, razgradni produkt penicilina, u koncentraciji od 44 mg L^{-1} (Li i sur., 2008b.). Međutim, koncentracija penicilina u toj otpadnoj vodi bila je relativno niska (1,68 μg L^{-1}) što i ne čudi s obzirom na izrazitu nestabilnost β-laktamskog prstena koji je sklon razgradnji. Novijim istraživanjem provedenim na obrađenim otpadnim vodama iz šest uređaja za pročišćavanje otpadnih voda farmaceutskih industrija izmjerene su koncentracije pojedinačnih antibiotika iz skupina makrolida, cefalosporina i linkozamida do 1 mg L^{-1} (Guo i sur., 2018a.). Osim Indije i Kine, visoke koncentracije antibiotika nađene su u industrijskim otpadnim vodama i u drugim azijskim državama. Tako su Sim i sur. (2011.) u otpadnim vodama farmaceutskih industrija u Koreji i Pakistanu detektirali linkomicin u koncentraciji do 44 mg L^{-1} te neke antibiotike iz skupina fluorokinolona, kinolona i tetraciklina u koncentracijama do 9,5 mg L^{-1} (Hussain i sur., 2016.). Nasuprot tome, Ashfaq i sur. (2017.) su u industrijskim otpadnim vodama u Pakistanu izmjerili puno niže koncentracije nekih fluorokinolona, do 81 μg L^{-1}. Suprotno azijskim zemljama, za Europu ima vrlo malo podataka o koncentraciji antibiotika u otpadnim vodama farmaceutske industrije i okolišu koji je pod utjecajem tih otpadnih voda. Prema dostupnoj literaturi samo su u jednoj studiji u otpadnoj vodi hrvatske farmaceutske industrije pronađene visoke koncentracije nekih veterinarskih antibiotika, pri čemu je koncentracija sulfametazina bila viša od 500 μg L^{-1} (Babić i sur., 2007.).

Što se tiče koncentracija antibiotika u okolišu koji je pod utjecajem otpadnih voda farmaceutske industrije, one su također često bile razmjerno visoke. Tako su u uzorcima pojedinih rijeka u Indiji detektirane visoke koncentracije nekih antibiotika, naročito fluorokinolona, pri čemu je koncentracija ciprofloksacina bila viša od 6 mg L^{-1}, a u riječnom sedimentu čak viša od 50 mg kg^{-1} (Fick i sur., 2009.; Kristiansson i sur., 2011.). Povrh toga, u nekim su uzorcima podzemnih voda i voda za piće pronađeni fluorokinoloni u povišenim koncentracijama (ukupno od 0,39 μg L^{-1} do 16 μg L^{-1}) (Fick i sur., 2009.; Rutgersson i sur., 2014.). Nadalje, u riječnoj vodi blizu ispusta industrijskih otpadnih voda iz proizvodnje oksitetraciklina u Kini detektiran je oksitetraciklin u koncentraciji od 641 μg L^{-1} na mjestu

ispusta te 377 μg L^{-1} oko 20 km nizvodno od ispusta (Li i sur., 2010.). U Pakistanu je sulfametoksazol detektiran u riječnoj vodi u najvišoj koncentraciji od 49 μg L^{-1} (Khan i sur., 2013.), dok je u Vijetnamu pak njegova koncentracija u rijeci/kanalu dosezala 250 μg L^{-1} (Thai i sur., 2018.). Neki su autori ukazali i na problem nepropisnog odlaganja farmaceutskog otpada, budući da su u podzemnim vodama u neposrednoj blizini nekadašnjeg odlagališta farmaceutskog otpada u Danskoj pronađene vrlo visoke koncentracije sulfagvanidina (čak do 1,6 mg L^{-1}) i sulfanilne kiseline, razgradnog produkta sulfonamida (do 6,5 mg L^{-1}) (Holm i sur., 1995.).

S obzirom na to da dozvoljena koncentracija antibiotika u otpadnim vodama i u okolišu nije propisana zakonom, Bengtsson-Palme i Larsson (2016.) predložili su tzv. PNEC vrijednosti (engl. *Predicted No Effect Concentractions*) za 111 pojedinačnih antibiotika koje predstavljaju granične koncentracije iznad kojih se očekuje selekcija antibiotičke otpornosti. Uzimajući u obzir predložene PNEC vrijednosti i gore spomenute koncentracije antibiotika izmjerene u industrijskim otpadnima vodama i recipijentnom vodenom okolišu, općenito se može reći da su te koncentracije bile uglavnom više od odgovarajućih PNEC vrijednosti i stoga selektivne za razvoj otpornosti među okolišnim bakterijama. Slijedom toga, otpadne vode farmaceutskih industrija mogu predstavljati prijetnju za zdravlje ljudi i okoliša te im je potrebno posvetiti posebnu pozornost.

2.3.2. Utjecaj industrijskih otpadnih voda na rezistom i bakterijske zajednice recipijentnog okoliša

Osim antibiotika, pokazalo se da otpadne vode farmaceutske industrije mogu biti izvor bakterija otpornih na antibiotike i njihovih gena za otpornost, jer se često miješaju sa sanitarnim otpadnim vodama (Šimatović i Udiković-Kolić, 2019.; Larsson, 2014a.). Tako je pokazano da otpadne vode iz proizvodnje β-laktama, kinolona i tetraciklina sadrže od 10^4 do 10^6 otpornih bakterija u 100 mL otpadne vode (Li i sur., 2009., 2010.; Tahrani i sur., 2018.). Osim toga, geni za otpornost na antibiotike nađeni su u industrijskim otpadnim vodama u maksimalnoj koncentraciji od 10^9 kopija gena u 100 mL otpadne vode (Guo i sur., 2018a.; Wang i sur., 2015a.; Zhai i sur., 2016.). Temeljem tih podataka procjenjeno je da se putem industrijskih ispusta svakodnevno u okoliš unese od 10^{12} do 10^{14} kopija gena otpornosti na antibiotike (Guo i sur., 2018a.). Premda su te koncentracije manje od koncentracija gena otpornosti koje se unesu putem komunalnih otpadnih voda (od 10^{14} do 10^{18} kopija gena po danu) (Manaia i sur., 2016.),

navedene spoznaje ipak upućuju da se industrijskim otpadnim vodama ti geni, kao i otporne bakterije unose u recipijentni okoliš.

Istraživanja učinaka otpadnih voda farmaceutskih industrija na recipijentni vodeni okoliš pokazala su značajan utjecaj na rezistom i bakterijske zajednice. Što se tiče rezistoma, zapaženo je značajno povećanje količine gena za otpornost u izloženim bakterijskim zajednicama (nizvodno od ispusta industrijskih otpadnih voda) u odnosu na neizložene zajednice (uzvodno od ispusta). Tako su Kristiansson i sur. (2011.) u metagenomima iz riječnih sedimenata onečišćenih fluorokinolonima detektirali povećanu zastupljenost gena za otpornost na sulfonamide (*sul*2) i aminoglikozide (*str*A i *str*B), ali ne i na fluorokinolone (*qnr*D, *qnr*S, *qnr*VC) koji su bili zastupljeniji na uzvodnim lokacijama. To je objašnjeno nesposobnošću gena *qnr* da pruže otpornost na visoke razine fluorokinolona prisutne na nizvodnim lokacijama. Međutim, analizirajući koncentracije šireg spektra *qnr* gena (*qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S i *qnr*VC) primjenom preciznije metode, qPCR-a, Rutgersson i sur. (2014.) pronašli su značajno više količine gotovo svih navedenih gena u indijskim sedimentima s nizvodnih lokacija u odnosu na riječni sediment izložen komunalnim otpadnim vodama u Švedskoj (kontrolni sediment), što je ipak potvrdilo značajan doprinos industrijskog otpada onečišćenju okoliša genima za antibiotičku otpornost. Osim u Indiji, značajno povišenje količine gena otpornosti zapaženo je u sedimentima pod utjecajem industrijskih otpadnih voda i u Pakistanu te u Europi (Španjolska) (Khan i sur., 2013.; Sidrach-Cardona i sur., 2014.). Povrh gena za otpornost na antibiotike, u svim navedenim studijama te u studijama provedenim u Kini (Li i sur. 2009., 2010.), zapažena je i povećana zastupljenost pokretnih genetičkih elemenata poput integrona skupine 1 ili plazmida koji bi mogli mobilizirati detektirane gene otpornosti i pridonijeti njihovom širenju među okolišnim bakterijama. To je i dokazano za širenje otpornosti putem plazmida u okolišu onečišćenom antibioticima. Naime, Flach i sur. (2015.). dokazali su povećani horizontalni prijenos plazmida koji su nosili otpornost na fluorokinolone, sulfonamide ili β-laktame među bakterijama u sedimentima izloženim industrijskim ispustima u Indiji u usporedbi s kontrolnim sedimentima u Švedskoj. Kao što je spomenuto u Poglavlju 2.1.7.2., izolirani mobilni plazmidi pripadali su IncA/C i IncN skupinama inkompatibilnosti te su nosili gene za otpornost na više skupina antibiotika u sklopu integrona skupine 1, insercijskih sekvenci ili transpozona. Slično tome, fenotipska otpornost na više skupina antibiotika bila je prisutna i kod većine bakterijskih izolata porijeklom iz industrijskih otpadnih voda i/ili recipijentnog vodenog okoliša u Kini i Indiji (Li i sur. 2009., 2010.; Marathe i sur., 2013.).

Osim učinaka na okolišni rezistom, pokazalo se da industrijski ispusti izazivaju nepovoljne učinke na okolišne bakterijske zajednice. Tako su Li i sur. (2009.) uočili razlike u taksonomskom sastavu kultivabilnih bakterijskih zajednica porijeklom iz riječne vode nizvodno i uzvodno od ispusta iz proizvodnje penicilina G u Kini. S druge strane, Kristiansson i sur. (2011.) su metagenomskom analizom indijskih riječnih sedimenata nizvodno i uzvodno od industrijskih ispusta zapazili razlike u sastavu tih bakterijskih zajednica na taksonomskoj razini roda. Međutim, iznenađujuće je da nisu uočene značajne razlike u broju bakterijskih vrsta između nizvodnih i uzvodnih sedimenata, što ukazuje na robusnost prirodnih zajednica, odnosno njihovu sposobnost da održe brojnost vrsta čak i uslijed izloženosti visokim koncentracijama antibiotika. S druge strane, utvrđene su puno izraženije razlike u funkcionalnom sastavu zajednica između uzvodnih i nizvodnih sedimenata. Pritom je zastupljenost gena odgovornih za otpornost na antibiotike, transport i metabolizam koenzima te replikaciju DNA bila povišena nizvodno, dok je zastupljenost gena za funkcije poput transporta i metabolizma aminokiselina, proizvodnje i pretvorbe energije te biosinteze sekundarnih metabolita nađena značajno smanjena na nizvodnim u odnosu na uzvodne lokacije (Kristiansson i sur., 2011.). Zaključeno je da bi se zapažene razlike u funkcionalnoj raznolikosti zajednica mogle odraziti na usluge koje okolišni mikroorganizmi pružaju ekosustavu. Povrh utjecaja na brojnost i raznolikost bakterijskih zajednica, neke studije upućuju na povezanost izloženosti visokim koncentracijama antibiotika i nastanka patogena otpornih na više skupina antibiotika (Lübbert i sur., 2017., Marathe i sur., 2013., 2017.; Li i sur., 2009., 2010.).

2.4. Metode za praćenje otpornosti na antibiotike u okolišu

Spoznaje o učincima antibiotika u okolišu u pogledu razvoja i širenja antibiotičke otpornosti bile su relativno oskudne sve do posljednjih desetak godina, za što je dijelom odgovorno i ograničenje primjenjenih metoda (Chee-Sanford i sur., 2001.; Koike i sur., 2007.; Li i sur., 2009., 2010.). U većini istraživanja upotrijebljena su dva glavna pristupa. Klasični mikrobiološki pristup temelji se na uzgoju bakterija otpornih na antibiotike na selektivnim krutim hranjivim podlogama (Korzeniewska i sur., 2013.; Li i sur., 2009., 2010.; Lim i sur., 2013.). Međutim, dobro je poznato da većinu okolišnih mikroorganizama (> 97 %) nije moguće uzgojiti u laboratoriju (Amann i sur., 1995.) pa ni analizirati primjenom metoda ovisnih o uzgoju. Drugi, moderniji pristup temeljen je na upotrebi molekularnih metoda kojima se zaobilaze ograničenja vezana uz uzgoj koristeći umnožavanje ciljanih gena otpornosti PCR-om iz ukupne DNA izolirane direktno iz okolišnog uzorka (metagenomska DNA) (Graham i sur., 2011.; Khan i sur., 2013.; Marti i sur., 2014.; Stoll i sur., 2012.). Primjenom tog pristupa i

dorađenog pristupa, qPCR-a koji omogućuje određivanje količine ciljanih gena, detektirani su i kvantificirani brojni geni otpornosti na antibiotike u različitim okolišnim uzorcima i na taj način dobivene vrijedne spoznaje o učincima antibiotika, ali i drugih selektivnih tvari poput metala na okolišni rezistom (Cacace i sur., 2019.; Khan i sur., 2013.; Guo i sur., 2018b.; Rutgersson i sur., 2015.; Sidrach-Cardona i sur., 2014.; Zhu i sur., 2018.). Međutim, ograničenje klasičnog PCR i qPCR pristupa leži u odabiru početnica koje se primjenjuju za umnožavanje ciljanih sekvenci. Budući da se te početnice dizajniraju na temelju poznatih sekvenci, primjenom PCR-a i qPCR-a mogu se detektirati i kvantificirati samo otprije poznati geni odgovorni za otpornost na antibiotike. Uvođenje novih metagenomskih pristupa koji se temelje na pretraživanju sekvenci i/ili funkcije otpornosti osiguralo je novi skup alata za proučavanje okolišnih spremnika otpornosti na antibiotike (Handelsman, 2004.).

2.4.1. Metode sekvenciranja

Sekvenciranje DNA obuhvaća skup postupaka i tehnologija kojima se određuje slijed nukleotida u molekuli DNA, a može se podijeliti u sekvenciranje prve generacije (Sangerova dideoksi metoda) te druge i treće generacije koje se zajednički nazivaju metode sekvenciranja sljedeće generacije (engl. *Next Generation Sequencing*, NGS). Sangerova metoda je uglavnom usmjerena na sekvenciranje pojedinačnih gena te se često primjenjivala za analizu različitih gena otpornosti u pojedinačnim bakterijskim izolatima te analizu gena 16S rRNA u svrhu identifikacije bakterija otpornih na antibiotike (Gorgani i sur., 2009.; Hatosy i sur., 2015.; Marathe i sur., 2018.; Wichmann i sur. 2014.; Willms i sur., 2019.). Unatoč zahtjevnosti i visokoj cijeni kad su u pitanju veliki projekti, ta je metoda još uvijek najprimjenjivanija tehnologija sekvenciranja zbog mogućnosti čitanja dugih sekvenci visokom preciznošću.

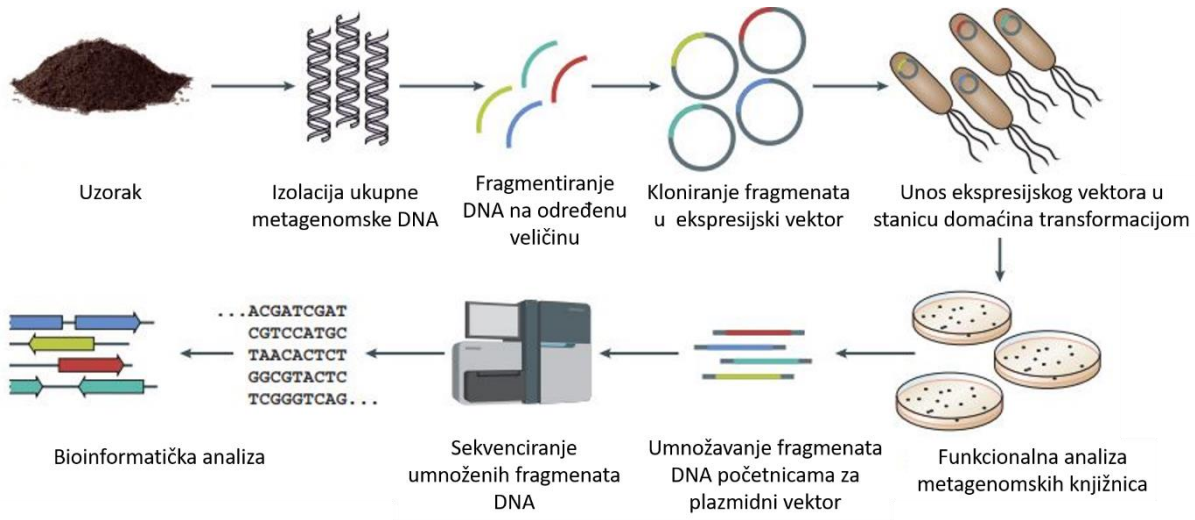
2.4.1.1. Metode sekvenciranja sljedeće generacije

Razvoj tehnologija sekvenciranja sljedeće generacije doveo je do mogućnosti sekvenciranja većih količina DNA u kraćem vremenu i uz sve niže troškove (Heather i Chain, 2016.). Kao glavna ograničenja ovih tehnologija ističu se manja duljina sekvenciranih sljedova i često manja pouzdanost točnosti svakog pročitanog nukleotida (Heather i Chain, 2016.). Razlikuju se tehnologije druge generacije kod kojih je prije samog izvođenja potrebno provesti PCR reakciju umnožavanja DNA kalupa i tehnologije treće generacije kod kojih to nije potrebno, već je moguće sekvenciranje pojedinačne molekule u realnom vremenu (engl. *Single Molecule Real-Time Sequencing,* SMRT). Umnožavanje PCR-om je potrebno kako bi se pojačao signal, jer detektor nije u mogućnosti detektirati dodatak samo jedne baze na razini

jedne molekule DNA. Tehnologije koje koriste umnožavanje PCR-om su Illumina, SOLiD i Roche 454 (Heather i Chain, 2016.). Usporedbom navedenih tehnologija došlo se do zaključka da Illumina ima najviše outpute uz najniže troškove, SOLiD sustav daje najveću točnost, dok je uz primjenu Roche 454 moguće analizirati najdulje sekvence (Liu i sur., 2012.). Sekvenciranje amplikona 16S rRNA na Illumina ili Roche 454 instrumentu učestalo se koristi za analizu raznolikosti bakterijskih zajednica u okolišima izloženim različitim antropogenim pritiscima, uključujući antibiotike (Kristiansson i sur., 2011.; Mittal i sur., 2019.; Udikovic-Kolic i sur., 2014.). S druge strane, Pacific Biosciences (PacBio) razvio je tzv. SMRT sustav koji omogućuje sekvenciranje duljih fragmenata DNA bez prethodnog umnožavanja, jer može detektirati signal na razini jedne molekule DNA (Rhoads i Au, 2015.). U posljednje vrijeme se PacBio tehnologija sve više koristi za sekvenciranje plazmida odgovornih za širenje otpornosti na antibiotike među bakterijama (Botts i sur., 2017.; Hazen i sur., 2017.; He i sur., 2016.; Liang i sur., 2019.).

2.4.2. Funkcionalna metagenomika

Funkcionalna metagenomika je jedini pristup koji ima mogućnost otkriti potpuno nove gene eksprimirajući ih u surogatnom domaćinu, tipično *E. coli* (Mullany, 2014.). Ova robusna metoda prvi put je opisana 2000. godine, a uključuje konstrukciju metagenomskih knjižnica koja započinje izolacijom ukupne okolišne (metagenomske) DNA te potom njezinim fragmentiranjem na određenu veličinu (najčešće od 1 do 5 kb) i kloniranjem dobivenih fragmenata u ekspresijski vektor (Slika 11.) (Adu-Oppong i sur., 2017.; Crofts i sur., 2017.). Takav rekombinantni vektor se potom transformacijom unosi u *E. coli*, nakon čega slijedi funkcionalna analiza probirom klonova koji ispoljavaju otpornost na antibiotike na selektivnim krutim hranjivim podlogama. Geni za otpornost na antibiotike identificiraju se iz aktivnih klonova Sangerovim sekvenciranjem inserta koristeći početnice dizajnirane za plazmidni vektor (Hatosy i sur., 2015.; Marathe i sur., 2018.; Wichmann i sur., 2014.) ili primjenom NGS-a (Crofts i sur., 2017.). Primjenom metode funkcionalne metagenomike identificirani su novi, ali i poznati geni za otpornost na antibiotike u različitim uzorcima, uključujući tlo (Udikovic-Kolic i sur., 2014.; Willms i sur., 2019.), aktivni mulj (Parsley i sur., 2010.), ljudska crijeva (Wang i sur., 2015b.), stajsko gnojivo (Wichmann i sur., 2014.), rijeku i riječni sediment (Amos i sur., 2014.; Marathe i sur., 2018.) te ocean (Hatosy i sur., 2015.).



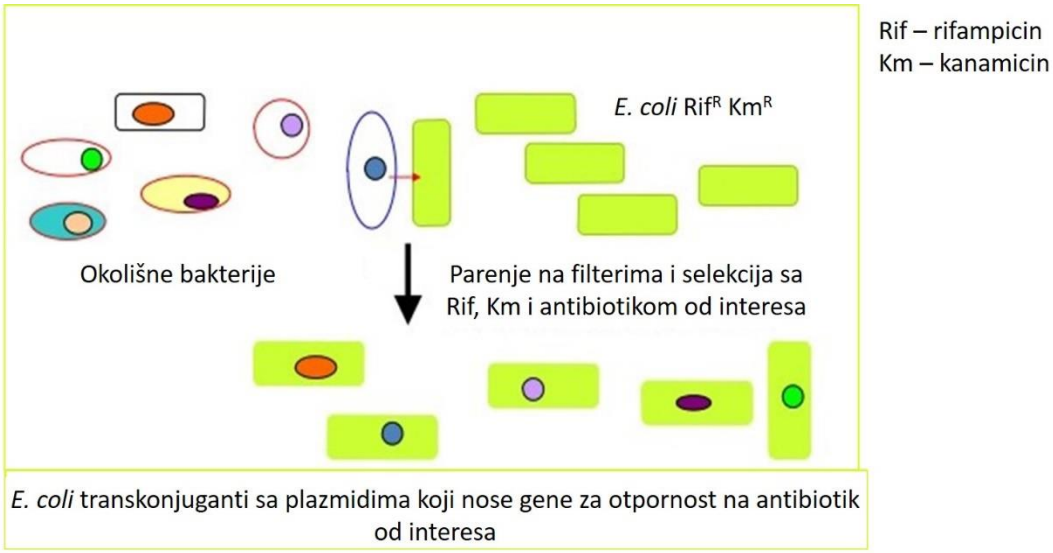
Slika 11. Shematski prikaz metode funkcionalne metagenomike. Preuzeto i prilagođeno iz Crofts i sur. (2017.).

Budući da se ova metoda ne temelji na otprije poznatoj sekvenci gena nego na funkciji koju taj gen eksprimira (otpornost na antibiotike), moguća je identifikacija potencijalno novih gena i mehanizama odgovornih za otpornost na antibiotike. Pored toga, primjenom funkcionalne metagenomike može se procijeniti i potencijal horizontalnog prijenosa identificiranih gena analizom genske okoline gena, tj. njegovih okolnih regija (Adu-Oppong i sur., 2017.; Pehrsson i sur., 2013.). Međutim, glavni nedostatak te metode je u tome što gen, da bi bio detektiran i identificiran, mora biti funkcionalan, odnosno eksprimiran u svom domaćinu. Upotreba domaćina kao što je Gram-negativna bakterija *E. coli* može dovesti do smanjene ekspresije gena koji potječu iz Gram-pozitivnih bakterija, a osim toga i urođena otpornost *E. coli* i drugih surogatnih domaćina na određene antibiotike može dati lažno pozitivne rezultate prilikom selekcije određenih fenotipova (Crofts i sur., 2017.). U konačnici, uslijed fragmentacije molekule DNA može doći do gubitka informacija o genskoj okolini i njegovoj povezanosti s pokretnim genetičkim elementima. Međutim, istraživanje okolišnog rezistoma pomoću funkcionalne metagenomike, posebice rezistoma okoliša onečišćenog visokim količinama antibiotika, može dovesti do važnih saznanja o raznolikosti gena otpornosti na antibiotike kao i do identifikacije potencijalno novih mehanizama otpornosti (dos Santos i sur., 2017.).

2.4.3. Egzogena izolacija i tipizacija plazmida

Podaci o mobilnosti gena za otpornost na antibiotike su vrlo važni za procjenu rizika za ljudsko zdravlje. Kao što je ranije spomenuto (Poglavlje 2.1.7.), prijenos gena otpornosti konjugacijom jedan je od najčešćih mehanizama kojima se otpornost na antibiotike širi među bakterijama. Takve analize mogu se provesti na okolišnim uzorcima primjenom pristupa neovisnog o uzgoju tzv. egzogenog „hvatanja" plazmida (engl. *Exogenous Plasmid Capture*) (Slika 12.) (Smalla i sur., 2015.). Tom metodom se prati prijenos plazmida s genima za otpornost na odabrane antibiotike direktno iz okolišnih bakterija (donori plazmida) u odabrani recipijentni bakterijski soj, najčešće *E. coli* CV601. Taj soj ne posjeduje plazmide i otporan je na rifampicin i kanamicin te obilježen zelenim fluorescentnim proteinom (engl. *Green Fluorescent Protein*, GFP) radi olakšane identifikacije transkonjuganata. Suspenzija okolišnog uzorka (npr. sedimenta) i kultura recipijentnog soja pomiješaju se u određenom omjeru i inkubiraju određeno vrijeme na membranskim filterima pri čemu dolazi do prijenosa plazmida. Potom se bakterijske stanice, prikupljene s membranskog filtera, nacjepljuju na selektivne krute hranjive podloge s rifampicinom, kanamicinom i antibiotikom od interesa, a nakon inkubacije se na spomenutim pločama broje transkonjuganti, tj. stanice *E. coli* koje su primile plazmid s genima za otpornost iz okolišnih bakterija (Blau i sur., 2020.). Prednost ove metode je u mogućnosti izolacije i identifikacije novih, još neotkrivenih plazmida, dok je glavni nedostatak nemogućnost identifikacije bakterije donora plazmida. Metoda egzogenog „hvatanja" ili izolacije plazmida je do sada uspješno primjenjena za praćenje plazmidnog širenja otpornosti na antibiotike u okolišu uključujući riječne sedimente izložene otpadnim vodama farmaceutskih industrija u Indiji (Flach i sur., 2015.), tlo tretirano stajskim gnojivom (Heuer i sur., 2009., 2012.) ili obrađenim aktivnim muljem (Wolters i sur., 2019.), kao i tla onečišćena pesticidima (Anjum i sur., 2012.) ili metalima (Garbisu i sur., 2018.).

Tipizacija mobilnih plazmida koji nose gene za otpornost na antibiotike, a izolirani su iz dobivenih transkonjuganata, obično se provodi primjenom metode tipizacije replikona PCR-om (engl. *PCR-Based Replicon Typing*, PBRT). Ta metoda se bazira na primjeni PCR-a ili qPCR-a, a koristi početnice specifične za određene skupine inkompatibilnosti plazmida. Svrstavanjem plazmida u Inc skupine dobiva se uvid u potencijal njihovog horizontalnog širenja (Smalla i sur., 2015.).



Slika 12. Shematski prikaz metode egzogene izolacije plazmida.

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Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries

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ABSTRACT

Effluents from pharmaceutical industries are recognized as significant contributors to aquatic pollution with antibiotics. Although such pollution has been mostly reported in Asia, knowledge on industrial discharges in other regions of the world, including Europe, and on the effects associated with such exposures is still limited. Thus, we performed chemical, microbiological and ecotoxicological analyses of effluents from two Croatian pharmaceutical industries during four seasons. In treated effluents of the company synthesizing macrolide antibiotic azithromycin (AZI), the total concentration of AZI and two macrolide by-products from its synthesis was 1–3 orders of magnitude higher in winter and springtime (up to 10.5 mg/L) than during the other two seasons (up to 638 μg/L). Accordingly, the highest total concentrations (up to 30 μg/L) in the recipient river were measured in winter and spring. Effluents from second company formulating veterinary antibiotics contained fluoroquinolones, trimethoprim, sulfonamides and tetracyclines ranging from low μg/L to approx. 200 μg/L. Low concentrations of these antibiotics, from below the limit of quantification to approx. few μg/L, have also been measured in the recipient stream. High frequency of culturable bacteria resistant to AZI (up to 83%) or sulfamethazine (up to 90%) and oxytetracycline (up to 50%) were also found in studied effluents. Finally, we demonstrated that toxicity to algae and water fleas often exceeded the permitted values. Most highly contaminated effluents induced multiple abnormalities in zebrafish embryos. In conclusion, using a wide array of analyses we have demonstrated that discharges from pharmaceutical industries can pose a significant ecological and public health concern due to their toxicity to aquatic organisms and risks for promoting development and spread of antibiotic resistance.

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1. Introduction

Pharmaceuticals are considered emerging pollutants since they are continuously being released into the environment and affect the biota on multiple levels. Consequently, global concern about their potential adverse impacts on both human health and the environment is growing. The primary sources of pharmaceuticals in the environment are municipal wastewater treatment plants, agricultural settings, aquaculture, hospitals and pharmaceutical production facilities (Andersson and Hughes, 2014). A significant amount of research in the area has focused on municipal effluents and data from municipal wastewater plants are relatively accessible. Complex cocktail of various pharmaceuticals is assumed to enter municipal sewage by human excretion and their concentrations are generally in ng/L in treated sewage effluents (Kostich et al., 2014; Luo et al., 2014). In contrast to this, predominant compounds in pharmaceutical effluents depend on the production site and research on such effluents is more problematic due to difficulties in accessing information. However, concentrations of pharmaceuticals in industrial effluents can be several orders of

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magnitude greater than the concentrations found in sewage effluents as reported recently (Cardoso et al., 2014; Larsson, 2014). Besides pharmaceuticals, industrial effluents can also contain various intermediate products and by-products such as solvents, acids and inorganic compounds (including heavy metals, nitrate and phosphate). Moreover, these effluents can have high level of chemical and biochemical oxygen demand and total suspended solids (COD, BOD and TSS, respectively) (Gadipelly et al., 2014). However, in contrast to some other physico-chemical parameters, the concentration of pharmaceuticals and their metabolites is currently not legally regulated, although some of them, such as macrolide antibiotics, have been included in watch lists of the regulation bodies (Cardoso et al., 2014; Decision, 2015; Rivera-Utrilla et al., 2013).

Among pharmaceuticals, special attention is given to antibiotics because environmental contamination with antibiotics facilitates the development and spread of antibiotic resistance among bacteria, including pathogens (Andersson and Hughes, 2014). High emissions of antibiotics from manufacturing plants have been reported mostly in Asian countries, while much less information is available for other regions of the world, including Europe (Larsson, 2014). In addition, metals and other biocides present in industrial effluents have the potential to co-select for antibiotic resistance (Baquero et al., 2008; Roose-Amsaleg and Laverman, 2016). Besides risks for promoting resistance, antibiotic pollution can induce direct biostatic or bio-toxic effects to diverse organisms and alter species distribution, which is harmful for the ecosystem as a whole (Halling-Sørensen, 2000; Harada et al., 2008; Isidori et al., 2005; Wang et al., 2014a).

Noteworthy, most studies, such as the ones mentioned above, predict the environmental effects of single chemicals. Such research is certainly useful, but does not provide much information about toxic effects of effluents as mixtures of pharmaceuticals that can interact synergistically, additively or antagonistically (Białk-Bielińska et al., 2013; Caldwell et al., 2016; Flaherty and Dodson, 2005; Spurgeon et al., 2010). Therefore, from the environmental risk assessment perspective, the whole effluent testing is of importance to account for unknown substances present in effluents and combined 'cocktail' effects. Existing studies investigating the effects of pharmaceutical effluents on aquatic environment mostly focus on only one or few endpoints, reporting for instance only chemical analyses of the pharmaceuticals in the wastewaters (Fick et al., 2009), only analyses of antimicrobial resistance (Guardabassi et al., 1998) or only toxicological data (Carlsson et al., 2009; Tišler and Zagorc-Končan, 1999). The work of Larsson et al. (2007) is the most detailed to date, including both chemical analyses of pharmaceuticals in the effluent and toxicity bioassays using bacteria (*Vibro fisheri*), invertebrates (*Daphnia magna*) and plants (*Lactuca sativa*) as models.

Thus, there is a clear need for more research in these areas to better understand the exposure routes and the effects pharmaceutical pollution might have on human and ecological health. In order to take a step toward this understanding, we have sampled final (treated or partially treated) effluents from two Croatian pharmaceutical industries that are involved in synthesis and formulation of antibiotics during four seasons in one year monitoring period. We have analyzed the physico-chemical properties and chemical composition of effluent samples, including quantification of antibiotics and heavy metals. We also measured antibiotic concentrations in the recipient water bodies. Finally, we have assessed the frequency of culturable antibiotic-resistant bacteria in effluents and the effect of effluents on various levels of biological organization: bacteria, freshwater algae, invertebrates (*Daphnia*) and vertebrates (zebrafish embryos).

2. Materials and methods

2.1. Sampling of the effluents

Effluents from two pharmaceutical industries situated in northern Croatia were collected (Company 1 and Company 2) four times a year over one year monitoring period.

Company 1 has a long tradition in manufacturing the macrolide antibiotic azithromycin via synthesis from another macrolide antibiotic, erythromycin. The final effluent from this industry (after treatment in industry's membrane bioreactor) is discharged into the Sava river together with effluent from baker's yeast production. Such mixed effluents were collected as grab samples at the discharge pipe located at the bank of the Sava river in winter (February), spring (May), summer (August) and autumn (November) 2016. In addition to effluent samples, surface river water was collected as grab sample from three locations along the river: at 3.5 km upstream and at 700 m and 4.5 km downstream of the discharge site.

Company 2 is involved in manufacturing of various human and animal health products, but is mostly known for formulation of veterinary antibiotics (sulfonamides, fluoroquinolones, tetracyclines and β-lactams) into doses. The technological effluent produced by this industry is discharged into the nearby small stream after being diluted with industrial sewage and passed through a primary treatment (mechanical removing of larger, floating solids). The 24-h flow proportional samples were collected directly with an automatic sampler that is connected to a compatible flow measuring device. Sampling was performed in winter (January), spring (April), summer (July) and autumn (October) 2016. Also, stream samples were collected as grab samples from three locations along the stream: at 300 m upstream and at 2 m and 3 km downstream of the discharge site.

Effluent and surface water samples were collected in sterile polyethylene wide mouth jars and stored at +4 °C or −20 °C, depending on analysis performed. Parts of samples were sterile-filtered through a 0.22 μm filter (Millipore, USA).

2.2. Chemical analyses

The samples were analyzed for a number of basic parameters within their holding time using internationally validated methods (ISO standards) for examination of water and wastewater (for more details see Table S1).

Antibiotics (azithromycin, AZI; dehydrated erythromycin, ERY-H_2O; enrofloxacin, ENR; oxytetracycline, OTC; sulfadiazine, SDZ; sulfamethazine, SMZ; trimethoprim, TMP; Sigma, Germany) and intermediate from AZI production (*N*-Desmethyl Azithromycin, N-DMA) were quantified using the previously described method based on liquid chromatography - tandem mass spectrometry (Senta et al., 2017, 2008). Briefly, samples were enriched using solid-phase extraction on Oasis HLB cartridges. Typical volumes were between 1 and 250 mL, depending on the sample type. It should be noted that erythromycin (ERY) was not analyzed in the original form but as its dehydrated transformation product ERY-H_2O.

Concentrations of heavy metals in effluent samples, arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn), were determined by inductively coupled plasma mass spectrometry (ICP-MS) following the methodology described by Dautović et al. (2014).

2.3. Bioassays

2.3.1. Determination of the frequency of culturable antibiotic-resistant bacteria

Serial dilutions of effluents were prepared in physiological saline (0.9% NaCl) and plated on R2A agar (R2A broth [Lab M Limited, UK] amended with 1.5% agar [BioLife Italiana, Italy]) supplemented with yeast extract (5 g/L) (BioLife Italiana, Italy) to enumerate total bacteria. In parallel, serial dilutions of effluents were plated on R2A agar with yeast extract and antibiotic AZI (15 mg/L) (Fluka, Germany) for the analysis of Company 1 effluents, or antibiotic SMZ (350 mg/L) (Sigma, Germany) and OTC (25 mg/L) (Sigma, Germany) for the analysis of Company 2 effluents, to enumerate resistant bacteria. The culture medium was supplemented with cycloheximide (100 mg/L final concentration; Sigma, Germany) to inhibit growth of fungi. The number of colony forming units (CFU) was determined after incubation at 28 °C for 5 days. The frequency of antibiotic-resistant bacteria was calculated as the ratio of resistant bacteria CFU and total CFU.

2.3.2. Freshwater algal growth inhibition test

Effect of effluents on the growth of planktonic unicellular freshwater green microalgae *Pseudokirchneriella subcapitata* was measured using the standardized ISO 8692:2012 method (ISO, 2012). The algae were exposed to effluent dilution series, as well as appropriate positive and negative control, and incubated for 72 h. The toxicity of effluents was expressed as the lowest ineffective dilution (LID) at which growth inhibition of <5% was observed.

2.3.3. Acute Daphnia magna toxicity test

Effect of effluents on motility of water flea *Daphnia magna* Straus (Cladocera, Crustacea) was determined using the standardized ISO 6341:2013 method (ISO, 2013). After 24 h exposure of water fleas to effluent dilution series, as well as appropriate positive and negative control, the toxicity of effluents was expressed as LID for which ≤10% of the fleas were immobile.

2.3.4. Mutagenicity to Salmonella

Mutagenicity was tested on two most contaminated effluent samples from Company 1 (winter and spring samples) and on two effluent samples from Company 2 that showed toxicity to algae (summer and autumn samples). The test was performed in triplicates (with or without S9 fraction, Sigma, Germany) using the standard Ames bacterial test (Maron and Ames, 1983; Sauerborn Klobučar et al., 2013) with *Salmonella typhimurium*, strains TA98 (*his D3052*, *rfa*, *uvrB*, pKM101) and TA100 (*his G46*, *rfa*, *uvrB*, pKM101). Revertants were scored after 48 h incubation at 37 °C. Distilled water was used as negative solvent control and positive controls were 9-aminoacridine for TA98 strain without metabolic activation (50 μg per plate), sodium azide for TA100 strain without metabolic activation (1 μg per plate), and 2-aminoantracene for TA98 and TA100 strains with metabolic activation (2 μg per plate).

2.3.5. Zebrafish embriotoxicity assay

Embriotoxicity assay on zebrafish *Danio rerio* (wild type) was performed on the same selected samples as mutagenicity according to Babić et al. (2017). Each sample was tested in triplicates per 10 embryos (amounting to a total of 30 embryos per sample/dilution) – undiluted, 2 × and 4 × diluted with artificial water (ISO, 1996). Toxicological endpoints, both lethal and sublethal, were recorded at 24, 48 and 72 h post fertilization (hpf) (ISO, 2007). The mortality in all negative control groups was less than 5%. Additionally, physiological endpoints, i.e. heart rate, pigmentation formation and hatching rate, were evaluated at 48 and 72 hpf.

3. Results

3.1. Physicochemical properties and chemical composition of pharmaceutical effluents, including heavy metal and antibiotic content

Effluent quality parameters are given in Table 1, Table 2 and Table S2. Effluents from Company 1 had a high level of organic pollution, especially in winter and spring, as COD, BOD5 and TOC values were far higher than permitted values (Table 1). Concentrations of nitrites were higher than maximal permitted value only in the winter sampling (1.5 mg/L), while nitrates were elevated in summer sample. Total phosphorus concentrations were above the limit for winter, spring and summer effluents from Company 1.

Company 2 effluents had satisfactory quality regarding organic and nutrient pollution; only BOD5 value in the summer sample was slightly above the limit (Table 2). However, BOD5/COD ratios were lower than 0.4 for effluents of both Companies indicating low biodegradability (Chamarro et al., 2001).

The measured values of temperature, pH and sulfates of effluents of both companies were in compliance with the permissible limits for wastewaters discharge in surface waters set by Croatian legislative (Official Gazette of the Republic of Croatia 80/13, 43/14, 27/15, 3/16; Table S2). Conductivity of effluents is not regulated by legislation but it can serve as a useful measure of dissolved inorganic materials. All effluents from Company 1 and Company 2 had high conductivity, 2–10 times higher than the values reported in the literature for pharmaceutical effluents (Idris et al., 2013; James et al., 2014).

The results on heavy metal content in analyzed effluents are given in Table 3. Concentrations of Cr, Cu, Pb, Ni and Zn are regulated by Croatian law and their concentrations in the effluents were lower or approx. the same as maximal permitted values (Official Gazette of the Republic of Croatia 80/13, 43/14, 27/15, 3/16). Only concentration of zinc in the autumn sample from Company 2 was four times higher than maximal permitted value.

In winter and spring, effluents from Company 1 had the highest total concentration of AZI and two macrolide by-products from its synthesis, N-DMA and ERY–H_2O ($\sum$ = 6.4 and 10.5 mg/L, respectively) (Table 4). These compounds were also present in summer and autumn effluents but in 1–3 orders of magnitude lower total concentration (up to 638 μg/L) than in winter and spring samples. Surface water samples from the Sava river taken upstream from the discharge mostly contained ng/L levels of the analyzed macrolides, which are much lower than in downstream locations. The concentrations of macrolides in downstream river generally decreased as the compounds were carried downstream, with an exception in autumn when higher concentration of macrolides was observed in the river at a greater distance from the discharge.

In effluents of Company 2 (Table 5), the concentrations of sulfonamides were measured in the range from 6.7 to 231.0 μg/L for SMZ, from 3.0 to 20.0 μg/L for SDZ, from 7.4 to 29.0 μg/L for OTC, from 3.6 to 98 μg/L for ENR and at approx. few μg/L for TMP. Concentrations of these antibiotics did not generally exceed 10 μg/L in the receiving stream near the discharge and were further decreased at 3 km downstream location ranging from below the limit of quantification to low μg/L. Nevertheless, these levels were generally 4–34 × higher than those detected at upstream location.

3.2. Antibiotic-resistant bacteria as biological pollutants in studied effluents

Our culturing data showed that studied effluents contained high numbers of total culturable bacteria (in the range of 10^5–10^7 CFU/mL) and high frequency of antibiotic-resistant bacteria (Fig. 1).

Table 1
Quality parameters of Company 1 effluents discharged into the Sava river and their permissible limits by Croatian legislative. Recorded values higher then permitted are marked in **bold**.

Parameters	Sampling campaigns				Maximal permitted value[a]
	Winter	Spring	Summer	Autumn	
COD[b] mg O_2/L	**1915**	**1166**	**398**	**596**	125
BOD5[c] mg O_2/L	**564**	**352**	**28**	**119**	20
BOD5/COD ratio	0.29	0.30	0.07	0.20	–
TOC[d] mg C/L	**616.2**	**177.6**	**86.2**	**134.9**	30
Total phosphorus mg P/L	**6.77**	**2.5**	**4.41**	4.2	1.5
Nitrite mg N/L	**1.5**	<0.01	<0.01	<0.01	1
Nitrate mg N/L	1.4	0.5	**3.8**	0.2	2

[a] Maximal permissible values for pharmaceutical effluents discharged into surface waters (Official Gazette of the Republic of Croatia 80/13, 43/14, 27/15, 3/16).
[b] Chemical oxygen demand.
[c] Biochemical oxygen demand.
[d] Total organic carbon.

Table 2
Quality parameters of Company 2 effluents discharged into nearby stream and their permissible limits by Croatian legislative. Recorded values higher then permitted are marked in **bold**.

Parameters	Sampling campaigns				Maximal permitted value [a]
	Winter	Spring	Summer	Autumn	
COD[b] mg O_2/L	37.0	54.0	99.0	27.0	125
BOD5[c] mg O_2/L	7.3	13.0	**33.0**	7.0	20
BOD5/COD ratio	0.20	0.24	0.33	0.26	–
TOC[d] mg C/L	7.9	5.4	11.7	4.84	30
Total phosphorus mg P/L	0.62	0.42	0.008	0.17	1.5
Nitrites mg N/L	0.07	0.09	<0.01	0.06	1
Nitrates mg N/L	0.84	1.2	<0.05	0.9	2

[a] Maximal permissible values for pharmaceutical effluents discharged into surface waters (Official Gazette of the Republic of Croatia 80/13, 43/14, 27/15, 3/16).
[b] Chemical oxygen demand.
[c] Biochemical oxygen demand.
[d] Total organic carbon.

Table 3
The heavy metal ions concentration in the effluents from two Croatian pharmaceutical companies, Company 1 (a) and Company 2 (b), during four sampling campaigns and their permissible limits by Croatian legislative. Recorded values higher then permitted are marked in **bold**.

Heavy metal ions (μg/L)	Winter	Spring	Summer	Autumn	Maximal permitted value[a]
a)					
Arsenic (As)	0.73	0.59	0.41	0.67	–
Cadmium (Cd)	0.28	0.79	0.07	0.71	–
Chromium (Cr)	7.23	2.62	15.73	1.98	50
Copper (Cu)	26.92	6.19	18.36	10.36	100
Lead (Pb)	4.24	3.73	2.70	3.30	500
Nickel (Ni)	20.66	13.62	12.42	24.77	50
Zinc (Zn)	90.94	**103.06**	56.35	70.42	100
b)					
Arsenic (As)	0.53	0.46	0.79	50.27	–
Cadmium (Cd)	0.02	0.02	0.05	0.12	–
Chromium (Cr)	0.51	0.16	1.13	0.38	50
Copper (Cu)	7.46	12.75	12.70	15.42	100
Lead (Pb)	2.33	1.55	9.3	1.97	500
Nickel (Ni)	1.25	0.81	2.12	1.31	50
Zinc (Zn)	33.88	28.72	47.64	**417.67**	100

[a] Maximal permitted value for pharmaceutical effluents discharged into surface waters (Official Gazette of the Republic of Croatia 80/13, 43/14, 27/15, 3/16).

Table 4
Concentration of three macrolide compounds, expressed in μg/L, in effluents of Company 1 and in the recipient Sava river.

Antibiotic	Sampling campaign	Effluent concentration	Sava Upstream 3.5 km	Sava Downstream 700 m	Sava Downstream 4.5 km
Azithromycin (AZI)	Winter	2137.00	0.13	3.40	0.39
	Spring	3776.00	0.01	3.40	1.60
	Summer	4.90	0.02	0.03	0.02
	Autumn	218.00	0.06	0.01	2.80
N-Desmethyl Azithromycin (N-DMA)	Winter	2341.00	0.37	13.00	1.50
	Spring	5660.00	0.06	8.60	6.20
	Summer	11.10	< LOD	< LOD	0.02
	Autumn	390.00	< LOD	< LOD	0.16
Erythromycin-H_2O (ERY-H_2O)	Winter	2009.00	3.40	4.20	22.00
	Spring	1069.00	0.13	18.00	10.00
	Summer	1.00	< LOD	< LOD	< LOD
	Autumn	30.0	< LOD	< LOD	0.02
Macrolides total, $\sum$	Winter	6487.00	3.90	20.60	23.89
	Spring	10 505.10	0.20	30.00	17.80
	Summer	17.00	0.02	0.03	0.04
	Autumn	638.00	0.06	0.01	2.98

< LOD – below detection limit.

Table 5
Concentration of five antibiotics, expressed in μg/L, in effluents of Company 2 and in the recipient stream.

Antibiotic	Sampling campaign	Effluent concentration	Stream Upstream 300 m	Stream Downstream 2 m	Stream Downstream 3 km
Sulfadiazine (SDZ)	Winter	20.00	0.07	6.60	2.40
	Spring	8.12	0.07	2.30	0.29
	Summer	7.10	0.04	< LOD	0.78
	Autumn	3.00	0.06	< LOD	0.70
Sulfamethazine (SMZ)	Winter	10.00	0.10	5.30	1.10
	Spring	44.70	0.79	12.00	1.70
	Summer	231.00	1.00	2.80	11.00
	Autumn	6.70	0.20	0.70	1.70
Trimethoprim (TMP)	Winter	5.60	< LOD	0.11	1.10
	Spring	1.57	< LOD	0.37	0.24
	Summer	5.40	< LOD	0.11	0.42
	Autumn	1.00	0.01	< LOD	0.12
Enrofloxacin (ENR)	Winter	98.00	< LOD	10.60	1.00
	Spring	6.66	< LOD	1.00	< LOD
	Summer	4.30	< LOD	0.40	0.21
	Autumn	3.60	< LOD	0.30	< LOD
Oxytetracycline (OTC)	Winter	17.00	< LOD	10.00	< LOD
	Spring	7.40	< LOD	< LOD	< LOD
	Summer	29.00	< LOD	8.00	< LOD
	Autumn	10.00	< LOD	1.00	0.43

< LOD – below detection limit.

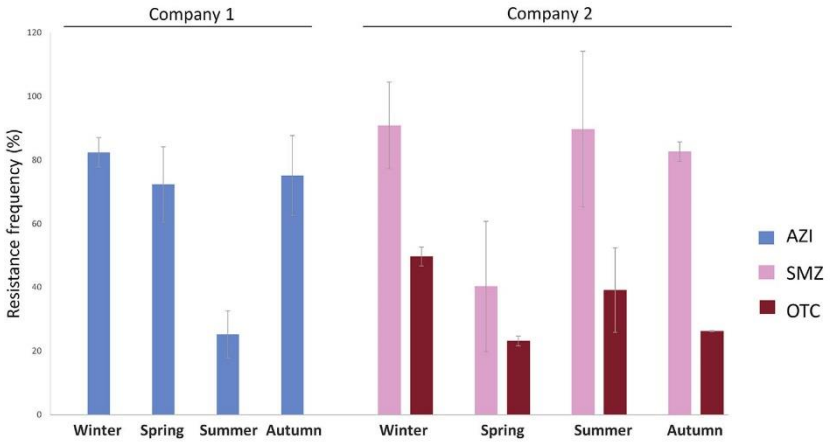

Fig. 1. The frequency of culturable antibiotic-resistant bacteria present in pharmaceutical effluents. The percentage of antibiotic-resistant bacteria was calculated as the ratio of resistant bacteria CFU and total CFU. The mean of calculated resistance frequencies is represented as column and the error bar indicates standard deviation ($n = 3$). AZI - azithromycin; SMZ - sulfamethazine; OTC – oxytetracycline.

Effluents of Company 1 contained up to 82.5% of AZI-resistant bacteria, whereas Company 2 effluents contained up to 91.0% of SMZ-resistant and up to 49.7% of OTC-resistant bacteria.

3.3. Effects on biota

We have tested the effects of our pharmaceutical effluents on various levels of biological organization: (i) bacteria (potential mutagenic effect on *Salmonella typhimurium* tester strains), (ii) freshwater algae (growth inhibition of green alga *Pseudokirchneriella subcapitata*), (iii) invertebrates (induction of immobility in water flea *Daphnia magna*), and (iv) vertebrates (lethal and sub-lethal toxicity to fish *Danio rerio* embryos).

The Ames *Salmonella* assay (Fig. S1) showed no mutagenic effect after exposure of TA98 and TA100 tester strains to Company 1 effluents. However, incubation with Company 2 effluents caused slight increase in the number of *S. typhimurium* TA98 revertants, especially pronounced after metabolic activation (up to 1.3 fold increase in comparison with negative control), indicating the occurrence of frameshift mutations.

Toxic effects of the effluents on *P. subcapitata* and *D. magna* are reported in Table 6. Company 1 effluents (especially winter and spring samples) were very toxic to algae as LID values of all samples were higher than permitted. When comparing acute toxicity to *Daphnia*, only winter and spring samples were toxic, with winter sample being more toxic than the spring one (LID values 16 and 5, respectively). Only summer and autumn effluent samples from Company 2 were toxic to green algae (LID values 4 and 10, respectively).

Embriotoxicity assay on zebrafish *Danio rerio* was performed on winter and spring effluent samples from Company 1 and summer and autumn samples from Company 2. All tested samples caused adverse effects on zebrafish embryos, which were manifested through lethal and sub-lethal alterations (Fig. 2, Fig. S2, Table S3). Most strikingly, all embryos exposed to undiluted effluents of Company 1 were coagulated at 24 hpf (see mortality rate in Fig. 2). In comparison, undiluted effluents of Company 2 induced much lower mortality rate (<10%). After we have diluted the samples we still observed some mortality after exposure of the embryos to Company 1 effluents. In overall, mortality was more pronounced after exposure to winter than to spring sample. Even when the samples were diluted 4 ×, effluents of Company 1 still caused up to 50% mortality at 72 hpf, while no mortality was observed during exposure to 2 or 4 × diluted Company 2 effluents.

To get a more detailed insight into the toxic effects of effluents to zebrafish embryos, type of observed abnormalities (Fig. S2) and some physiological endpoints (heart beat rate, pigmentation formation, hatching rate, Table S3) were followed upon exposure. Type of abnormalities depended on the sample. Exposure to Company 1 winter sample induced multiple malformations including heart and yolk edema (Fig. S2; f), scoliosis (Fig. S2; g, h, i) and lack of pigmentation formation (Fig. S2; e). During exposure to Company 1 spring sample, embryos often exhibited delayed development (e.g. reaching the 24 h stage at 72 hpf), yolk deformity and pericardial edemas (Fig. S2; a, b, d, i). Observed developmental delay was in correlation with reduced hatching rate, which was most pronounced after exposure of embryos to 2 × diluted spring sample. Among other explored endpoints, embryo pigmentation was significantly reduced upon exposure to 2 and 4 × diluted winter sample at 48 hpf (Table S3). Further, heart beat rate was also significantly reduced upon exposure to dilutions of winter sample at 48 and 72 hpf. In contrast to diluted winter sample, 2 and 4 × diluted spring sample significantly increased heart beat frequency at 72 hpf.

The only observed abnormality during exposure to Company 2 summer and autumn effluents was pericardial and yolk sack edema. Additionally, those samples did not affect pigmentation formation, but significantly increased heart beat rate at 48 and 72 hpf. Undiluted autumn sample caused the largest hatching rate reduction at 72 hpf (Table S3), which was also revealed through constant twitching of embryo body.

4. Discussion

The present study evaluated chemical composition and ecotoxicity of final effluents from two antibiotic-manufacturing plants in Croatia as well as antibiotic contamination of receiving surface waters.

Our chemical analyses of effluents from Croatian Company 1 revealed occasionally (winter and spring season) high concentrations of AZI and two other macrolides associated with AZI production (N-DMA and ERY-H_2O), in the order of few mg/L. It is important to stress that both identified macrolide by-products have antimicrobial activity (Shepard et al., 1990; Wang et al., 2014b). The observed mg/L-levels of AZI in Company 1 effluents are three orders of magnitude greater than the levels generally found in treated hospital and sewage effluents (around or below 1 μg/L) (Michael et al., 2013; Verlicchi et al., 2012). These findings together with high levels of organic, phosphorus and nitrate compounds detected in the effluents are indicative of unsatisfactory wastewater treatment of Company 1 and of unauthorized discharges. They also indicate that these effluents are sources of macrolide pollution observed in the recipient river downstream from the discharge point. Indeed, during the winter and springtime, all studied macrolides were found in the river at enhanced concentrations (up to approx. 30 μg/L total). The observation of a declining gradient of macrolide concentration from the discharge site might be due to further dilution of macrolides with the river water and/or some elimination processes such as sorption and degradation. However, reverse situation observed occasionally indicates that the industrial

Table 6
Toxicity of effluents to freshwater organism. Toxicity to algae is expressed as the lowest ineffective dilution (LID) at which growth inhibition of <5% was observed. Toxicity to *Daphnia* is expressed as the LID for which ≤10% of the fleas were immobile. Recorded values higher then permitted[a] are marked in **bold**.

	Sampling campaign	Alga *Pseudokirchneriella subcapitata*	Water flea *Daphnia magna*
Company 1	Winter	**>32**	**16**
	Spring	**>32**	**5**
	Summer	**12**	2
	Autumn	**4**	2
Company 2	Winter	1	1
	Spring	3	1
	Summer	**4**	2
	Autumn	**10**	2
Maximal permissible value[a]		3	2

[a] Maximal permissible value for pharmaceutical effluents discharged into surface water (Official Gazette of the Republic of Croatia 80/13, 43/14, 27/15, 3/16).

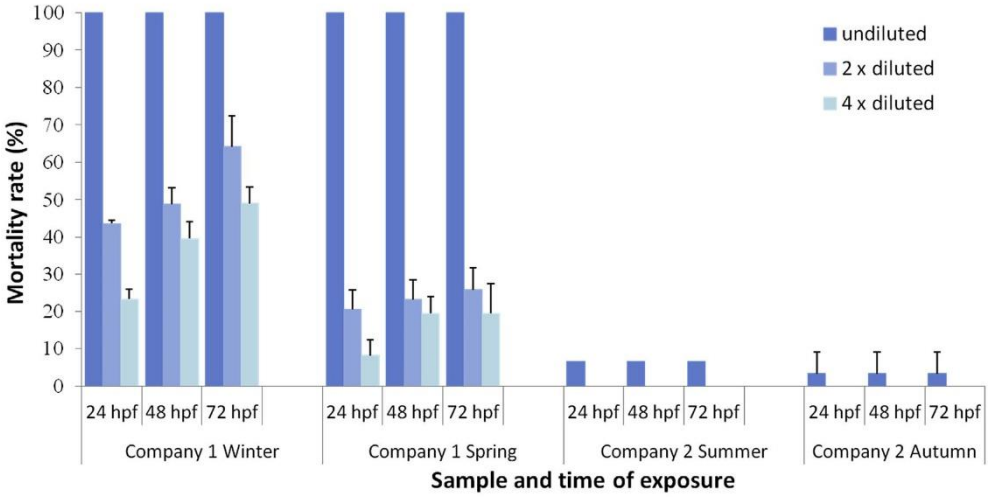

Fig. 2. Mortality of *D. rerio* embryos exposed to effluent samples, expressed as mean ± SD (n = 30).

effluents are not the only source of macrolide pollution of studied river. Discharge from the wastewater treatment plant of the City of Zaprešić located between two studied downstream locations may be responsible for this.

In contrast to mg/L concentrations of macrolides in effluents from AZI production, the concentrations of sulfonamides, trimethoprim, enrofloxacin and oxytetracycline antibiotics in effluents from Company 2 were much lower and ranging from low μg/L to approx. 200 μg/L. Even though, peak values were approx. one order of magnitude higher than the levels observed in the receiving studied stream and the levels that are regularly found in treated sewage effluents (Michael et al., 2013). Noteworthy, concentrations of the same antibiotic in Company 2 effluents varied significantly, sometimes even by approx. 30 times as observed for sulfamethazine (between 7 and 231 μg/L). Such fluctuations in antibiotic concentrations were observed for effluents of both Companies and could be due to multiple reasons, including different production cycles and washing of reaction/formulating tanks (Larsson, 2014; Pruden et al., 2013).

The high public concern associated with release of antibiotics from pharmaceutical production is promotion of antibiotic resistance, an event not restricted to the area or even country of emergence but a potential threat to human health worldwide. Indeed, enrichment of resistant bacteria or their resistance genes has already been shown in aquatic environment polluted with antibiotics from pharmaceutical manufacturing (Kristiansson et al., 2011; Li et al., 2011, 2009, 2010). In addition to this, antibiotic resistance can be promoted even at antibiotic levels far below the inhibitory concentrations, similar to those found in some aquatic environments (Andersson and Hughes, 2014; Berendonk et al., 2015; Gullberg et al., 2011). Despite this, there are still no regulation regarding surface water levels for any antibiotic, although AZI and ERY were recently added to the so-called watch list' within the European Water Framework Directive due to their toxicity, persistence and bioaccumulation potential [Decision, 2015/495/EU of 20 March 2015 (Barbosa et al., 2016)]. In the current lack of regulatory system, Bengtsson-Palme and Larsson (2016) have recently estimated Predicted No Effect Concentrations (PNECs) for resistance selection for 111 antibiotics. AZI concentration in our effluents can be 4 orders of magnitude higher then estimated PNEC (250 ng/L), suggesting that it might be sufficient to specifically promote resistance in the recipient Sava river. Indeed, AZI concentrations in the river at two downstream locations measured during winter and springtime exceeded the corresponding PNEC values, suggesting the risk for selecting macrolide resistance in the environment. Although much lower antibiotic concentrations were detected in effluents of antibiotic-formulating Company 2 and in the receiving stream near discharge, they were still above the corresponding PNECs and thus predicted to be selective for resistance (Bengtsson-Palme and Larsson, 2016). However, antibiotic concentrations measured in the stream 3 km downstream of discharge were mostly lower than PNEC values.

Additionally, metals detected in effluents have the potential to co-select for antibiotic resistance (Baker-Austin et al., 2006; Pal et al., 2015). Despite the fact that the concentrations of most ecotoxic heavy metals (As, Cd, Cr, Cu, Pb, Ni) were lower than the legally set upper limit, they were still up to 85 times higher than minimum concentrations that were reported to have co-selective potential in surface waters (Seiler and Berendonk, 2012; Stepanauskas et al., 2006). This suggests that co-selection could be at play, at least in effluents. However, in current lack of data on metal concentrations in the receiving water bodies, we do not know if metal concentrations in the receiving waters are still selective.

Effluents from both industries were found to contain high frequency of antibiotic-resistant bacteria i.e. up to 91% of SMZ-resistant bacteria and 50% of OTC-resistant bacteria in effluents from Company 2 and up to 83% of AZI-resistant bacteria in effluents from Company 1. The frequencies of AZI-resistant bacteria in Company 1 effluents and OTC-resistant bacteria in Company 2 effluents were found to be positively associated with the occurrence of AZI and OTC suggesting that increased antibiotic concentrations in these effluents created a breeding ground for proliferation of resistant bacteria. In addition, the fact that effluents from Company 2 are mixed with human sewage within the industry implies a risk that pathogens are exposed to antibiotics at concentrations selecting for resistance. Therefore, discharge of such poorly treated effluents into the receiving stream offers a potential route for dissemination of resistant bacteria, including human pathogens. This suggests that there is a need for better management of discharges from antibiotic manufacturing which should involve limits on both antibiotic-resistant bacteria and antibiotic levels.

The direct effects of effluent exposure on biota was assessed using eukaryotic algae, crustacean invertebrates and zebrafish embryo models. In overall, majority of effluent samples induced some level of toxic effects, but Company 1 effluents proved to be more toxic to all tested organisms. For example, clearly detectable effects on *D. magna* motility were found after exposure to Company 1 winter effluents diluted up to 16 ×. On the other hand, Larsson et al. (2007) demonstrated the same effects after exposure to higher dilutions of Patancheru industrial effluents contaminated with fluoroquinolones, suggesting even higher toxicity of Patancheru effluents on *D. magna* in comparison with our macrolide-contaminated effluents. Similarly, lower dilutions of our Company 1 winter and spring effluents (2 and 4 ×) caused high zebrafish embryo mortality, reduced the heart rate and embryo pigmentation, whereas Carlsson et al. (2009) observed the same effects at higher dilutions of Patancheru industrial effluents. In comparison to *D. magna* and zebrafish embryos, alga *P. subcapitata* was shown to be more sensitive to exposure to tested effluents because even more than 32 times diluted winter and spring Company 1 samples still caused growth inhibition. However, majority of the observed lethal and sub-lethal toxic effects can not be attributed to any single antibiotic, since previously reported EC50 values for antibiotic toxicity to algae, daphnids and fish (Table S4) are mostly higher than antibiotic concentrations we measured in the effluents (Tables 3 and 4). The only exception is AZI: we have measured approx. 100 × higher concentrations of AZI in winter and spring Company 1 effluents than its EC50 value for algae (19 μg/L) (Harada et al., 2008). Therefore, presence of macrolides could partly explain the toxic effects of Company 1 effluents to *P. subcapitata*. It is also possible that some other undetected potent compound(s) in effluents are causative agent(s) of the observed effects, but a mixture effect from multiple compounds is also possible. Indeed, it was reported that synergism often occurs in antibiotic mixtures and that certain combinations of antibiotics, such as mixtures of different macrolides, present an additional ecological risk for aquatic ecosystems (González-Pleiter et al., 2013; Yang et al., 2008). Also, Zhang et al. (2012) have showed that antibiotics (OTC and CIP) make complexes with heavy metals (Cu, Zn and Cd) which are more toxic to bacterium *Vibrio fischeri* and alga *Scenedesmus obliquus* than individual compounds. Such scenarios likely contributed to the high toxicity of pharmaceutical effluents to various organisms presented in this study and in the previous studies (Carlsson et al., 2009; Larsson et al., 2007).

Finally, the results from the *Salmonella* mutagenicity assay showed a mild mutagenicity of Company 2 effluents which suggests that these effluents contained compounds with mutagenic effects. This could be potentially ascribed to the presence of ENR in those samples as fluoroquinolones were reported to induce genotoxic effects (Gocke, 1991; Hayasaki et al., 2006). However, other compounds or mixture of compounds cannot be excluded as well.

Together, our data indicate that effluents from local antibiotic manufacturing plants can be contaminated to the point where they affect aquatic wild-life. Additional studies are required to elucidate adverse effects for human health which may rather be related to indirect risks coupled to potential promotion and spread of antibiotic resistance as a consequence of the environmental pollution with antibiotics.

5. Conclusions

This study demonstrates the multiple negative potential effects of antibiotic-contaminated pharmaceutical effluents for the receiving waters. Observed high concentrations of macrolides (up to few mg/L) along with high load of antibiotic-resistant bacteria (10^5–10^7 CFU/mL) and high organic and nutrient loads in winter and spring suggest that discharge of studied effluents can pose a great risk for environmental and human health. Studied effluents can be seen as a reservoirs from which resistance traits, in the presence of residual antibiotics and other co-selective agents as well as nutrients, may be further spread, with the potential to ultimately reach human pathogens.

Most importantly, this study emphasizes the environmental risk of macrolides that are recognized as antibiotics with high toxicity, persistence and bioaccumulation potential (Decision, 2015/495/EU of 20 March 2015). We report high levels of macrolides in final, treated effluents from azithromycin-production during winter and spring season. Even after dilution of the effluent with the receiving river water, the concentrations of macrolides are likely to be selective for antibiotic resistance and to have toxic effect on freshwater green algae.

Finally, our work prompts for further research and strategies for improved management of discharges from antibiotic production, including more efficient wastewater treatment technologies and defining emission limits for individual antibiotics, with emphasis on the compounds that were shown to pose a greatest environmental and health risk, such as macrolides.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.09.019.

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Functional Repertoire of Antibiotic Resistance Genes in Antibiotic Manufacturing Effluents and Receiving Freshwater Sediments

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Environments polluted by direct discharges of effluents from antibiotic manufacturing are important reservoirs for antibiotic resistance genes (ARGs), which could potentially be transferred to human pathogens. However, our knowledge about the identity and diversity of ARGs in such polluted environments remains limited. We applied functional metagenomics to explore the resistome of two Croatian antibiotic manufacturing effluents and sediments collected upstream of and at the effluent discharge sites. Metagenomic libraries built from an azithromycin-production site were screened for resistance to macrolide antibiotics, whereas the libraries from a site producing veterinary antibiotics were screened for resistance to sulfonamides, tetracyclines, trimethoprim, and beta-lactams. Functional analysis of eight libraries identified a total of 82 unique, often clinically relevant ARGs, which were frequently found in clusters and flanked by mobile genetic elements. The majority of macrolide resistance genes identified from matrices exposed to high levels of macrolides were similar to known genes encoding ribosomal protection proteins, macrolide phosphotransferases, and transporters. Potentially novel macrolide resistance genes included one most similar to a 23S rRNA methyltransferase from *Clostridium* and another, derived from upstream unpolluted sediment, to a GTPase HflX from *Emergencia*. In libraries deriving from sediments exposed to lower levels of veterinary antibiotics, we found 8 potentially novel ARGs, including dihydrofolate reductases and beta-lactamases from classes A, B, and D. In addition, we detected 7 potentially novel ARGs in upstream sediment, including thymidylate synthases, dihydrofolate reductases, and class D beta-lactamase. Taken together, in addition to finding known gene types, we report the discovery of novel and diverse ARGs in antibiotic-polluted industrial effluents and sediments, providing a qualitative basis for monitoring the dispersal of ARGs from environmental hotspots such as discharge sites of pharmaceutical effluents.

Keywords: antibiotic resistance, effluent, manufacturing, antibiotic pollution, sediment, macrolides, functional metagenomics

INTRODUCTION

Antibiotic resistance is one of the most serious global public health threats of the twenty-first century (Carlet et al., 2012; ECDC, 2016; O'neill, 2016). This phenomenon is strongly associated with hospitals and other clinical environment (Brown et al., 2006; Rodriguez-Mozaz et al., 2015), because the extensive use of antibiotics in clinical settings is the driving force for increasing antibiotic resistance. However, there is a growing awareness that anthropogenic inputs of antibiotics into the environment through effluents, use of manure and biosolids in agriculture, and aquaculture contribute to this problem. The selection pressure imposed by antibiotics and other selective agents has promoted the propagation of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (collectively known as the resistome) in the environment, creating vast reservoirs of ARGs with potential to be transferred to pathogens (Bengtsson-Palme et al., 2014; Cabello et al., 2016; Tao et al., 2016; Peng et al., 2017; Su et al., 2017). Understanding these reservoirs and behaviors of ARGs is crucial to control the emergence of resistant pathogens at a global scale.

Direct discharge of pharmaceutical effluents in receiving water bodies has been recognized as an important source of pollution, as they may contain high concentrations of antibiotics, ARB, heavy metals, and other hazardous materials (Babić et al., 2007; Larsson et al., 2007; Li et al., 2009; Sim et al., 2011; Larsson, 2014; Bielen et al., 2017). High concentrations of antibiotics which are above the minimum inhibitory concentrations could cause death of many susceptible environmental microorganisms and enrich those genetically adapted, while sub-inhibitory concentrations exert a selective pressure, which act as a moving force in horizontal dissemination of ARGs (Baker-Austin et al., 2006; Tacão et al., 2014; Di Cesare et al., 2016; Navon-Venezia et al., 2017). Therefore, environments impacted by discharges from manufacturing of antibiotics are high risk environments for antibiotic resistance selection and dissemination into human or animal pathogens. Hence, it is essential to understand the contribution of manufacturing sites to the environmental resistome in more detail.

Studies addressing the impact of effluent discharge from antibiotic production on the resistome of the receiving environment are still scarce and limited to Asian countries. Several studies using culture- and PCR-based methods have reported the presence of multidrug resistant bacteria (MDR) in rivers receiving effluents from antibiotic production (Li et al., 2009, 2010; Sidrach-Cardona et al., 2014; Lübbert et al., 2017). Although these methods provide valuable information, their major limitations are that the bacteria need to be culturable under laboratory conditions or screening is limited to known ARGs. Sequence-based metagenomics has enabled the exploration of the total DNA of a sample, providing a broad spectrum of known ARGs. This methodology has been used to study river and lake sediments highly polluted with antibiotics (mostly fluoroquinolones) from bulk drug production in India, revealing a high diversity and promotion of resistance genes to several classes of antibiotics as well as their mobilization elements (Kristiansson et al., 2011; Bengtsson-Palme et al., 2014). Despite these extremely valuable findings, metagenomic sequencing cannot identify potentially novel ARGs nor gives information on the expression of the ARGs (Mullany, 2014). Functional metagenomics, which consists of heterologous expression of metagenomic DNA in a surrogate host and activity-based screening, is a useful approach to identify different types of functional ARGs, both known and novel. Furthermore, the genes discovered by functional metagenomics are, by definition, candidates for horizontal transfer, as they must be functional in a heterologous host (Crofts et al., 2017). This method has been successfully applied in exploring the ARGs in different matrices including the human microbiome (Sommer et al., 2009), soil (Udikovic-Kolic et al., 2014), manure (Wichmann et al., 2014), activated sludge (Parsley et al., 2010), river (Amos et al., 2014), and ocean (Hatosy and Martiny, 2015).

Recently, our study on effluents from two Croatian pharmaceutical industries showed that they are contaminated with high levels of antibiotics and culturable ARB (Bielen et al., 2017). Treated effluent from an azithromycin (AZI)-synthesizing factory contained occasionally high, mg L^{-1} concentrations of macrolide antibiotics, which are critical for use in human medicine (WHO, 2017). On the other hand, partially treated effluent from a formulation industry contained a range of antibiotics (fluoroquinolones, trimethoprim, sulfonamides, and tetracyclines) from low to high $\mu g\ L^{-1}$ concentrations (Bielen et al., 2017). These effluents are discharged into the nearby river and stream providing an opportunity for selection of antibiotic resistance. There is also the possibility that these environments harbor yet undescribed resistance genes, some of which we may face in pathogens in the hospitals tomorrow. Previous studies, applying metagenomic sequencing, have shown high levels of known and mobile ARGs, particularly those conferring resistance to quinolones and sulphonamides, in Indian sediments heavily polluted with fluoroquinolone antibiotics (Kristiansson et al., 2011; Bengtsson-Palme et al., 2014). In this study, using functional metagenomics we have explored the diversity of ARGs in industrial effluents and sediments polluted with macrolide or different veterinary antibiotics. We have also assessed the genetic context of the identified resistance genes by analyzing their flanking DNA.

MATERIALS AND METHODS

Study Areas and Sampling Procedures

Study areas lie in the northwest of Croatia where two local pharmaceutical industries are situated. Industry 1 has a long tradition in synthesizing the macrolide antibiotic AZI and discharges its final, treated effluent into the Sava river. Industry 2 formulates various veterinary antibiotics, including antibiotics from the tetracycline, sulfonamide and beta-lactam classes, and discharges its partially treated effluent into the nearby small stream. More detailed information about these two industries and properties of their effluents have been recently published (Bielen et al., 2017).

Industrial effluents and sediment samples from the receiving river and stream were collected in January and February 2016. Effluent from Industry 1 was collected as grab sample from the

discharge pipe and effluent from Industry 2 consisted of a 24-h composite sample. Both effluent samples were collected in a sterile 2 L bottle and kept at 4°C. Immediately upon return to the laboratory, aliquots of 50–100 mL were vacuum-filtered through mixed cellulose ester filters (0.22 μm pore diameter) (GE Healthcare Life Sciences) to collect the bacterial cells, and filters were stored at −80°C until DNA extraction. Surface sediment samples (0–10 cm) were taken at effluent discharge locations and at reference locations situated upstream of the discharge areas (4 samples in total). Four sub-samples were collected at each location and merged to a composite sample (10 g of each sub-sample) within 4 h of collection. These samples were used fresh for immediate culturing or stored at −80°C until DNA was extracted.

Culturing Bacteria from Sediments

To culture bacteria from fresh sediment samples, 1 g of the composite sediment was suspended in physiological saline (0.9% NaCl) by vortexing. Serial 10-fold dilutions were cultured on three replicate R2A agar plates to enumerate total bacteria. To enumerate resistant bacteria, serial dilutions were cultured in triplicates on plates supplemented with AZI (15 mg L^{-1}) (Fluka, Germany) for Industry area 1 samples; or sulfamethazine (SMZ; 350 mg L^{-1}) (Sigma, Germany) or oxytetracycline (OTC; 25 mg L^{-1}) (Sigma, Germany) for Industry area 2 samples. Colony forming units (CFU) were counted after a 5 day incubation at 28°C. ARB cultured from sediments from discharge locations were scraped from the plates, pooled and stored in R2A broth containing antibiotic and 15% glycerol at −80°C.

Small Insert-Size Metagenomic Library Construction

DNA for the construction of libraries was isolated from the filters, sediment samples and from pools of cultured sediment bacteria using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's recommendations. DNA was either partially digested with *Pst*I (NEB, USA) and cloned into the pCF430 vector (Newman and Fuqua, 1999) or digested with *Hind*III (NEB, USA) and cloned into the pZE21-MCS vector (Lutz and Bujard, 1997; **Table 1**). Ligation products were dialyzed using 0.2-μm filter membranes (Millipore, Ireland) and electroporated into *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA) using a Micropulser (Biorad, Hercules, CA). After a 1 h incubation in SOC media, cells were plated on LB plates supplemented with 5 mg L^{-1} tetracycline (Industry area 1) or 50 mg L^{-1} kanamycin (Industry area 2), and incubated at 37°C overnight. Library storage and size estimation were performed according to previously published protocols (Wichmann et al., 2014). Briefly, the average insert size for each library was determined by restriction digest analysis of 10 randomly picked clones using *Pst*I (pCF430) or *Hind*III (pZE21-MCS). After insert size analysis, all clones were pooled together by scraping them from plates into LB supplemented with 20% glycerol and tetracycline or kanamycin followed by storage at −80°C.

Identification of Antibiotic Resistance Genes

Metagenomic libraries (10 μL of the pooled clones) were grown in 5 mL of LB supplemented with the appropriate antibiotic (either tetracycline or kanamycin) for 2 h at 37°C and 200 rpm. Appropriate dilutions were plated on LB plates containing an antibiotic of interest: AZI (16 mg L^{-1}) or erythromycin (ERI; 100 mg L^{-1}) for Industry area 1 libraries; trimethoprim (TRM; 20 mg L^{-1}), tetracycline (TET; 20 mg L^{-1}), OTC (20 mg L^{-1}) ampicillin (AMP; 100 mg L^{-1}), cefotaxime (CTX; 8 mg L^{-1}), or ciprofloxacin (1 and 0.5 mg L^{-1}) for Industry area 2 libraries, and incubated overnight at 37°C. For screening of libraries from Industry area 2 on SMZ (350 mg L^{-1}) and TRM, instead of LB media, Mueller-Hinton broth or agar was used because it is low in sulfonamide and trimethoprim inhibitors. The antibiotic concentrations used inhibited growth of *E. coli* DH5α transformed with empty pCF430 or pZE21-MCS plasmid. The proportion of resistant clones in each library was calculated as the ratio of the number of resistant clones (grown on plates containing an antibiotic of interest) and the total number of clones (grown on plates containing vector antibiotic). The diversity of individual resistant clones was assessed with *Hind*III and *Bam*HI digestion (libraries derived from Industry area 1) or with *Pst*I and *Bam*HI digestion (libraries derived from Industry area 2). Plasmids containing inserts with distinct restriction patterns were sent to Macrogen DNA Sequencing Service (Macrogen, Netherlands) for bi-directional Sanger sequencing using vector-targeting forward and reverse primers (Sommer et al., 2009; Wichmann et al., 2014). Additional specific primers were designed as necessary for extension of the obtained sequences. Sequencing data was processed using the DNASTAR Lasergene software package (version 14) and nucleotide sequences of the identified open reading frames (ORFs) were compared to the publicly available sequences using BLASTX search against the NCBI nr/nt database. Active gene was considered to be unique if it did not have identical nucleotide sequence to any other gene in the same library.

Determination of Minimum Inhibitory Concentration (MIC)

MIC assays were performed on unique resistant clones by broth microdilution in Mueller-Hinton broth (Difco, USA) according to previously published protocols (Donato et al., 2010). The MIC was defined as the lowest concentration of the antibiotic that inhibited visible growth of 10^5 cells of tested clone. As a control we used DH5α cells transformed with the empty vector (pCF430 or pZE21-MCS).

Phylogenetic Analysis

The Geneious software (version 6.0.5) (Kearse et al., 2012) was used for sequence comparisons and phylogenetic analyses. For sequence alignments, we used CLUSTALW (Thompson et al., 2002), and the phylogenetic trees were inferred using maximum likelihood (Jones et al., 1992). Bootstrap values were calculated based on 100 replications. Trees were adapted using the FigTree program (v1.4.3.) (Rambaut, 2009).

TABLE 1 | Features of the metagenomic libraries constructed in this study.

Study area	Library name	Origin	Vector/AR marker	Average insert size (kb)	Amount of cloned DNA (Gb)
Industry area 1	S_US_C1	Sediment at upstream site	pCF430/Tet^R	4.40	5.47
	F_WW_C1	Pharmaceutical effluent	pCF430/Tet^R	4.20	19.20
	B_DS_C1	Culturable AZI-resistant bacteria from sediment at discharge site	pCF430/Tet^R	3.40	5.50
	S_DS_C1	Sediment at discharge site	pCF430/Tet^R	2.80	1.54
Industry area 2	S_US_C2	Sediment at upstream site	pZE21-MCS/Kan^R	3.70	2.20
	F_WW_C2	Pharmaceutical effluent	pZE21-MCS/Kan^R	3.38	4.56
	B_DS_C2	Culturable SMZ and OTC-resistant bacteria from sediment at discharge site	pZE21-MCS/Kan^R	2.90	1.96
	S_DS_C2	Sediment at discharge site	pZE21-MCS/Kan^R	2.50	4.58

Nucleotide Sequence Accession Numbers

The metagenomic insert sequences from Industry area 1 are shown in **Table 2** and were deposited in GenBank under accession numbers MG585943 to MG585960. Sequences from Industry area 2 are shown in Supplementary Table 2 and were deposited under accession numbers MG585961 to MG586044.

RESULTS AND DISCUSSION

Selection of Antibiotic Resistance in River and Stream Sediments Receiving Effluents from Antibiotic Manufacturing

Culturing of sediment bacteria showed a considerably higher proportion of AZI-resistant or SMZ- and OTC-resistant bacteria in sediments at the discharge vs. upstream sites, indicating an enrichment of resistant populations in the effluent-impacted environment (**Figure 1**). Higher proportion of ARB has also been observed in the Indian sediment samples in comparison with samples from reference locations, most likely caused by the emissions of high concentrations of antibiotics from local drug manufacturers (Flach et al., 2015). In a recent study, we showed that effluents from two manufacturing sites studied here contained high concentrations of antibiotics and a high proportion of culturable ARB (Bielen et al., 2017). For example, mg L^{-1} levels of macrolide antibiotics along with high frequencies of AZI-resistant bacteria (up to 83%) were found in effluents from Industry area 1. Furthermore, effluents from Industry area 2 were found to contain high levels of SMZ- and OTC-resistant bacteria (up to 90 and 50%, respectively) and several antibiotics including sulfonamides, fluoroquinolones, trimethoprim and tetracyclines in concentrations up to about 230 μg L^{-1}. Consequently, it would be reasonable to expect higher levels of these antibiotics in sediments at the discharge compared with upstream sites, as shown in our preliminary analyses (unpublished data). Therefore, the observed higher proportion of culturable ARB at both discharge sites could be due to a pollution of the river and stream with antibiotics that select for resistant bacteria already resident in the sediment. Alternatively, the released effluent-associated resistant bacteria may proliferate in the sediment, or a combination of these contributors may take place. Receiving sediments may thus act as a reservoir where known circulating resistant bacteria and their genes are maintained as well as new resistant strains and genes may emerge and spread.

Metagenomic DNA extracted from sediment and effluent samples was used to build eight small-insert libraries (2 from effluents, 2 from upstream sediments, and 4 from discharge sediments, **Table 1**). The libraries from Industry area 1 had an average insert size of 3.7 kb and an average total size of 32 Gb. The libraries from Industry area 2 contained a total of 13 Gb with an average insert size of 3.1 kb. The proportion of resistant clones selected on eight antibiotics was generally lower in libraries originating from upstream sediments compared with libraries from discharge sediments and effluents (Supplementary Figure 1), suggesting a selection of resistance genes in matrices impacted by pollution with antibiotics and ARB.

Identification of Macrolide Resistance Genes in Effluent and River Sediments Near Industry Area 1

Screening of the libraries of Industry area 1 yielded 17 different macrolide resistant clones with 18 resistance genes, 16 of which were unique based on their nucleotide sequence (**Table 2**, Supplementary Table 2). These genes represented one unknown resistance mechanism mediated by a GTPase binding protein and three known mechanisms, such as efflux

TABLE 2 | Summary of all macrolide resistance genes from clones with distinct restriction digest patterns in functional metagenomic libraries built from effluent and sediments of Industry area 1.

Antibiotic used for selection	Clone designation/origin	MIC (mg L^{-1})	Gene length (bp)	Gene annotation (Closest BLASTX hit in NCBI)	% amino acid identity	GenBank Accession No.
Azithromycin	AZI1_S_US_C1/ Upstream sediment	<16 (AZI) 128 (ERI)	1,281	GTPase binding protein HflX (*Emergencia timonensis* WP_067543614.1)*	63	MG585943
	AZI2_S_US_C1/ Upstream sediment	<16 (AZI) 128 (ERI)	1,281	GTPase binding protein HflX (*Emergencia timonensis* WP_067543614.1)*	63	MG585944
	AZI1_F_WW_C1/ Effluent	64 (AZI) 512 (ERI)	1,476	ABC-F type ribosomal protection protein Msr(E) (*Klebsiella pneumoniae* YP_003754030.1)*	100	MG585948
	AZI4_F_WW_C1/ Effluent	32 (AZI) 512 (ERI)	1,476	ABC-F type ribosomal protection protein Msr(E) (*Klebsiella pneumoniae* YP_003754030.1)*	99	MG585949
	AZI1_B_DS_C1/ Sediment bacteria	512 (AZI) 2,048 (ERI)	1,476	ABC-F type ribosomal protection protein Msr(E) (*Klebsiella pneumoniae* YP_003754030.1)*	100	MG585952
			885	Macrolide 2′-phosphotransferase Mph(E) (*Klebsiella pneumoniae* YP_003754029.1)*	100	MG585954
	AZI4_B_DS_C1/ Sediment bacteria	512 (AZI) 2,048 (ERI)	348	SMR family, quaternary ammonium compound efflux QacEΔ1 (*Salmonella enterica* NP_511227.1)*	100	MG585956
	AZI1_S_DS_C1/ Discharge sediment	256 (AZI) 1,024 (ERI)	1,476	ABC-F type ribosomal protection protein Msr(E) (*Acinetobacter baumannii* YP_724476.1)*	99	MG585957
Erythromycin	ERI1_S_US_C1/ Upstream sediment	64 (ERI) <16 (AZI)	1,281	GTPase binding protein HflX (*Emergencia timonensis* WP_067543614.1)	63	MG585945
	ERI2_S_US_C1/ Upstream sediment	128 (ERI) <16 (AZI)	1,278	GTPase binding protein HflX (*Emergencia timonensis* WP_067543614.1)*	62	MG585946
	ERI9_S_US_C1/ Upstream sediment	64 (ERI) <16 (AZI)	1,281	GTPase binding protein HflX (*Emergencia timonensis* WP_067543614.1)*	62	MG585947
	ERI1_F_WW_C1/ Effluent	512 (ERI) 32 (AZI)	1,224	MFS macrolide efflux protein Mef(C) (*Colwellia chukchiensis* WP_085286200.1)*	100	MG585951
	ERI4_F_WW_C1/ Effluent	1,024 (ERI) 64 (AZI)	1,473	ABC-F type ribosomal protection protein Msr(E) (*Klebsiella pneumoniae* YP_003754030.1)*	100	MG585950
	ERI2_B_DS_C1/ Sediment bacteria	1,024 (ERI) 256 (AZI)	1,476	ABC-F type ribosomal protection protein Msr(E) (*Acinetobacter baumannii* YP_724476.1)	100	MG585953
	ERI9_B_DS_C1/ Sediment bacteria	1,536 (ERI) <16 (AZI)	903	23S ribosomal RNA methyltransferase (*Clostridium* sp. CAG:780 CCZ18576.1)*	67	MG585955
	ERI1_S_DS_C1/ Discharge sediment	1,024 (ERI) 128 (AZI)	885	Macrolide 2'-phosphotransferase Mph(E) (*Acinetobacter baumannii* YP_001736317.1)*	100	MG585958

(Continued)

TABLE 2 | Continued

Antibiotic used for selection	Clone designation/origin	MIC (mg L^{-1})	Gene length (bp)	Gene annotation (Closest BLASTX hit in NCBI)	% amino acid identity	GenBank Accession No.
	ERI2_S_DS_C1/ Discharge sediment	1,024 (ERI) 64 (AZI)	1,224	MFS macrolide efflux protein Mef(C) (*Colwellia chukchiensis* WP_085286200.1)*	100	MG585959
	ERI7_S_DS_C1/ Discharge sediment	512 (ERI) <16 (AZI)	885	Macrolide 2′-phosphotransferase Mph(G) (*Colwellia chukchiensis* SEL95196.1)*	100	MG585960

*Unique genes (based on their nucleotide sequence) from the same library are marked with *. MIC, Minimal inhibitory concentration.*

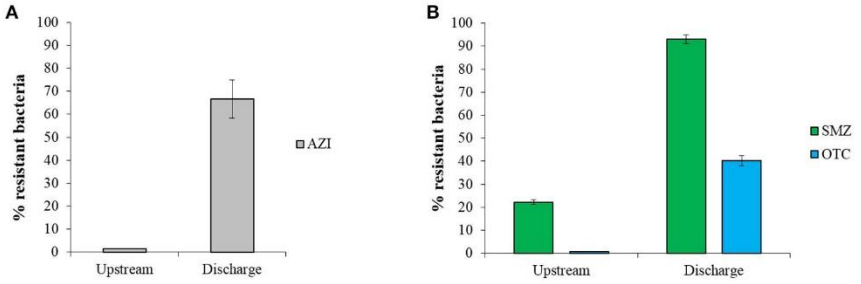

FIGURE 1 | The proportion of resistance (mean ± SD) of culturable bacteria to **(A)** azithromycin (AZI) and **(B)** sulfamethazine (SMZ) and oxytetracycline (OTC) in sediments from discharge and upstream sites of two study areas. The percentage of antibiotic resistant bacteria was calculated as the ratio of resistant bacteria CFU and total CFU.

pumps, macrolide inactivation by phosphotransferases and target modification/protection by ribosomal RNA methyltransferases or ribosomal protection proteins (**Table 2**). To classify our annotated genes as novel genes, we set a cut-off of 80% protein sequence identity (Zhao et al., 2014).

The majority of unique genes (12/16) matched known ARGs encoding macrolide efflux pumps, ribosomal protection proteins and macrolide 2′-phosphotransferases (Supplementary Figure 2, Supplementary Table 1A). Efflux pump genes included two major facilitator superfamily (MFS) transporter genes with high sequence identity to *mef*(C) from *Colwellia chuchiensis* and one small multidrug resistance (SMR) transporter, highly similar to the quaternary ammonium compound efflux gene (*qacEΔ1*) from *Salmonella enterica*. The *mef* genes have largely been found among clinically relevant macrolide resistant pathogens (Fyfe et al., 2016). Ribosomal protection proteins were encoded by six ABC-F protein genes with high similarity to *msr*(E) previously found in *Klebsiella pneumoniae* and *Acinetobacter baumannii*. Recently, Sharkey et al. (2016) provided strong evidence that these proteins interact with the ribosome and displace the drug from its binding site, thus revealing a novel role for ABC-F proteins in antibiotic resistance. Macrolide inactivating enzymes included phosphotransferase genes, *mph*(E) and *mph*(G) which were identical (100% of amino acid identity) to those from *A. baumanii*, *K. pneumoniae,* and *C. chuchiensis*, respectively. All of these known genes (*mef, msr, mph*) were obtained from libraries deriving from antibiotic-impacted matrices (effluent and receiving sediment), suggesting their relation to selection pressure from macrolide antibiotics and possibly other co-selecting agents from AZI production.

Only one gene (*erm*, clone ERI9_B_DS_C1) deriving from the library from the discharge site shared low amino acid sequence identity (67%) with a 23S rRNA methyltransferase from *Clostridium* sp. (**Table 2**). This suggests that it encodes for a potentially novel member of this methyltransferase family, which confers clinically relevant levels of ERI resistance (MIC = 1,536 mg L^{-1}; **Table 2**) through ribosomal methylation. The mechanism mediated by *erm* genes remains the most widespread mechanism of macrolide resistance in clinically important pathogens (Fyfe et al., 2016).

Contrary to most known genes in polluted matrices, all unique genes deriving from upstream reference sediment (4/16) had ≤63% protein sequence identity with their best hit in the NCBI, a GTPase HflX from *Emergencia timonensis*, indicating potential novelty of these genes (**Table 2**, Supplementary Table 1A, Supplementary Figure 2). Although the exact mechanism mediated by HflX remains to be unraveled, Lau et al. (2017) proposed that the GTPase HflX acts as an alternative ribosome splitting factor which disassembles the 70S ribosomes into its subunits and in this way helps to overcome the translational arrest caused by macrolides.

Based on MIC results, the HflX-mediated macrolide resistance seems less effective than the other identified mechanisms, which conferred a higher level of macrolide resistance (ERI 64–128 vs. 512–2,048 mg L^{-1}; AZI 16 vs. 32–512 mg L^{-1}, **Table 2**). This indicates that the bacteria living in sediment at the

discharge site have evolved or acquired increasing resistance to macrolides in response to exposure to high macrolide selection pressure. In contrast, sediment bacteria from the upstream site that has had no known anthropogenic exposure to antibiotics are source of different, yet unknown mechanisms of macrolide resistance. These might evolve coincidentally in the presence of selective forces other than antibiotics that may cause accumulation of mutations that incidentally also confer antibiotic resistance (Knöppel et al., 2017). Other studies also showed that environments (i.e., sediments) not subjected to anthropogenic antibiotic pollution could be reservoirs of novel ARGs (Kristiansson et al., 2011; Amos et al., 2014; Nesme et al., 2014).

In addition, the discovery of potentially novel macrolide resistance genes originating from *Emergencia* and *Clostridium* genera in *E. coli* further demonstrates the power of functional metagenomics to identify resistance genes from Gram-positive bacteria in Gram-negative host.

Identification and Phylogeny of Sulfonamide, Tetracycline, Trimethoprim, and Beta-Lactam Resistance Genes in Effluent and Environment Near Industry Area 2

Of 82 clones with 84 genes conferring resistance to sulfonamides, tetracyclines, trimethoprim and beta-lactams, we obtained 66 unique ARGs (Supplementary Tables 1B, 2). No clones resistant to fluoroquinolones (ciprofloxacin) were obtained in this study, as has also been reported in one other study of soil (Charles et al., 2017), which may be due to incompatibility resulting from the use of *E. coli* as surrogate host. The predicted protein sequences of identified ARGs shared between 49 and 100% amino acid sequence identity with proteins in the NCBI database (Supplementary Figure 3), although the average sequence identity differed among the types of ARGs. For example, all of the genes conferring resistance to SMZ, OTC, and TET were highly similar (amino acid identity ≥94%) with previously known genes. SMZ resistant clones contained the dihydropteroate synthase genes (*sul1* and *sul2*), which are also found in pathogens such as *Enterobacter cloacae* and *A. baumannii* (Supplementary Table 2). The fact that the *sul1* gene was detected in all of the four libraries suggests a wide distribution of this gene in background sediment and industrial effluent. In contrast, the *sul2* was detected in sediment only at the discharge site, suggesting that its presence may have resulted from effluent discharge. However, both genes (*sul1* and *sul2*) have been previously reported in antibiotic polluted (Luo et al., 2010; Kristiansson et al., 2011; Bengtsson-Palme et al., 2014) and unpolluted sediments (Czekalski et al., 2015; Archundia et al., 2017), which is likely due to their genetic localization on mobile elements that could be easily transferred among bacteria (Heuer et al., 2011; Hu et al., 2016; Johnson et al., 2016; Koczura et al., 2016).

All functional genes from OTC and TET resistant clones matched previously reported tetracycline transporters that belong to the MFS, indicating that efflux is the predominant mechanism of resistance to tetracyclines in Industry area 2 (Supplementary Table 2). Phylogenetic analysis (Supplementary Figure 4) showed that the majority of tetracycline and oxytetracycline resistance genes (14/21), identified from all four libraries, cluster closely together with a *tet* transporter gene from *Flavobacterium psychrophilum* which is not similar to any annotated group of *tet* resistance genes. The detection of these genes in all four libraries suggests their natural distribution in the sediment and industrial effluent. The rest of the sequences are related to *tet*(39), *tet*(A), or *tet*(C) from the *γ-Proteobacteria*. These genes have also been found in a highly antibiotic polluted lake sediment, with the *tet*(39) being the most abundant (Bengtsson-Palme et al., 2014). Of these genes, we only detected *tet*(C) in our upstream sediment, suggesting that it may occur naturally in the studied sediment or it comes from nearby agricultural sources. However, other studies detected *tet* resistance genes, including *tet*(A) and *tet*(C), in environments not subjected to anthropogenic antibiotic pollution (Andersen and Sandaa, 1994; West et al., 2011; Durso et al., 2016), implying their wide distribution in the environment.

Screening of libraries with TRM resulted in a substantial proportion (11/35) of potentially novel genes (≤80% amino acid identity) along with the known genes (Supplementary Table 2). Both known and all potentially novel genes were found in libraries of both upstream and discharge sediment indicating that sediment itself is a natural reservoir for TRM-resistant bacteria carrying a diverse set of known and unknown TRM resistance genes (Supplementary Table 2). All identified genes were predicted to encode target-modified dihydrofolate reductases (DHFR) or thymidylate synthases (TYMS). Phylogenetic clustering showed that the majority of identified genes are distributed in the cluster containing type I DHFRs from the *Proteobacteria*, *Firmicutes,* and *Bacteroidetes* (**Figure 2**). Within this cluster, known resistance genes were mainly related to *dfr14, dfrA1,* and *dfr17* genes from pathogenic *γ-Proteobacteria* or *dfr* from *Bacteroidetes*. In contrast, novel resistance genes formed a separate clusters with DHFR type I proteins from *β-Proteobacteria, Firmicutes,* and *Bacteroidetes*. Only one gene (TRM6_F_WW_C2) originating from effluent was identified as type II DHFR gene from *Pseudomonas aeruginosa* (99% homology). In addition, the TYMS group of sequences included both known and novel *thy* genes found in the *Proteobacteria* and *Bacteroidetes*.

Ampicillin screens led to the identification of 15 unique beta-lactamase genes from all four Amber molecular classes (A–D) (Bush, 2017) (Supplementary Table 1B). The majority of these genes (11) were known beta-lactamase genes (Supplementary Figure 3; Supplementary Table 2) originating mostly from industrial effluent and receiving stream sediment. Three known genes (AMP2_S_DS_C2, AMP3_S_DS_C2, AMP10_S_DS_C2) cluster with the beta-lactamase genes *bla*GES-1 and *bla*VEB-9 (class A) from *K. pneumoniae* and *P. aeruginosa* (**Figure 3B**), and *bla*CMY-10 (class C) from *A. baumannii* (Supplementary Table 2), all of which are clinically relevant gene families (Paterson and Bonomo, 2005; Jacoby, 2009). In addition to high-level resistance to AMP (MIC > 1,024 mg L^{-1}), these enzymes displayed activity against CTX, a 3rd generation cephalosporin (MIC = 8–32 mg L^{-1}, Supplementary Table 2).

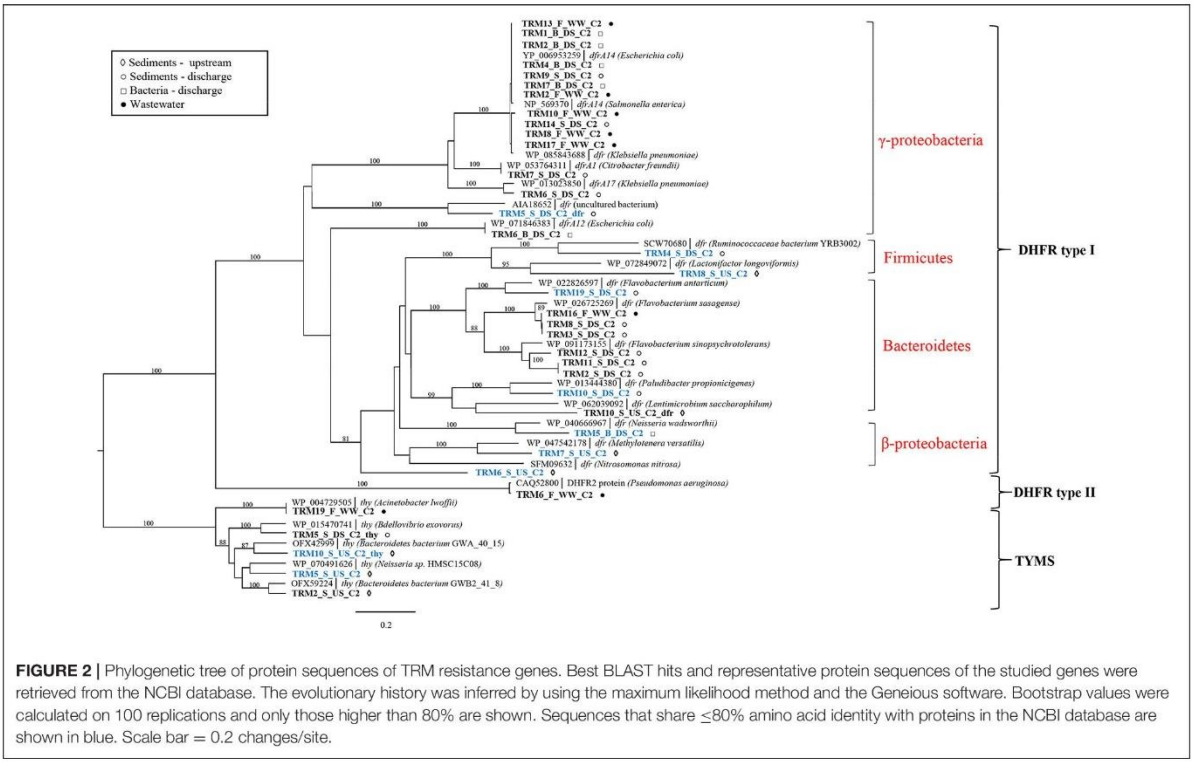

FIGURE 2 | Phylogenetic tree of protein sequences of TRM resistance genes. Best BLAST hits and representative protein sequences of the studied genes were retrieved from the NCBI database. The evolutionary history was inferred by using the maximum likelihood method and the Geneious software. Bootstrap values were calculated on 100 replications and only those higher than 80% are shown. Sequences that share ≤80% amino acid identity with proteins in the NCBI database are shown in blue. Scale bar = 0.2 changes/site.

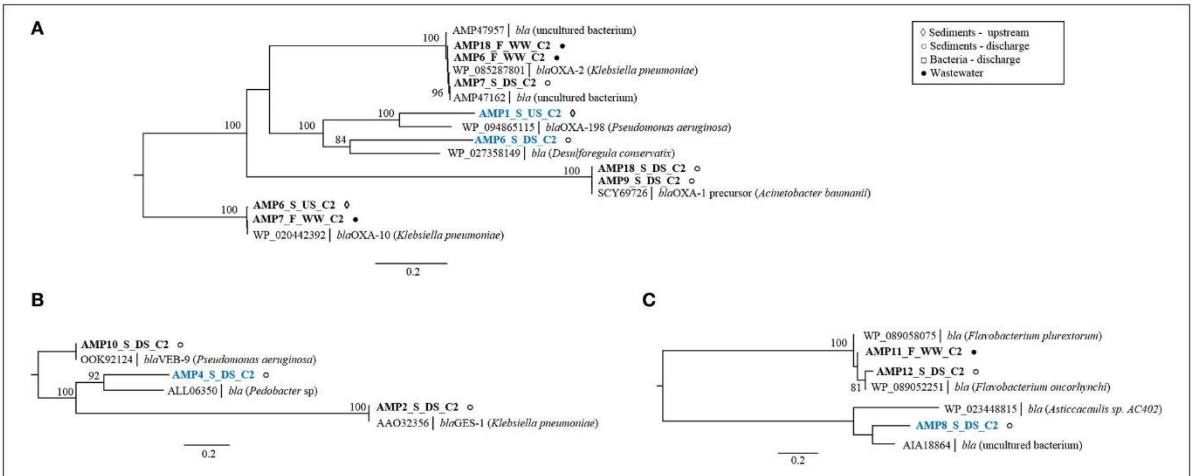

FIGURE 3 | Phylogenetic trees of protein sequences of class D beta-lactamases **(A)**, class A beta-lactamases **(B)**, and class B beta-lactamases **(C)**. Best BLAST hits and representative protein sequences of the studied gene were retrieved from the NCBI database. The evolutionary history was inferred by using the maximum likelihood method and the Geneious software. Bootstrap values were calculated on 100 replications and only those higher than 80% are shown. Sequences that share ≤80% amino acid identity with proteins in NCBI database are shown in blue. Scale bar = 0.2 changes/site.

Moreover, the *bla*GES-1 variant is known to display activity against carbapenems, a class of last resort antibiotics (Stewart et al., 2015). All of these observations indicate that the studied effluent-impacted sediment can act as a reservoir of pathogen-borne extended-spectrum beta-lactamases such as the GES, VEB, and CMY-10 types. The detection of these genes in

sediment only at the discharge site suggests their accumulation in the environment because of effluent discharge. As effluent from Industry area 2 is mixed with human sewage within the industry, we suspect that these genes could have derived from human sources. Recently, Marathe et al. (2017) showed that untreated urban waste enriches river sediment with GES-type carbapenemases.

Nine remaining known genes cluster with either class D OXA-type genes from *K. pneumoniae* and *A. baumannii* (AMP6_F_WW_C2, AMP18_F_WW_C2, AMP7_S_DS_C2, AMP9_S_DS, AMP18_S_DS_C2, AMP6_S_US_C2, AMP7_F_WW_C2,) or subclass B1-metallo beta-lactamases from *Flavobacterium* sp. (AMP11_F_WW_C2, AMP12_S_DS_C2) (**Figures 3A,C**). Some of the class D, OXA-type beta-lactamase genes (*bla*OXA-10 and *bla*OXA-198) were obtained from upstream sediment suggesting that the occurrence of these genes was not limited to the release of industrial effluents. As the studied stream flows through the rural area and might be impacted by livestock fecal runoff, the source of the observed *bla*OXA genes is likely attributed to fecal pollution, rather than antibiotic selection pressure from effluent, although the latter cannot be excluded (Agga et al., 2015).

Along with the known genes, four potentially novel beta-lactamases were identified encoding putative class A beta-lactamases (AMP4_S_DS_C2; **Figure 3B**), two class D beta-lactamases (AMP6_S_DS_C2, AMP1_S_US_C2; **Figure 3A**) and one class B beta-lactamase (AMP8_S_DS_C2; **Figure 3C**), sharing 55–74% amino acid sequence identity with known enzymes (Supplementary Table 2). All clones with these genes conferred high-level resistance to AMP (MIC $>$ 1,024 mg L^{-1}) and two of them, with class A and class B beta-lactamases (AMP4_S_DS_C2, AMP8_S_DS_C2), conferred additional resistance to CTX (MIC = 8 mg L^{-1}, $>$16 mg L^{-1}), suggesting their increased spectrum of activity. Selection on CTX resulted in the identification of a single, known, AmpC beta-lactamase gene originating from discharge sediment (Supplementary Table 2). This gene displayed a high sequence similarity to the *bla*MOX-9 gene from carbapenem-hydrolyzing *Citrobacter freundii*, isolated from a hospital wastewater plant in central Italy (Antonelli et al., 2015). This suggests that the *bla*MOX-9 gene may have originated from human bacteria present in industrial effluent.

Collectively, these results provide a survey of those ARGs in effluents and sediments that are accessible by functional metagenomics. It is also likely that these matrices contain resistance determinants that are not expressed in *E. coli*. Nevertheless, our findings indicate that sediments impacted by antibiotic polluted pharmaceutical effluents could be important sources of clinically relevant known and novel resistance genes, including those conferring resistance to antibiotics that are critically important for human medicine, such as penicillins, 3rd generation cephalosporins, and carbapenems (WHO, 2017).

Organization and Mobility of Identified ARGs

To assess the genomic context of identified ARGs, we studied the available flanking DNA in more detail. Identification of ORFs in macrolide resistant clones revealed that many clones from the libraries of effluent and receiving sediment carried more than one macrolide resistance mechanisms, sometimes on the same mobile element (**Figure 4**). For example, clone AZI1_B_DS_C1 contained a cluster comprised of genes that encode for a ribosomal protection protein, [*msr*(E)] and a macrolide phosphotransferase [*mph*(E)], separated just by a 55 bp spacer. As observed here, this gene cluster was previously found to be flanked by an IS6 family transposase and is localized on plasmids in different hosts (Schlüter et al., 2007b; Kadlec et al., 2011; Zhang et al., 2013), suggesting that these vectors may play an important role in the dissemination of the *msr*(E)-*mph*(E) cassette. Clone ERI2_S_DS_C1 contained a similar gene cassette composed of the genes *mph*(G) and *mef*(C), which encode a macrolide phosphotransferase and a macrolide efflux pump, respectively. This gene cluster has been found on plasmids from different hosts in Asia (Nonaka et al., 2012; Sugimoto et al., 2017), suggesting its potential for dissemination across species. This seems to be the first time that this gene cassette is reported in Europe. Each gene cluster [*mef*(C)-*mph*(G) and *msr*(E)-*mph*(E)] might be collectively involved in a phenotype of observed high-level resistance to ERI (MIC $\geq$ 1,024 mg L^{-1}) and AZI (MIC $\geq$ 64 mg L^{-1}) as has been previously shown (Schlüter et al., 2007a; Nonaka et al., 2015). The fact that clones with identical gene clusters, either *msr*(E)-*mph*(E) or *mef*(C)-*mph*(G) were found in libraries of effluent and receiving river sediment (Supplementary Figure 5), indicates that industrial effluent is a point source of these gene clusters in river sediment.

Besides carrying genes for two different macrolide resistance mechanisms, some macrolide resistant clones (i.e., clone ERI2_S_DS_C1) carried additional genes, such as *sul2* (sulfonamide resistance). This suggests the potential for co-selection of macrolide and sulfonamide resistance genes as well as their co-transfer under the selection pressure of macrolides and/or other factors.

In addition to clones harboring macrolide resistance genes and deriving from Industry area 1, clones deriving from Industry area 2 and conferring resistance to TRM, sulfonamides and beta-lactams also harbored clusters of ARGs (**Figure 4**). For example, the TRM resistant clone TRM4_S_DS_C2 contained a cluster comprised of two different genes involved in TRM resistance, *thy* and *dfr*, which have previously been found in bacteria (Kehrenberg and Schwarz, 2005). Clones TRM10_F_WW_C_2 and TRM3_S_DS_C2 harbored clusters containing genes involved in TRM resistance (*dfr14*) and aminoglycoside resistance or TRM resistance (*dfr*) and ubiquinone biosynthesis (methyltransferase). Little is known about the involvement of this methyltransferase in bacterial resistance to antibiotics, though Baisa et al. (2013) reported that the deletion of the *ubiE* gene led to bacterial insensitivity to D-cycloserine, a second-line drug in the treatment of MDR *Mycobacterium tuberculosis* infections. The SMZ resistant clone, SMZ5_B_DS_C2, harbored clustered genes encoding sulfonamide (*sul1*) and aminoglycoside resistance (*aac*). Similarly, genes encoding beta-lactamases were usually clustered with aminoglycoside resistance genes (*ant* or *aac*) or chloramphenicol resistance genes (*cmlA5* or *mdtL*), or co-localized with other beta-lactamase genes (*bla*VEB

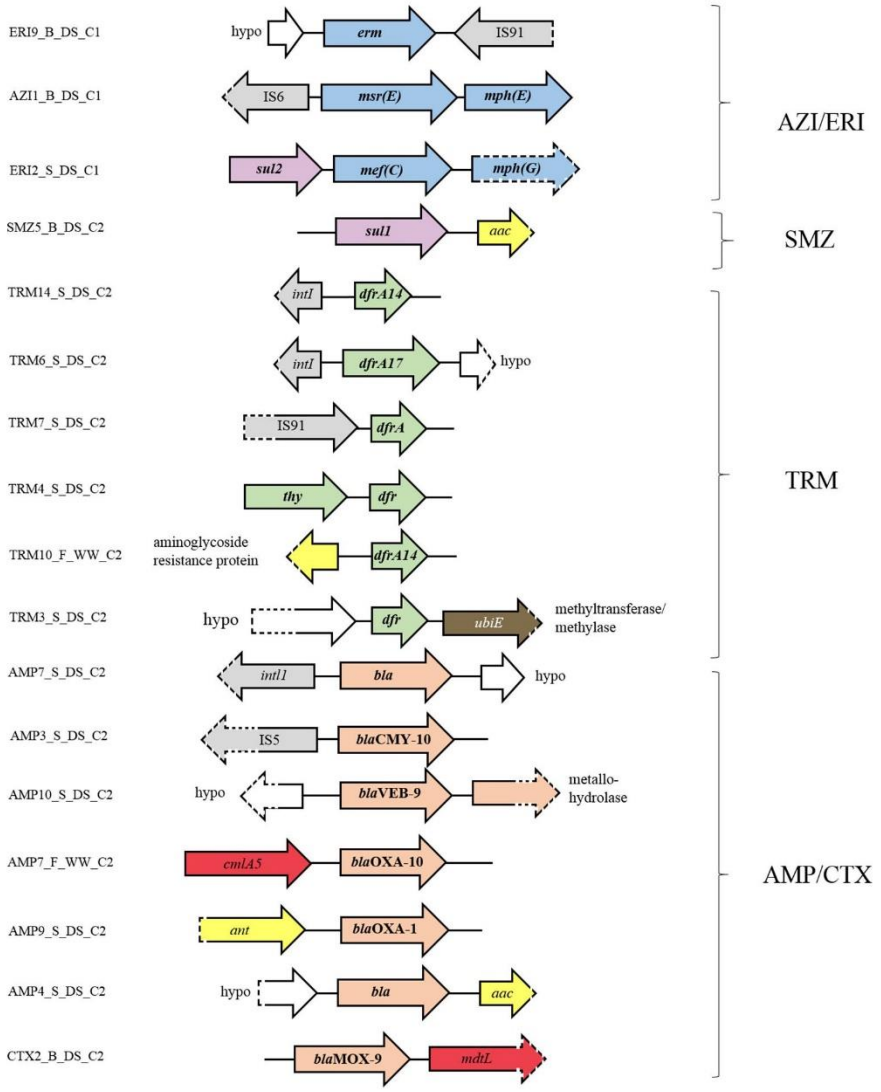

FIGURE 4 | Genetic context of the resistance genes and flanking ORFs identified in the metagenomic libraries. Orientation of the annotated genes in comparison to their genetic context is given by the direction of the arrow. ORFs involved in antibiotic resistance to macrolides are shaded in blue, sulfonamides in purple, trimethoprim in green, beta-lactams in pink, aminoglycosides in yellow, chloramphenicol in red, and D-cycloserine in brown. ORFs connected to gene dissemination are shaded in gray and ORFs annotated as hypothetical proteins in white. Dashed parts of arrows indicate incomplete sequences.

and metallo hydrolase gene). Finally, we also found that many resistance genes are flanked by mobile genetic elements such as insertion sequence (IS) elements (e.g., IS91, IS5, and IS6) and integron elements (e.g., *int*L and *int*I1 integrase genes).

Taken together, our results and previous studies of sediments subjected to industrial pollution (Kristiansson et al., 2011; Bengtsson-Palme et al., 2014) suggest that ARGs selected in such settings are candidates for dissemination to other bacteria in the environment, including pathogens. Further quantitative studies are needed to assess the transfer of identified genes to other environmental reservoirs or clinical settings. Such studies will provide the basis for future mitigation efforts.

CONCLUSIONS

The present study is the first effort to catalog the resistome from antibiotic polluted pharmaceutical effluents and receiving sediments using functional metagenomics. We highlight these polluted matrices as an important sources of diverse functional

ARGs, both known and novel. The association of many of these resistance genes with mobile genetic elements raises the concern that they may spread among bacteria with the potential to reach human pathogens and ultimately lead to clinical failure. Today's traveling habits and trade practices can cause a quick and worldwide spread of any of these resistant bacteria (Zhu et al., 2017). It is of utmost importance to set discharge limits for antibiotics and antibiotic-resistant bacteria from manufacturing sites, in order to limit further evolution of antibiotic resistance in pathogens or commensal bacteria. Furthermore, we need global metagenomic surveys of resistance within high risk habitats such as these impacted by pharmaceutical waste as a prerequisite for proper risk assessment and future mitigation efforts.

AUTHOR CONTRIBUTIONS

NU-K: Designed the research; NU-K, JG-P, MM, AŠ, and AB: Collected the samples; JG-P, MM, AŠ, and AB: Performed the experiments; NU-K, JG-P, AŠ, MM, and FW: Analyzed the data and prepared the figures; NU-K, JG-P, and AŠ: Wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02675/full#supplementary-material

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Pollution from azithromycin-manufacturing promotes macrolide-resistance gene propagation and induces spatial and seasonal bacterial community shifts in receiving river sediments

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ABSTRACT

Effluents from antibiotic manufacturing may contain high concentrations of antibiotics, which are the main driving force behind the selection and spread of antibiotic resistance genes in the environment. However, our knowledge about the impact of such effluent discharges on the antibiotic resistome and bacterial communities is still limited. To gain insight into this impact, we collected effluents from an azithromycin-manufacturing industry discharge site as well as upstream and downstream sediments from the receiving Sava river during both winter and summer season. Chemical analyses of sediment and effluent samples indicated that the effluent discharge significantly increased the amount of macrolide antibiotics, heavy metals and nutrients in the receiving river sediments. Quantitative PCR revealed a significant increase of relative abundances of macrolide-resistance genes and class 1 integrons in effluent-impacted sediments. Amplicon sequencing of 16S rRNA genes showed spatial and seasonal bacterial community shifts in the receiving sediments. Redundancy analysis and Mantel test indicated that macrolides and copper together with nutrients significantly correlated with community shift close to the effluent discharge site. The number of taxa that were significantly increased in relative abundance at the discharge site decreased rapidly at the downstream sites, showing the resilience of the indigenous sediment bacterial community. Seasonal changes in the chemical properties of the sediment along with changes in effluent community composition could be responsible for sediment community shifts between winter and summer. Altogether, this study showed that the discharge of pharmaceutical effluents altered physicochemical characteristics and bacterial community of receiving river sediments, which contributed to the enrichment of macrolide-resistance genes and integrons.

1. Introduction

Since antibiotics are naturally produced by microorganisms in the environment, bacterial communities maintain a large collection of resistance genes, called the resistome (D'Costa et al., 2006; Surette and Wright, 2017). However, continuous environmental pollution by antibiotics and other selective agents, such as heavy metals, has perturbed the dynamics of natural systems (Di Cesare et al., 2016a; Martins et al., 2014). The selection pressure imposed by these pollutants has promoted the proliferation and spread of resistant populations and their resistance genes, thus creating a pool of resistance genes for pathogens to acquire (Li et al., 2017; Xu et al., 2017; Zhang et al., 2018). This acquisition of resistance genes is often facilitated by mobile genetic elements such as plasmids often carrying integrons which are therefore considered as key contributors in the dissemination of antibiotic resistance genes (ARGs) and promoters of multidrug resistance (Gillings et al., 2015; Heuer et al., 2012; Smalla et al., 2015; Zhang et al., 2011). Highly similar or even identical ARGs have been found in both

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environmental- and pathogenic bacteria (Forsberg et al., 2012; Nordmann and Poirel, 2005), emphasizing a potentially shared resistome. It has been shown that sub-inhibitory concentrations of antibiotics, high bacterial densities and increased nutrient availability facilitate plasmid-mediated horizontal gene transfer (HGT) (Jutkina et al., 2018; Rizzo et al., 2013). Elevated levels of antibiotics in the environment are also expected to increase the intensity of resistance gene exchange through plasmid- (Flach et al., 2015) or phage-mediated transfer. However, antibiotic discharges into the environment have been rarely regulated directly, although discharge limits for individual antibiotics have recently been proposed (Bengtsson-Palme and Larsson, 2016). Therefore, understanding the role of antibiotic-polluted environments in the evolution and dissemination of ARGs is crucial to develop effective and sustainable mitigation strategies to reduce the rising threat of antibiotic resistance on a global scale.

Effluents from antibiotic manufacturing plants have been reported to be a significant point source and dissemination route for antibiotics, antibiotic-resistant bacteria (ARB) and ARGs into the aquatic environment (Larsson et al., 2007; Li et al., 2008; Marathe et al., 2013; Rutgersson et al., 2014). For example, high concentrations (in the mg/L range) of oxytetracycline, lincomycin or fluoroqinolone antibiotics have been reported in treated effluents from pharmaceutical industries in China, Korea or India (Larsson, 2014). Discharges of such effluents have led to antibiotic contamination of surface, ground and drinking water bodies (Fick et al., 2009), and to an enrichment of multidrug-resistant bacteria, ARGs and elements facilitating their transfer (Flach et al., 2015; Kristiansson et al., 2011; Li et al., 2010).

Similarly to the above-mentioned reports from Asian countries, we have also recently investigated effluents from the local pharmaceutical industry, located near Zagreb in Croatia, which produces the macrolide antibiotic azithromycin (AZI). Macrolide antibiotics act as inhibitors of bacterial protein synthesis and have been used widely to combat respiratory tract infections and soft-tissue infections. They are highly potent against a wide variety of Gram-positive and Gram-negative organisms, and they are used as penicillin substitutes (Kaneko et al., 2007; Wierzbowski et al., 2005). Macrolides have recently been ranked as one of most important antibiotics for human medicine by the World Health Organization (WHO, 2017). Thus, it is important to preserve their efficacy in the treatment of human infections. Our chemical analyses of effluents from AZI production sites revealed very high concentrations of macrolides (up to 10 mg/L total) and a high proportion of AZI-resistant bacteria (> 80%) (Bielen et al., 2017). We also reported high concentrations of macrolides in effluent-receiving Sava river downstream from industrial discharge (up to 30 µg/L total). Furthermore, functional metagenomic analysis of polluted effluents and Sava river sediments identified mostly known (*msr, mph, mef*) and potentially novel (*erm*) macrolide-resistance genes, which were frequently organized in gene clusters and flanked by IS elements (González-Plaza et al., 2018).

Along with its impact on the environmental resistome, antibiotic pollution can affect the microbiome of aquatic ecosystems, by altering its diversity and/or functional properties, as has been demonstrated for different antibiotic classes including macrolides (Grenni et al., 2018).

The present study aimed to comprehensively explore the impact of the discharge of AZI-production effluents on the abundance of macrolide-resistance genes and class 1 integrons, as well as on the composition of bacterial communities in sediments along the receiving Sava river. We then correlated bacterial community with the macrolide-resistance genes and additionally, measured physicochemical properties of sediments, including macrolides, heavy metals and nutrients, to assess environmental factors contributing to community shifts in space and time.

2. Materials and methods

2.1. Study area and sampling

This study was carried out in a section of the Sava River situated in the northwest Croatia. This stretch has been selected due to pollution from an azithromycin-manufacturing industry, which is located approximately 25 km northwest of the city of Zagreb. The final effluent from this industry (after treatment in industry's membrane bioreactor) is discharged, together with effluent from baker's yeast production, into the Sava river near the city of Zaprešić, which makes this location a hot spot for dissemination of antibiotics and ARB/ARGs into the environment (Bielen et al., 2017; González-Plaza et al., 2018). Therefore, we chose to sample surface sediments (0–10 cm) from six locations along the river during winter (February; high-flow, average 1194 m^3/h) and summer (August; low-flow, average 421 m^3/h) of 2016. The sampling sites were 7.5 km upstream of the discharge (UP7500), immediately below the discharge point (DW0) and then progressively downstream at 300 m (just prior to where small river Krapina, which receives effluents from wastewater treatment plant of the city of Zaprešić, enters the Sava river; DW300), 700 m (DW700), 4.5 km (DW4500) and 11 km (DW11000) below the discharge point. Sampling site UP7500 was chosen to represent reference site without any known anthropogenic antibiotic pollution.

From each sampling site, four replicates (about 250 g each) were sampled with a plastic core tube and within approximately 2 square meters at each site. In addition, four effluent samples (about 5 L each) were collected as grab samples at the discharge outflow located at the bank of the Sava river. All samples were stored on ice and transported to the laboratory for immediate processing. Aliquots of effluent (50–100 mL) were vacuum-filtered through a 0.22 µm pore-size membrane (GE Healthcare Life Sciences) to collect bacterial cells; filters were stored at −80 °C until DNA extraction. Subsamples from each of the four replicate sediment samples (2 g) were stored at −80 °C for subsequent DNA extractions. The remaining of the subsamples were composited (10 g of each subsample) and air-dried at ambient temperature for subsequent physicochemical analyses.

2.2. Sediment physicochemical properties

The water content of the sediment samples was determined by the difference in mass after oven drying at 105 °C to constant mass. Dried samples were coarse ground to < 2 mm, and the physicochemical properties, including pH, concentration of total organic carbon (TOC), total carbon (TC), total nitrogen (TN), total phosphorus (TP), nitrite, nitrate and ammonia nitrogen were determined using internationally validated methods (ISO standards) as shown in Table S1. Size fractions were measured with a Laser Coulter LS 13320 diffractometer (Beckman Coulter, USA).

2.3. Chemical analyses of antibiotics and metals

The target macrolides were extracted from the sediments using pressurized liquid extraction and subsequently analyzed by reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) as previously described (Senta et al., 2008; Senta et al., 2013). The heavy metals, including Cr, Co, Ni, Cu, Zn, As, Cd and Pb were determined by inductively coupled plasma mass spectrometry (ICP-MS) following the methodology described previously (Dautović et al., 2014).

2.4. DNA extraction and quantitative PCR of macrolide-resistance genes and class 1 integrons

Total DNA was extracted directly from sediment samples and from frozen filters using the Power Soil DNA isolation kit (MoBio, USA)

according to the manufacturer's recommendations. Non-template sample (DNA-free water) was included as a negative control during the whole workflow. The extraction yield and quality of the DNA were assessed by spectrophotometry (BioSpec Nano, Shimadzu, Japan); DNA quantity was measured by fluorometry (Qubit Fluorometer 3.0, Thermo Fisher Scientific, USA). All extractions were stored at −20 °C until used. Quantitative PCR (qPCR) was used to quantify five macrolide-resistance genes (*mef*C, *mph*G, *mph*E, *msr*E, and *erm*B) that were previously identified in this study area by functional metagenomics (González-Plaza et al., 2018) and the class 1 integron-integrase gene (*intI*1). In addition, the 16S rRNA gene copy numbers (*rrn*) were determined to assess the total bacterial load and to calculate the relative abundance of the resistance genes targeted in the collected samples. All qPCR assays were performed on the ABI 7300 Real-time PCR System (Applied Biosystems, USA) using SYBR Green detection chemistry. The primers used for the ARGs, *rrn* and *intI*1 gene quantification are listed in Table S2. Each reaction was carried out in a total volume of 20 µL containing 10 µL Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 1 µM of each primer, and 2 ng of DNA template. For all ARGs, except *erm*B, the thermal cycling conditions were as follows: 95 °C for 15 min, 30 cycles at 95 °C for 15 s, annealing temperature (T_m) specific for each gene and primer pair (Table S2) for 30 s, and 72 °C for 30 s. For quantification of *erm*B, thermal cycling conditions were settled in accordance to Chen et al. (2007), for *rrn* according to López-Gutiérrez et al. (2004), and for *intI*1 in accordance with Barraud et al. (2010). Standard curves were constructed as previously described (López-Gutiérrez et al., 2004). Briefly, the target gene fragments were amplified from the positive metagenomic clone (González-Plaza et al., 2018), separated by 1.5% agarose gel electrophoresis and recovered with a QIAquick Gel Extraction Kit (QIAGEN, Germany), and then cloned into pGEM®-T Easy Vector (Promega, France) according to the manufacturer's recommendation. The recombinant plasmids were transformed into JM109 competent cells (Promega, France). Positive clones were checked with PCR and confirmed by Sanger sequencing (Macrogen, Netherlands). The plasmids extracted from them were used to prepare standards for qPCR assays. The efficiency and sensitivity of each qPCR assay (Table S2) was determined by generating a standard curve using 10-fold serial dilutions of the plasmid DNA (10^2–10^8). All detections in the qPCR assay were conducted with four technical replicates, and non-template control were likewise included in each assay. Possible qPCR inhibition was assessed by conducting an inhibition test using samples diluted to 1 ng/µL and 0.01 ng/µL, as previously described (Petric et al., 2011), and no inhibition was observed. Quantitative PCR data were expressed as the ratio of ARG/*intI*1 gene copy number per *rrn* copy number to evaluate the relative proportion of target gene in the bacterial community of each sample.

2.5. Amplicon sequencing and data processing

To profile bacterial communities, the V1-V2 region of 16S rRNA genes was amplified with universal primers 27F and 357R (Klindworth et al., 2013). Amplicon library was prepared as described previously (Gschwendtner et al., 2016) with some modifications. All samples was purified with Nucleospin Gel and PCR Clean-up kit (MACHEREY NAGEL GmbH & Co., Germany), and fragment size and concentration were validated using a FragmentAnalyzer device on a Standard Sensitivity NGS Fragment Analysis kit (Advanced Analytical, USA). Samples were sequenced on a MiSeq instrument (Illumina, United Kingdom, Chesterford) using the MiSeq Reagent Kit v3 for 600 cycles. Sequencing adapters were removed using AdapterRemoval (Schubert et al., 2016). Reads were analyzed using QIIME 2 v2018.2.0 (https://qiime2.org) and denoised using the DADA2 (Callahan et al., 2016) plugin. Resulting amplicon sequencing variants (ASVs) were compared to the 99% identical clustered SILVA database v132 (Quast et al., 2013) using a naive Bayes classifier trained on the amplified region. Alpha diversity was described for each sample using the metrics of observed species (i.e. ASVs), and rarefaction curves were generated to compare the level of bacterial ASVs diversity.

Non-metric multidimensional scaling (NMDS) analysis was performed to evaluate the overall composition of the bacterial community among different sites and seasons based on Bray-Curtis distance by using Canoco software (v5.1). A heat map visualization of the relative abundance of taxa (number of ASVs per number of sequences expressed in percentage) significantly increased at DW0 site compared to UP7500 reference site was performed with the heatmap.2 function of the 'gplots' package (Warnes et al., 2016).

2.6. Statistical analyses

qPCR data (ARG/*intI*1 copy number per *rrn* copy number) were log10 transformed and subjected to a Shapiro-Wilk test to evaluate its normal distribution. This was performed in R studio v1.1.383 with the 'fitdistrplus' and 'stats' packages. These data were further compared using the one-way analysis of variance (ANOVA) or Kruskal-Wallis test (if data were not normally distributed) and Tukey's multiple comparison test. Paired *t*-test was performed to compare the relative abundance of macrolide-resistance genes at each site between two seasons. Data were analyzed using GraphPad Prism V6.01. The package DESeq2 (Love et al., 2014; Jonsson et al., 2016) version 1.22.1 was applied to analyze the differences in relative abundance of bacterial community at phylum and at genus level between each DW site and upstream (UP7500) site. All statistical tests were considered significant at $p < 0.05$. Redundancy analysis (RDA) and Mantel test based on Bray Curtis distance were performed to determine the correlation between bacterial community and physicochemical parameters as well as between bacterial community and macrolide-resistance genes by using Canoco software (v5.1). Shannon diversity, Mantel test and Adonis test were performed using R studio software v1.1.383 with 'vegan' package (Oksanen et al., 2018).

3. Results

3.1. River sediment characteristics

Physicochemical characteristics of river sediments including particle grain-size and nutrient content are summarized in Table S3. The sediment samples from all sampling sites were characterized by alkaline pH values (7.7–9.1) and were generally of sandy-silt texture (Wenthworth, 1922), with sand and silt content ranging from 47 to 85% and 12 to 47%, respectively. In general, discharge of industrial effluents influenced most measured parameters at sites in close proximity to the discharge point (DW0-DW700). The discharge site (DW0) was unique among all sites having the highest concentrations of total carbon (up to 9.06%), total nitrogen (up to 0.26%), nitrite (up to 10 mg/kg) and ammonium (up to 296 mg/kg) as well as conductivity (up to 951 mS).

The concentrations of macrolides (azithromycin, AZI, and erythromycin-H_20, ERY-H_2O) were measured in the sediments to estimate the presence of selection pressure from antibiotics (Table 1). Both macrolides could not be detected in sediments sampled upstream (UP7500 site), except AZI in very low level during winter. In contrast, very high levels of AZI were detected at DW0 site during both seasons (9 and 23 mg/kg). These concentrations decreased with distance further downstream but were still high (> 1 mg/kg) at sites located within 700 m of the effluent discharge (DW300 and DW700). The concentrations of ERY-H_2O were much lower compared to AZI, being the highest at DW0 (approximately 1 mg/kg).

Heavy metals (As, Cd, Cr, Co, Cu, Pb, Ni and Zn) were found in sediments in a wide range of concentrations (Table 1). In general, most of the measured metals exhibited slightly higher concentrations at DW0 and downstream (DW) sites compared to the upstream site during both seasons. Exceptions were Cu, which increased during both seasons about 5 times at DW0 (48 mg/kg, two-season average) compared to

Table 1
Quantification of macrolides and heavy metals in sediments from different sites along the Sava river in winter and summer sampling campaigns.

			Sampling sites					
Macrolide compound (μg/kg of dry sediment)		Season	UP7500	DW0	DW300	DW700	DW4500	DW11000
Azithromycin (AZI)		Winter	4.60	9307	2686	1240	174	251
		Summer	< LOD	23,685	3598	1271	940	203
Erythromycin-H_2O (ERY-H_2O)		Winter	< LOD	869	24	13	6.60	3.30
		Summer	< LOD	939	71	11	6.80	6.00
Metal (mg/kg of dry sediment)	TEC*/MCC**							
Arsenic (As)	9.79/–	Winter	5.81	7.31	6.38	6.44	8.30	5.57
		Summer	5.11	5.00	5.40	9.48	7.23	5.44
Cadmium (Cd)	0.99/1.0	Winter	0.24	0.27	0.28	0.33	0.16	0.23
		Summer	0.27	0.37	0.24	0.46	0.26	0.25
Chromium (Cr)	43.4/–	Winter	37	34	*57*	41	*76*	26
		Summer	26	*74*	29	*58*	41	28
Cobalt (Co)	–/–	Winter	5.17	4.03	5.52	6.02	11	4.30
		Summer	4.42	4.92	4.29	12	6.15	5.03
Copper (Cu)	31.6/11.5	Winter	11	***41***	**18**	**16**	**28**	11
		Summer	9.42	***55***	**17**	**25**	**13**	**13**
Lead (Pb)	35.8/–	Winter	26	26	25	27	35	19
		Summer	15	27	18	*43*	21	18
Nickel (Ni)	22.7/–	Winter	17	*29*	18	20	*47*	13
		Summer	13	20	15	*44*	20	17
Zinc (Zn)	121/42.5	Winter	**79**	***148***	**76**	**83**	***150***	**49**
		Summer	***357***	***165***	**68**	**104**	**72**	**66**

Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream. LOD, limit of detection; the values in italic represent the concentration of the heavy metals above the threshold effect concentration (TEC*). The values in bold represent the concentration of heavy metals above the minimum co-selective concentration (MCC**), above which selection of antibiotic resistance is expected to occur (Seiler and Berendonk, 2012).

UP7500 (10 mg/kg, two-season average) and Zn, which increased about 2 times at DW0 (148 mg/kg) compared to UP7500 (80 mg/kg) only during winter. A surprisingly high concentration of Zn was measured in sediments from UP7500 site in summer (357 mg/kg), being even higher than that measured at DW0 site (165 mg/kg; Table 1), but the source of this pollution is unknown. Notably, concentrations of both Cu and Zn in the sediment at all DW sites exceeded in most cases concentrations needed to co-select for metal resistance and antibiotic resistance, i.e. the minimum co-selective concentrations (MCC) (Seiler and Berendonk, 2012). Additionally, levels of both Cu and Zn in the sediment at DW0 were higher than concentrations expected to induce adverse effects on sediment dwelling organisms, i.e. threshold effect concentrations (TEC) (MacDonald et al., 2000).

3.2. Elevated abundance of macrolide-resistance genes and class 1 integrons in sediments along the Sava River

We assessed the relative abundances of five macrolide-resistance genes (*mph*G, *mph*E, *msr*E, *mef*C and *erm*B) and an integrase gene (*intI*1) of class 1 integrons in effluents and sediments of the receiving river collected in winter and summer sampling campaigns using qPCR (Fig. 1). All values were normalized to 16S rRNA gene (*rrn*) abundances to minimize the variance in background bacterial abundances and to compare our data with ARGs quantitative data from other studies.

Among macrolide-resistance genes in effluents, the most abundant resistance genes in both seasons were *msr*E (up to 5.6×10^{-2} copies/*rrn* copies), *mph*G (up to 2.5×10^{-2} copies/*rrn* copies) and *erm*B (up to 1.1×10^{-2} copies/*rrn* copies), while the abundances of *mef*C and *mph*E were in most cases 10-times lower (approximately 10^{-3} copies/*rrn* copies in winter and 10^{-4} copies/*rrn* copies in summer) (Fig. 1). The relative abundances of all target ARGs, except *erm*B, were significantly higher in winter than in summer effluents ($p < 0.05$; paired *t*-test). Interestingly, *intI*1 gene was even more abundant than ARGs in effluents, with an average value of the two seasons of 6.6×10^{-1} copies/*rrn* copies (Fig. 1).

In sediment samples from UP7500 site, only *mph*G, *msr*E and *erm*B genes were detected at relative abundances of about 10^{-4}–10^{-5} gene copies/*rrn* copies, with *msr*E abundance being the highest (Fig. 1). However, in sediments from DW0 and DW sites, all five resistance genes targeted were detected at significantly higher relative abundances compared with UP7500 site ($p < 0.05$; ANOVA). Highest relative abundances occurred at DW0 site (up to 10^{-1} to 10^{-2} gene copies/*rrn* copies), while at DW sites (DW300 - DW11000), the relative abundance of up to 10^{-3} ARG copies/*rrn* copies was determined. In general, no significant difference was found between relative abundances of ARGs from winter and summer samples from all sites ($p > 0.05$; paired *t*-test).

Compared to ARGs, higher relative abundances of *intI*1 gene were observed in the samples from UP7500 site, with 8.6×10^{-3} copies/*rrn* copies in winter samples and 2.1×10^{-1} copies/*rrn* copies in summer samples indicating higher background levels of *intI*1 gene. The relative abundance of this gene was significantly increased at all DW sites ($p < 0.05$; ANOVA), except DW700 during the winter (Fig. 1). Values were in the range of 10^{-1} gene copies/*rrn* copies at locations DW0 and DW300, and about 10 times lower at other DW sites. The level of *intI*1 in the UP7500 sediment was considerably higher during summer compared to winter (Fig. 1).

3.3. Effects of effluent discharge on bacterial communities

A total of 6,140,241 sequences of 16S rRNA genes amplified from a total of 50 effluent and sediment samples were analyzed. Of that, 6,123,931 sequences were high-quality reads after adapter removal, which corresponded to a range of 70,509 to 207,244 sequences per sample. These sequences were assigned to 26,147 ASVs at 99% similarity level. The number of sequences obtained for the non-template control ranged from 71 to 64,287. Rarefaction curves indicated that the number of sequences was sufficient to cover the vast majority of species in the bacterial community within each analyzed sample (Fig. S1).

Shannon-Wiener diversity index indicated that effluent discharge did not significantly ($p > 0.05$, Kruskal-Wallis) affect the number of taxa in receiving sediments (species richness) during both seasons,

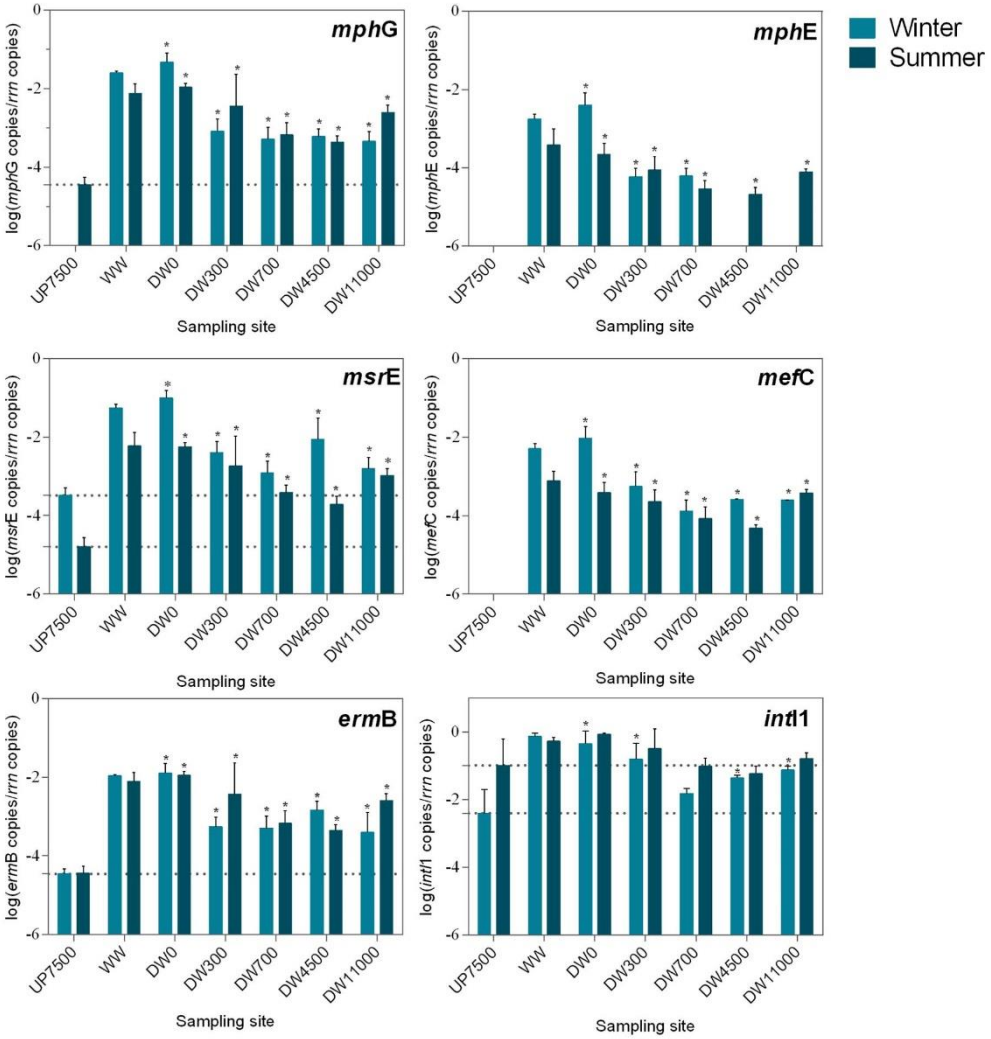

Fig. 1. Relative abundance of macrolide-resistance genes (*mph*G, *mph*E, *msr*E, *mef*C, *erm*B) and class 1 integron-integrase gene (*intI*1) in azithromycin-manufacturing effluents (WW) and sediments of the receiving Sava river from different sites. Each value is the mean ± SD of four replicates. Dotted lines indicate the relative abundances of each gene in background sediment. Asterisks represent a significant difference ($p < 0.05$; ANOVA) between each DW site and reference UP7500 site. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream; WW, effluent.

although sediments from the discharge site tended to have the lowest diversity among all samples (Fig. S2). In addition, the NMDS plots based on Bray Curtis similarity showed a clearly distinct bacterial community composition between sediments from DW0 and the other (UP and DW) sites (Fig. 2; adonis: $R^2 = 0.8675$, $p < 0.05$). Furthermore, bacterial communities varied between the sampling time points at DW0 and the other (UP and DW) sites (Fig. 2; adonis: $R^2 = 0.8772$, $p < 0.05$), implying a seasonal effect on the sediment community composition.

Proteobacteria were dominating in all samples, followed by *Actinobacteria* and *Acidobacteria* in winter upstream and downstream sediment samples and by *Acidobacteria* and *Bacteroidetes* in summer upstream and downstream sediment samples (Fig. S3). In contrast, in effluents and sediments at the discharge site *Bacteroidetes, Epsilonbacteraeota* and *Firmicutes* were, besides *Proteobacteria*, the most abundant phyla independent from sampling season. To pinpoint those phyla which significantly differed between UP7500 and the other sites, DESEq2 analysis was applied ($p < 0.05$). As evident from Fig. 3, effluent discharge significantly decreased relative abundance of a number of phyla at DW0, but not at downstream sites. Compared to UP7500, the highest decrease in relative abundance at DW0 was observed for *Actinobacteria* (23.65% winter, 7.85% summer), *α-Proteobacteria* (11.2% winter, 6.5% summer), *Acidobacteria* (9.21% winter, 7.26% summer), and *Cyanobacteria* (6% summer), whereas for the other three affected phyla (*Chloroflexi, Gemmatimonadetes* and *Nitrospirae*), the decrease in relative abundance was ≤3% for both seasons. In contrast, several phyla showed a significantly higher relative abundance at DW0 compared to UP7500 site during both seasons, including *Epsilonbacteraeota* (11% winter, 5% summer), *Bacteroidetes* (15% winter, 21% summer) and *Firmicutes* (12% winter, 14% summer). Other phyla, such as *Spirochaetae* and *Synergistetes* were detected in significantly higher relative abundance at DW0 in both seasons, but their actual

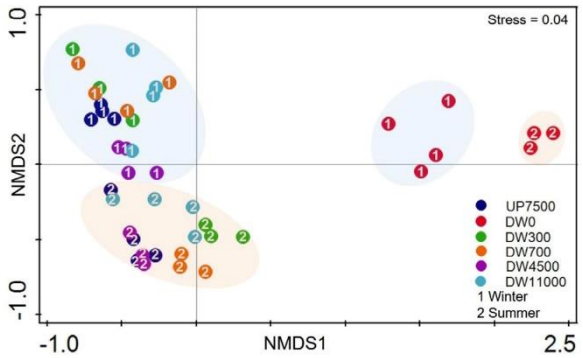

Fig. 2. Spatial and seasonal changes in sediment community composition across three to four replicates of each sampling site along the river. The replicate samples obtained from the same sampling site were marked with the same color, and from the same season with the same number. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream.

relative abundances were, at most, 3% in winter and 4% in summer samples. In addition to this, γ-*Proteobacteria* (12.4%) and *δ-Proteobacteria* (4%) was significantly higher in relative abundance at DW0 only during winter, whereas *Cloacimonetes* (4%) were higher only in samples taken in summer. As *Proteobacteria, Bacteroidetes, Epsilonbacteraeota* and *Firmicutes* were the most abundant phyla in both, effluents and sediments at the discharge site, we further investigated which genera within these phyla were significantly higher at DW0 compared to UP7500. We postulated that they may have represented bacterial taxa that acquired macrolide-resistance genes or are intrinsically resistant, and therefore able to survive and proliferate despite the strong selective pressure from macrolides (and potentially other toxic chemicals). Within *Bacteroidetes*, genera including *Bacteroides, Paludibacter* and vadinBC27 wastewater-sludge group that originated from effluent were significantly higher in relative abundance in both winter- and summer sediment samples; additionally, *Macellibacteroidetes* was more abundant only in winter sediment (Fig. 4 and Tables S4 and S5). Within the *Firmicutes,* the genus *Trichococcus,* that was found in both effluent and background sediment, dominated at DW0 site during both seasons (Fig. 4 and Tables S4 and S5). In addition, ASVs linked to *Erysipelothrix* and *Fusibacter,* which were more abundant in effluent than in background sediment, as well as *Anaerovorax* and *Christensenellacae* R-7 group, specific to effluent, were enriched in winter sediment. However, ASVs which were closely related to *Enterococcus*, *Anaerovorax* and uncultured genera of the order *Selenomonadales,* which originated from effluent, were significantly enriched in sediment samples from the summer sampling (Tables S4 and S5; Fig. 4). Among *Proteobacteria* and *Epsilonbacteraeota*, populations that were enriched at DW0 compared to UP7500 differed between winter and summer sediment samples. The genus *Thauera* was the most abundant in sediment from DW0 site in both seasons but was present at low abundance in the UP7500 sediment (0.01%) and at relatively high abundance (approximately 1%) in effluents (Fig. 4 and Tables S4 and S5). In addition, genera found to be abundant in winter effluent (> 0.5%; e.g. *Commamonas, Variovax)* (Fig. 4a; Table S4) were also highly abundant in winter DW0 sample (Fig. 4a; Table S4). Some genera such as *Rhizobacter, Thiobacillus* and *Methylotenera* that were higher in relative abundance in DW0 compared to the UP7500 sediment in winter, originated from UP7500 sediment, whereas the other genera such as *Gemmobacter, Paracoccus, Propionivibrio, Desulfobulbus, Sulfuricurvum, Arcobacter, Pseudomonas* and *Acinetobacter* were specific to effluent or more abundant in effluent compared to UP7500 sediment. Further, genera such as *Falsirhodobacter, Desulfobacter,* and *Arcobacter,*

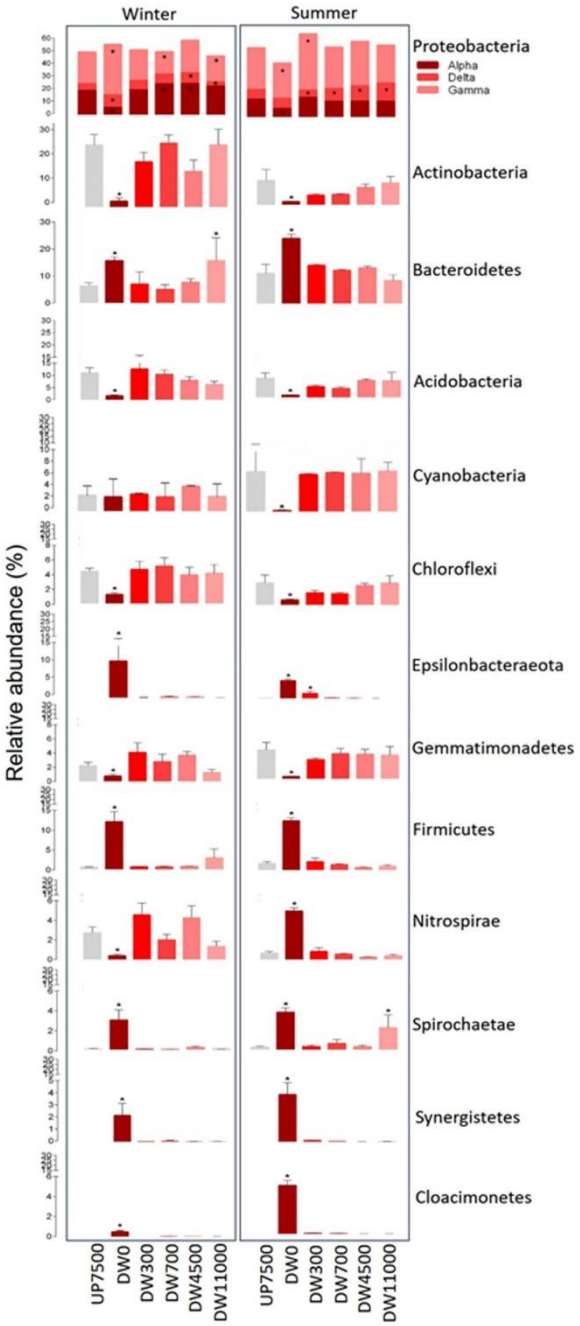

Fig. 3. Phylum-level changes in community composition in sediments from different sites along the Sava river during winter and summer. Relative abundances of phylotype reads are shown, identified to their closest match in the SILVA Database. Asterisks represent a significant difference ($p < 0.05$; DESeq2) between reference UP7500 and each DW site. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream.

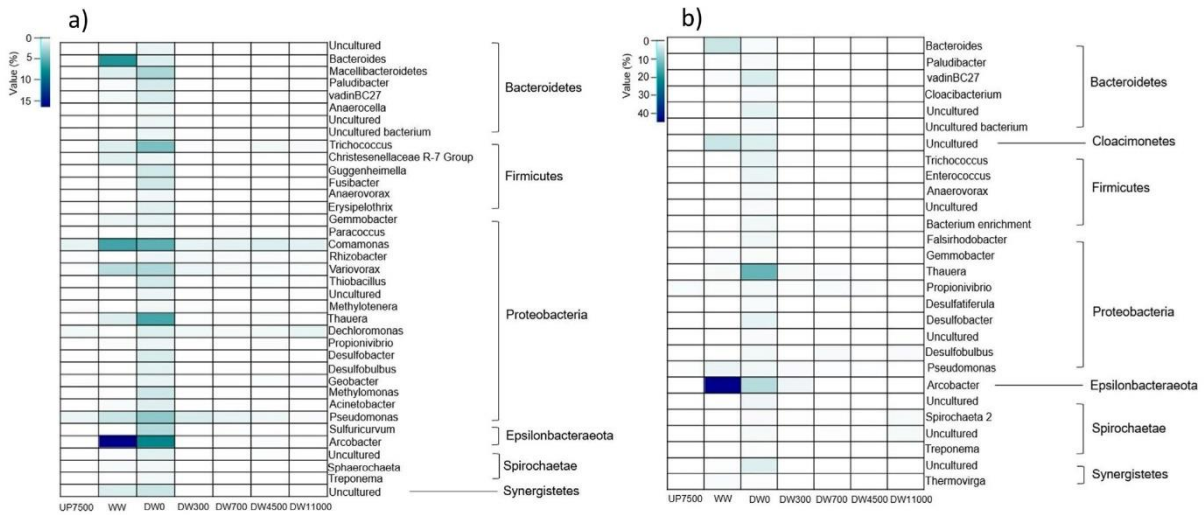

Fig. 4. Heatmaps showing changes in the relative abundance of taxa that were significantly increased at DW0 site compared to UP7500 site during a) winter and b) summer season. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream; WW, effluent.

that were enriched in DW0 during summer originated only from effluent (Fig. 4b; Table S5).

Overall, despite seasonal differences in sediment community composition at DW0 site, the abundance of nearly all taxa higher in relative abundance at this site dramatically decreased at downstream sites, without significant differences in abundance even at site DW300 compared with UP7500 site (Fig. 4). An exception is an effluent-associated *Arcobacter,* which remained significantly enriched at DW300, in addition to DW0, during summer.

3.4. Roles of environmental factors for bacterial community shifts and alteration of macrolide-resistance genes

We performed redundancy analysis (RDA) to study the relationship of sediment physicochemical parameters and bacterial community composition at the genus level during winter and summer (Fig. 5). In line with the NMDS plot (Fig. 2), RDA analysis confirmed shifts in bacterial community structure at DW0 site, which was significantly positively correlated with macrolides (AZI and ERY-H_2O), Cu, and nutrients (TC, TN, NH_4^+ and $N0_2^-$) during both seasons (Mantel test, $p < 0.05$), but negatively correlated with pH (Mantel test, $p < 0.05$). However, bacterial communities from winter DW0 samples were significantly correlated with Zn and NO_3^- (Fig. 5a), whereas communities from summer DW0 samples significantly correlated with TOC, TP, Cr and Cd (Fig. 5c). Surprisingly, Mantel test revealed no significant correlation between these bacterial communities and temperature (not shown). Therefore, seasonal changes of above-mentioned parameters, in addition to changes in bacterial community composition of effluents (Fig. 4), could be responsible for sediment community shifts between winter and summer.

RDA of the bacterial communities and macrolide-resistance genes showed that *mph, mef, msr* and *erm* genes were significantly correlated to community composition at DW0 site during both seasons (Mantel test, $p < 0.05$; Fig. 5b and d). Among the identified genera, Gram-positive *Trichococcus* and Gram-negative *Thauera* and *Arcobacter* showed a significant positive correlation with analyzed resistance genes at DW0 site during both seasons. In addition, genera such as *Macellibacteroides* (Gram-positive) and *Sulfuricurvum* and *Commanomas* (Gram-negative) positively correlated with macrolide-resistance genes at DW0 during winter (Fig. 5b), whereas vadinBC27 wastewater-sludge group and uncultured populations from *Bacteroidetes, Cloacimonetes* and *Synergistetes* exhibited a significant positive correlation with macrolide-resistance genes during summer (Fig. 5d).

4. Discussion

This study provided comprehensive insights into the effects of the discharge of treated effluents from AZI-manufacturing on macrolide-resistance genes and bacterial communities of the receiving Sava river sediment. We recently showed that effluents from this industry contained high numbers of AZI-resistant bacteria and macrolide antibiotics at concentrations clearly selective for antibiotic resistance (up to few mg/L; Bielen et al., 2017). Accordingly, direct discharge of these effluents could significantly affect the bacterial communities and the macrolide-resistance genes of the receiving river, which might pose a risk for human and environmental health. Although sediments act as a drain for pollutants, they can also act as a source of pollutants under certain environmental conditions, especially during high water-flow events (Herrero et al., 2018). We, therefore, investigated river sediments impacted by industrial effluents during both high-flow (winter) and low-flow (summer) sampling conditions.

4.1. Chemical pollution of river sediments by industrial effluent discharge

We detected high levels of macrolide antibiotics, especially AZI, in the effluent-impacted sediments, with the highest levels at the discharge site (up to 23 mg/kg) and a sharp decrease to about 1 mg/kg at the site located 700 m downstream. This indicated contribution of industrial waste to macrolide pollution of sediments from the Sava river, in addition to pollution of river surface water (Bielen et al., 2017). Although higher macrolide concentrations were previously found in winter than in summer effluent samples (Bielen et al., 2017), the reverse situation was observed for sediment samples. Lower concentrations of macrolides detected in sediment samples taken in winter compared to summer could be due to the higher flow rate of the river and increased sediment transport during winter or lower amount of effluent discharge during winter compared to summer. Although it is difficult to estimate the degree of bioavailability of the detected macrolides in the sediment, it has been shown using data generated for tylosin that macrolides retain their antimicrobial activity even when

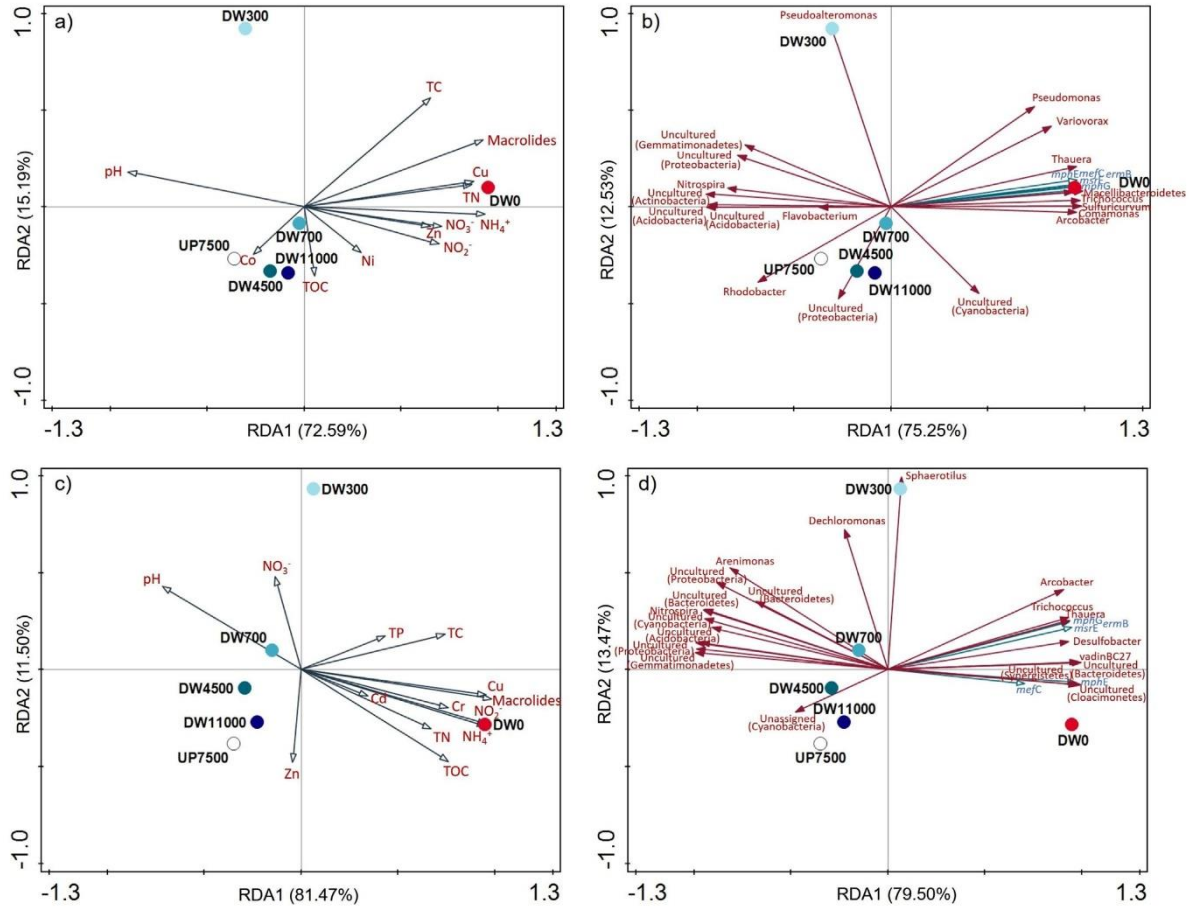

Fig. 5. Redundancy analysis (RDA) of the quantitative correlation between bacterial communities at genus level (> 2% in any sample) and physicochemical sediment parameters during a) winter and c) summer. RDA for the relationship between bacterial communities and relative abundance of macrolide-resistance genes detected in Sava river sediments during b) winter and d) summer season. Sampling sites: UP7500, 7500 m upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW4500, 4500 m downstream; DW11000, 11,000 m downstream.

tightly adsorbed by clay particles (Chander et al., 2005). Therefore, measured macrolide levels, at least at the most polluted DW0 site, may exert a strong selective pressure to select for macrolide resistant bacteria. Macrolide levels detected in river sediments in this study were lower than levels of fluoroquinolones discharged from bulk drug production in the Indian river sediments from Patancheru area (up to 54 mg/kg), but higher than fluoroquinolone levels observed in the Musi river sediments in India (up to 3 mg/kg), which were also exposed to effluents from drug manufacturing (Gothwal and Shashidhar, 2017). In addition to antibiotics, direct discharge of analyzed effluents led to the accumulation of heavy metals, especially Cu and Zn, in exposed sediments, suggesting metal pollution from incoming industrial waste. Based on the levels of Cu and Zn detected in sediments, which were above their MCCs (Seiler and Berendonk, 2012), they might contribute to co-selection of metal resistance and antibiotic resistance. There are numerous studies where metals have been reported to promote antibiotic resistance via co-occurrences of metal and antibiotic resistance genes (Di Cesare et al., 2016b; Guo et al., 2018; Pal et al., 2015; Song et al., 2017). For example, resistance to Cu in bacteria has already been linked with resistance to macrolides and to other antibiotic classes (Amachawadi et al., 2011; Hasman and Aarestrup, 2002). Therefore, the resulting selection pressure from Cu and Zn may have contributed to the development of macrolide resistance via co-selection. In addition to this, the concentration of Cu, Zn, Ni, and Cr were above their TEC values suggesting that these metals could have toxic effects on sediment-dwelling organisms. Together with antibiotics and metals, significant amounts of organic material, phosphorous and nitrogen compounds were released through effluent discharge into the Sava river (Bielen et al., 2017), elevating concentrations of C, N and P compounds in receiving sediments, especially at DW0 site, and modifying the structure of bacterial communities, promoting horizontal gene transfer and the abundance of ARGs.

4.2. Enrichment of macrolide-resistance genes and class 1 integrons in effluent-receiving sediments

Among the five targeted macrolide-resistance genes, three genes encoding macrolide phosphotransferase (*mph*G), ribosome protection protein (*msr*E) and ribosomal methylase (*erm*B) were the dominant subtypes detected in the analyzed effluents, and their relative abundance was significantly elevated in effluent-receiving sediments. In addition, the *mef*C gene and the *mph*E gene encoding for efflux and macrolide phosphotransferase, respectively, were found somewhat less prevalent in effluents compared to *msr*E, *mph*G and *erm*B genes, but

were also significantly more abundant in exposed sediments. Therefore, the discharge of industrial effluents contributed to increased levels of macrolide-resistance genes in the sediments of the receiving river. All resistance genes targeted were found to be persistent in sediments until 11 km downstream. Surprisingly, we found no significant effect of the season on the distribution of target resistance genes in sediments, despite higher river water flow in winter compared to summer. It has been shown that the relative abundance of ARGs in severely polluted areas can reach values of 10^{-2}–10^{-1} copies/*rrn* copies (Gao et al., 2018), which is comparable to the relative abundances observed in effluents and river sediments at the discharge site in this study. However, although the average relative abundance of the most prevalent resistance genes (*mph*G, *msr*E and *erm*B) was lower in downstream sediments (up to 10^{-3} gene copies/*rrn*), the complete downstream section of the Sava river was generally polluted by macrolide-resistance genes. The observed levels at downstream sites were comparable to those reported by Rutgersson et al. (2014), who quantified quinolone-resistance genes, especially *qnr*VC gene, at approximately 10^{-3} gene copies/*rrn* copies in Indian river sediments polluted with mg/L levels of fluoroquinolones. Other studies also showed that highly polluted sites often have > 10^{-4} ARG copies/*rrn* copies (Gao et al., 2018; Graham et al., 2011). Many macrolide-resistance genes including those targeted here have commonly been found on conjugative plasmids that often carry other ARGs (Sugimoto et al., 2017; Zhang et al., 2013). These plasmids can transfer between different bacterial populations and therefore promote the acquisition and spread of multiple resistance genes, contributing to their persistence in the environment.

In addition to target *msr, mph, erm* and *mef* genes, the relative abundance of the *int*I1-associated class 1 integrons was significantly higher in sediments sampled at the discharge and downstream sites during the winter, which indicated contribution of industrial effluent to increased abundance of class 1 integrons. The class 1 integron-integrase gene, *int*I1, was previously proposed as a genetic marker for anthropogenic pollution (Gillings et al., 2015) and its plasmid-localization would facilitate potential for HGT in these sediments. However, investigated sites, especially the highly impacted ones (DW0-DW300) were similar in both seasons regarding *int*I1 gene levels. Thus, the lack of *int*I1 genes at significantly elevated levels above background during summer is due to its increased level at the upstream site.

4.3. Changes in sediment bacterial community in response to pharmaceutical pollution

At the phylum level, the composition of bacterial communities in sediments of Sava river is typical for river sediment, with *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Acidobacteria* among the major groups (Ibekwe et al., 2016; Wang et al., 2018; Xie et al., 2016). The effluent discharge was found to shift community composition in sediments at the discharge site. *Firmicutes, Bacteroidetes* and *Epsilonbacteraeota* were significantly increased in relative abundance and dominated together with *Proteobacteria* at DW0 site and in effluents during both seasons. These results are consistent with the observation of Kristiansson et al. (2011), who demonstrated high abundance of the same three phyla in antibiotic-polluted river sediments in India. Our RDA analysis further suggested that macrolide and Cu concentration together with the concentration of some nutrients (TC, TN, NH_4^+ and NO_2^-) at least partially contributed to this community shift at the discharge site. The number of taxa was significantly increased, including those originated from effluent (*Macellibacteroidetes, Sulfuricurvum* or vadinBC27 group) and those that were found in UP7500 sediment and effluent (*Trichococcus, Thauera, Arcobacter, Pseudomonas, Variovax* and *Comamonas*) (Fig. 5). Therefore, deposition of effluent-associated bacteria together with the proliferation of resident sediment bacteria in nutrient-rich conditions under selection pressure from antibiotics and heavy metals evidently took place at the discharge site during both seasons. However, the proportion of taxa that were significantly increased in abundance at DW0 decreased sharply (proportion < 1%) at the downstream sites. Already at the site 300 m downstream, abundances found were comparable to the upstream UP7500 site. These observations are consistent with NMDS data (Fig. 2) showing the similar structure of bacterial communities in upstream and downstream sediment samples. These findings suggested that populations that were significantly increased in relative abundance at DW0 site were either not transported further downstream or could not survive and establish at downstream sites. Indeed, the majority of introduced effluent-associated bacteria might not be very well adapted to sediment, allowing the resilience of the indigenous sediment bacterial community. Furthermore, seasonal changes in effluent community composition along with changes in the chemical properties of sediments could be responsible for community shifts between winter and summer.

We found that both Gram-positive (*Macellibacteroidetes, Trichococcus)* and Gram-negative genera (*Macellibacteroidetes, Sulfuricurvum, Thauera, Arcobacter, Pseudomonas, Variovorax, Comamonas*) that had a significantly higher relative abundance at the discharge site were positively correlated with the distribution of *mph, msr* and *mef* genes, suggesting that they might at least partially influence the abundance of these genes in sediments. However, we cannot exclude the possibility that some or all of these bacteria are intrinsically resistant to macrolides. Identified genera were previously reported in different wastewater treatment facilities (Cydzik-Kwiatkowska and Zielińska, 2016; Dichosa et al., 2015; Zhang et al., 2017), and in river sediments polluted with effluents from these facilities (Lu and Lu, 2014; Martínez-Santos et al., 2018), antibiotics (Nakayama et al., 2017) or heavy metals (Suhadolnik et al., 2017; Zhao et al., 2014).

Despite the resilience of the bacterial communities in river sediments, the abundances of target macrolide-resistance genes were still maintained at elevated levels at downstream sites. This may be an indication of horizontal transfer of ARGs to new hosts or the persistence of extracellular ARGs or combination of both. Our previous analysis of the regions flanking macrolide-resistance genes targeted here suggested that these genes likely originated from plasmids (González-Plaza et al., 2018). This further suggests that analyzed resistance genes are candidates for dissemination to other bacteria in river sediment. This is also supported by our most recent exogenous isolation of conjugative broad host range plasmids conferring macrolide resistance from sediments downstream of industrial discharge point (unpublished data). Together, these data indicate that the transferable resistome is likely the primary mechanism for persistence of macrolide-resistance genes in downstream sediments.

Finally, this study together with studies on Asian production facilities provides further evidence for the importance of aquatic environments receiving effluents from antibiotic production for the selection and dissemination of antibiotic resistance. Therefore, to mitigate resistance dissemination, improvements in the management of pharmaceutical discharges are urgently needed also in Europe. These improvements should include increasing the efficiency of existing wastewater treatment technologies through implementation of advanced posttreatment methods to lower the concentration of both antibiotics and ARB/ARGs in the treated effluents (Gadipelly et al., 2014; Rizzo et al., 2013). In addition, improved management of industrial discharges also requires defining and implementing emission limits for both antibiotics and ARB/ARGs to reduce their releases into recipient environments.

5. Conclusion

In conclusion, this study revealed that discharge of insufficiently treated effluents from azithromycin production contributed to the chemical and biological pollution of sediments from receiving river, which resulted in a spatial and seasonal shift of sediment bacterial community, and led to the enrichment of resistance genes to clinically important macrolide antibiotics. The latter means more opportunity for

capture of these resistance genes by pathogens, exacerbating the risk of human exposure to resistant pathogens through drinking water, the food chain or recreation activities in a polluted river. To reduce such risks, more efforts should be devoted to better management strategies for keeping down the antibiotic and ARB/ARGs pollution levels from antibiotic production.

Data accessibility

The 16S rRNA gene sequences that support the findings of this study have been deposited in GenBank within the BioProject with the accession code PRJNA508592.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.12.050.

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Antibiotic-manufacturing sites are hot-spots for the release and spread of antibiotic resistance genes and mobile genetic elements in receiving aquatic environments

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ABSTRACT

High antibiotic releases from manufacturing facilities have been identified as a risk factor for antibiotic resistance development in bacterial pathogens. However, the role of antibiotic pollution in selection and transferability of antibiotic resistance genes (ARGs) is still limited. In this study, we analyzed effluents from azithromycin-synthesis and veterinary-drug formulation facilities as well as sediments from receiving river and creek taken at the effluent discharge sites, upstream and downstream of discharge. Culturing showed that the effluent discharge significantly increased the proportion of antibiotic resistant bacteria in exposed sediments compared to the upstream ones. Quantitative real-time PCR revealed that effluents from both industries contained high and similar relative abundances of resistance genes [*sul1*, *sul2*, *qacE/qacEΔ1*, *tet*(A)], class 1 integrons (*intI1*) and IncP-1 plasmids (*korB*). Consequently, these genes significantly increased in relative abundances in receiving sediments, with more pronounced effects being observed for river than for creek sediments due to lower background levels of the investigated genes in the river. In addition, effluent discharge considerably increased transfer frequencies of captured ARGs from exposed sediments into *Escherichia coli* CV601 recipient as shown by biparental mating experiments. Most plasmids exogenously captured from effluent and polluted sediments belonged to the broad host range IncP-1ε plasmid group, conferred multiple antibiotic resistance and harbored class 1 integrons. Discharge of pharmaceutical waste from antibiotic manufacturing sites thus poses a risk for development and dissemination of multi-resistant bacteria, including pathogens.

1. Introduction

Antibiotic resistance (AR) is rising to dangerously high levels in all parts of the world, threatening not only our ability to treat common infectious diseases, but also progress in many fields of medicine (Nicolau, 2011; O'Neill, 2016). While the overuse and misuse of antibiotics in humans and animals is undoubtedly a primary driving force for this serious problem, the environmental dimension of AR has also been recognized to play an important role in the emergence and spread of AR (Berendonk et al., 2015; Bengtsson-Palme et al., 2018; Larsson et al., 2018; Smalla et al., 2018). Antibiotic resistant bacteria (ARB) were present in the environment long time before the clinical and agricultural use of antibiotics started (D'Costa et al., 2011). These ARB and their antibiotic resistance genes (ARGs) were reported to increase in abundance whenever exposed to a sufficiently high antibiotic selection pressure (Heuer et al., 2009, 2012; Kristiansson et al., 2011; Larsson, 2014; González Plaza et al., 2018). An increasing abundance of ARGs may increase the potential for their spread to pathogenic bacteria by horizontal gene transfer (HGT) (Ghosh and LaPara, 2007). Plasmids and other mobile genetic elements (MGEs) such as integrons are regarded as key contributors in the dissemination of ARGs and promoters of multi-drug resistance (Heuer et al., 2009; Heuer and Smalla, 2012; Jechalke et al., 2013a; Flach et al., 2015; Blau et al., 2018). These MGEs are assumed to promote bacterial adaptation to rapidly changing environments (Heuer and Smalla, 2012). Plasmids belonging to the incompatibility groups IncP-1, IncN, IncQ, and IncW are of particular

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importance when studying ARGs as they have a broad host range (BHR) and may be exchanged among phylogenetically distant bacteria (Pukall et al., 1996; Suzuki et al., 2000; Shintani et al., 2010, 2014; Klümper et al., 2015). Specifically, IncP-1 plasmids, which are important in the clinical and environmental context, are highly promiscuous plasmids and were reported to often carry multiple ARGs, suggesting their important role in ARG dissemination (Popowska and Krawczyk-Balska, 2013; Wolters et al., 2015; Heuer et al., 2012). High abundance of populations carrying these plasmids seemed to be related to pollution (Smalla et al., 2006; Heuer et al., 2012). In addition, antibiotic pollution from manufacturing facilities has been shown to promote horizontal mobility of plasmids carrying ARGs among environmental bacteria (Flach et al., 2015).

In addition to plasmids, integrons are suspected to play a major role in disseminating ARGs. They are genetic platforms specialized in capturing and expressing genes in the form of gene cassettes and are usually associated with the plasmid groups mentioned above (Thorsted et al., 1998; Bahl et al., 2007; Eikmeyer et al., 2012; Heuer et al., 2012; Gaze et al., 2013). In addition, they frequently contain arrays of gene cassettes which can be excised and incorporated in new genetic contexts within a genome or between cells via MGEs (Stokes and Hall, 1989). Class 1 integrons are the best studied integrons which are minimally constituted of an *intI1* gene encoding a site-specific recombinase and of a recombination site *attI* where the integrase IntI1 catalyzes the insertion of gene cassette. They are not only restricted to the clinical context, but also widespread in different environments, particularly those exposed to antibiotics (Gaze et al., 2011; Heuer et al., 2011; Kristiansson et al., 2011; Moura et al., 2012; Li et al., 2009, 2010).

In recent years industrial discharges from antibiotic manufacturing have raised concerns since such discharge is often not controlled, but has been recognized as a risk factor for fostering AR development and dissemination (Larsson, 2014; Flach et al., 2015; Bielen et al., 2017; González Plaza et al., 2018). High, mg/L-levels of antibiotics, particularly fluoroquinolones, tetracycline or penicillins, have been detected in effluents from manufacturing facilities in countries like India, China and Korea (Larsson, 2014), underlying the importance of studying the consequences of this strong selection pressure on exposed microbial communities. Such industrial discharges that polluted receiving aquatic environments are assumed to contribute to an enrichment of ARGs and MGEs facilitating their spread, such as integrons, transposons and plasmids (Li et al., 2009, 2010; Kristiansson et al., 2011; Flach et al., 2015). Enriched ARGs represented several classes of resistance mechanisms even though only one single antibiotic class was measured at high levels at each site (Li et al., 2010; Kristiansson et al., 2011). It was therefore hypothesized that the enrichment of diverse ARGs was due to co-resistance that may be genetically linked, in combination with an increased frequency of HGT events.

However, unacceptable practices of discharging hazardous waste from antibiotic manufacturing are not restricted to Asian countries. We have recently investigated effluents from two Croatian pharmaceutical industries that were involved in synthesis of macrolide antibiotic azithromycin (AZI) or formulation of different veterinary drugs, including sulfonamides and tetracyclines. We showed high concentrations of macrolides (mg/L) and high levels of corresponding macrolide ARGs in effluents of AZI-production and in the recipient Sava river sediments (Bielen et al., 2017; Milaković et al., 2019). In contrast, lower levels of sulfonamides and oxytetracycline (μg/L) were detected in effluents from a formulation industry (Bielen et al., 2017). Antibiotic exposure may induce recruitment of ARGs from effluent-receiving sediments to human and animal pathogens by HGT; however, the potential for plasmid-mediated spread of ARGs in environments polluted by industrial discharges has rarely been studied (Flach et al., 2015).

The present study thus aimed to comprehensively explore the extent to which industrial discharges from two antibiotic production facilities in Croatia impact the promotion and spread of AR. We used quantitative real-time PCR to determine the relative abundance of MGEs such as BHR IncP-1 plasmids (*korB*) and class 1 integrons (*intI1*) as well as various resistance genes encoding sulfonamide (*sul1*, *sul2*), quaternary ammonium compound (*qacE/qacEΔ1*) and tetracycline resistance [*tet*(A)] in effluents and receiving river and creek sediments. Plasmid-mediated spread of resistance from effluent and sediment bacteria into *E. coli* recipient was investigated by exogenous plasmid isolation and captured plasmids were characterized with respect to their replicon type, presence of ARGs and integrons and AR phenotype.

2. Material and methods

2.1. Sampling

We used the same effluent samples of two Croatian pharmaceutical industries situated in the northwest of Croatia as described recently (Bielen et al., 2017; González Plaza et al., 2018; Milaković et al., 2019). Briefly, industrial facility 1 manufactures the macrolide antibiotic azithromycin through synthesis from another macrolide, erythromycin and discharges its effluent into the Sava river, near the city of Zapresic. Industrial facility 2 formulates veterinary drugs including sulfonamide, tetracycline, fluoroquinolone and beta-lactam antibiotics, and discharges its effluent into the nearby creek. Effluent from industrial facility 1 was collected as grab sample from the discharge pipe, and effluent from industrial facility 2 consisted of a 24 h composite sample. Both effluent samples were collected in sterilized screw cap bottles (2 L) and kept at +4 °C. Immediately upon return to the laboratory, aliquots of 50–100 mL were vacuum-filtered through a 0.22 μm pore-size membrane (GE Healthcare Life Sciences) to collect the bacterial cells, and filters were then stored at −80 °C until DNA extraction. In addition, cells for biparental mating assays were collected from 1 mL of each effluent by centrifuging for 5 min at 11,000 × *g*. After discarding the supernatant, the remaining cell pellets were resuspended in 10% TSB containing 15% glycerol and stored at −80 °C.

Sediment samples were collected from the Sava river and creek at the same time as effluent samples (January and February 2016). Sava river sediments were taken at the discharge site near the city of Zapresic (DW0), one site upstream of (UP, 7500 m) and 3 sites downstream of discharge point (DW300, 300 m; DW700, 700 m and DW4500, 4500 m) (Fig. 1). The sites DW300 and DW700 are solely under the impact of discharges from industrial facility 1. Site DW700 is located just upstream of where the small river Krapina, which receives effluents from wastewater treatment plant of the city of Zapresic, enters the Sava river. These municipal effluents thus influence the site DW4500, which is located downstream from the Zapresic, in the Zagreb city area (ca. 1 million inhabitants). Sampling site UP, situated upstream of discharge point, close to Slovenian border, represented reference site which was most likely not subjected to any significant anthropogenic antibiotic pollution. Sediment samples of the creek were collected at the discharge site of effluents from industrial facility 2 (DW0), one site upstream (UP, 300 m) and one site downstream (DW 3000, 3000 m) of effluent discharge. Site UP served as a background control. Both UP and DW0 sites are located in agricultural area, while DW300 site is located in forested area.

From each site, four sub-replicates (top 10 cm) were collected within approximately 1–2 m apart using the plastic core tube. Collected sediment samples were stored in sterile plastic bags; each replicate was about 500 g (fresh weight). Immediately upon return to the laboratory, aliquots of sediments (5 g) were stored at −80 °C until DNA was extracted. Composite sediment samples (10 g of each replicate) were also prepared and used for immediate culturing or stored at −80 °C for biparental mating assays after suspension of 1 g of sediment in 9 mL of 10% Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ, USA) containing15% glycerol (v/v). The remaining of the composite sediment samples were air-dried at ambient temperature for subsequent chemical analyses of antibiotics.

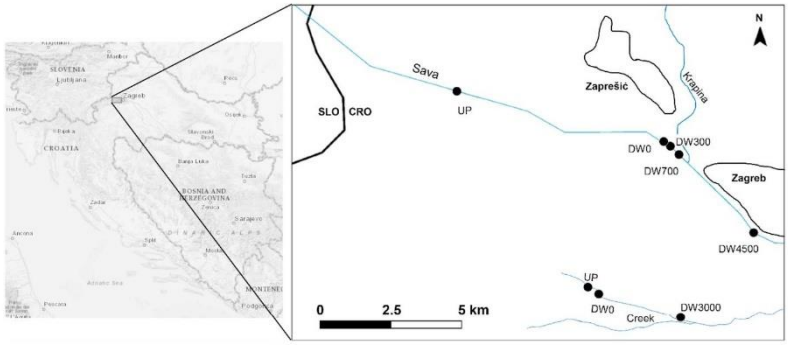

Fig. 1. Location of sampling sites in the Sava river and creek analyzed in this study.

2.2. Chemical analyses of antibiotics

The sulfonamide antibiotics (sulfadiazine and sulfamethazine) were extracted from creek sediments using pressurized liquid extraction and subsequently analyzed by reversed-phase liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) as previously described (Senta et al., 2008, 2013). The concentration data presented for Sava river sediments are based on previous analysis of macrolide antibiotics in those sediment samples (Milaković et al., 2019).

2.3. Culturing bacteria from sediments

One gram of each composite sediment sample was suspended in sterile saline (0.85% NaCl) by vortexing. To enumerate total bacteria, serial 10-fold dilutions were cultured on three replicate R2A agar plates containing cycloheximide (CYC; 100 mg/L, Sigma, Steinheim, Germany). To enumerate resistant bacteria, serial dilutions were cultured on the same plates supplemented with azithromycin (AZI; 15 mg/L) (Fluka, Germany) for river sediment samples or oxytetracycline (OTC; 25 mg/L) (Sigma, Steinheim, Germany) for creek sediment samples. Colony forming units (CFU) were counted after a five-day incubation incubation at 28 °C.

2.4. Extraction of total community DNA from sediments and effluents and quantification of target genes

Total community (TC-) DNA was extracted directly from sediment samples and filters using the Power Soil DNA isolation kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's recommendations. Quantitative real-time PCR (qPCR) 5′-nuclease assay was conducted with extracted TC-DNA to quantify *korB* as gene marker for IncP-1 plasmids, class 1 integron-integrase gene *intI1*, quaternary ammonium compound resistance gene *qacE/qacEΔ1*, tetracycline resistance gene *tet* (A), sulfonamide resistance genes *sul1* and *sul2* as well as bacterial 16S rRNA genes. The primers and probes targeting these genes and qPCR conditions are listed in Table 1. To adjust for differences in bacterial DNA extraction and amplification efficiency between samples, the relative gene abundance was calculated using the following equation: $\log_{10} 2^{-(\text{Ct 16S rRNA gene} - \text{Ct target gene})}$. All qPCR assays were performed in a CFX96 real-time (RT) PCR detection system (Bio-Rad, Hercules, USA). Differences between relative abundance of target genes at each DW site and reference UP site were tested for significance by Kruskal-Wallis test ($p < 0.05$).

2.5. Exogenous plasmid isolation through biparental assay

The kanamycin- and rifampicin-resistant *Escherichia coli* CV601 *gfp* +, carrying the *gfp* gene coding for green fluorescence protein (GFP) (Heuer et al., 2002; Wolters et al., 2015) was used as recipient strain for biparental exogenous plasmid capturing as described previously (Binh et al., 2007). Briefly, the recipient strain was cultured overnight in TSB supplemented with kanamycin (KAN; 50 mg/L), and rifampicin (RIF; 50 mg/L) at 28 °C. Cells were pelleted by centrifugation, washed (three times) and finally resuspended in 1 mL of 0.1 X TSB. Defrosted sediment or effluent samples were shaken for 2 h in 0.1 X TSB at 28 °C. Large particles were settled out, 3.5 mL of suspension was mixed with 50 µL of *E. coli* cells. This mixture was centrifuged, washed three times in 0.1 X TSB and transferred to a membrane filter placed on plate count agar (PCA; Merck; Darmstadt, Germany) supplemented with CYC (100 mg/L). Controls with only recipient or sediment bacteria were also prepared as described above. After overnight incubation at 28 °C, the suspended mating mixtures were spread plated on PCA with CYC (100 mg/L), KAN (50 mg/L) and RIF (50 mg/L) to enumerate the total number of recipients as well as on the same plates supplemented with tetracycline (TET; 15 mg/L) (creek and river sediments) or erythromycin (ERY; 50 mg/L) (only river sediments) to enumerate transconjugants. Transconjugants were confirmed by fluorescence emission and BOX-PCR fingerprints (Martin et al., 1992). Transfer frequencies were calculated based on the following formula:

Transfer frequency:CFU mL^{-1} of transconjugants/CFU mL^{-1} of recipients.

2.6. Isolation and digestion of plasmid DNA

Plasmid DNA was isolated from transconjugants using the QIAprep miniprep buffers (Qiagen, Hilden, Germany) and chloroform extraction followed by isopropanol precipitation (Wichmann et al., 2014). Plasmid DNA was digested with restriction enzyme *Not*I (Thermo Fisher Scientific, Waltham, MA, USA), analyzed on a 1% agarose gel and grouped into restriction profile patterns according to the number of different-sized fragments.

2.7. Characterization of captured plasmids by end-point and RT-PCR as well as Southern blot analysis

In order to assign captured plasmids to known plasmid groups, plasmid DNA from transconjugants was analyzed by RT-PCR for the presence of IncP-1 (*korB*), IncP-1ε (*trfA*), IncI and IncF (*traI*) plasmids. Only IncN plasmids (*rep*) were identified by end-point PCR. In order to determine which resistance genes are present on captured plasmids, plasmid DNA was screened by RT-PCR for the presence of the same resistance genes (*qacE/qacEΔ1*, *tet*(A), *sul1* and *sul2*) and integron-integrase gene (*intI1*) as described above. Detection of sequences specific for mercury resistance operon (*merRTΔP*) was performed by end-point PCR. A summary of primer sets and end-point or RT-PCR conditions used for targeting the above mentioned genes is given in Table 1.

Plasmids harboring *korB* (IncP-1 subgroup) were additionally

Table 1
Primer systems used in this study.

Target gene/plasmid group	Primers	Sequence [5′-3′]	Annealing temp.	Reference
korB (IncP-1)	korB-F	TCATCGACAACGACTACAACG	54 °C	Jechalke et al., 2013a,b
	korB-Fz	TCGTGGATAACGACTACAACG		
	korB-R	TTCTTCTTGCCCTTCGCCAG		
	korB-Rge	TTYTTCYTGCCCTTGGCCAG		
	korB-Rd	TTCTTGACTCCCTTCGCCAG		
	tp_korBgz	TSAGGTCGTTGCGTTGCAGGTTYTCAAT		
	tp_korB	TCAGYTCRTTGCGYTGCAGGTTCTCGAT		
traI (IncI1)	IncI1_traI_Fwd	TTCTTCTTCCCCTACCATC	60 °C	Blau et al., 2018
	IncI1_traI_Rev	CATTTTCCAGCGTGTTTC		
	IncI1_traI_TP	CGGCTTTTCACTTCGTGGTT		
traI (IncI2)	IncI2_traI_Fwd	CAAGAACAGAAACAGGCA	60 °C	Blau et al., 2018
	IncI2_traI_Rev	TCCCGCAGATAACAGATA		
	IncI2_traI_TP	CCAAACCAACCACAACCA		
traI (IncF)	682_F	CACGGTATGTGGGARATGCC	60 °C	Blau et al., 2018
	1073_R	TCCGGCGGCAGYATVCCRAC		
	973_P	CAGCAGGCGGTGRCRCAGGC		
trfA (IncP-1ε)	941f	ACGAAGAAATGGTTGTCCTGTTC	60 °C	Bahl et al., 2009; Dealtry et al., 2014
	1014r	CGTCAGCTTGCGGTACTTCTC		
	tp_trfAε_965	CCGGCGACCATTACAGCAAGTTCATTT		
rep (IncN)	rep-1	AGTTCACCACCTACTCGCTCCG	55 °C	Götz et al., 1996
	rep-2	CAAGTTCTTCTGTTGGGATTCCG		
intI1	intI1-LC1	GCCTTGATGTTACCCGAGAG	60 °C	Barraud et al., 2010
	intI1-LC5	GATCGGTCGAATGCGTGT		
	intI1-P	ATTCCTGGCCGTGGTTCTGGGTTTT		
tet(A)	tetA-qfw	CCGCGCTTTGGGTCATT	60 °C	Guarddon et al., 2011
	tetA-qrv	TGGTCGCGTCCCAGTGA		
	q-tetA-P	TCGGCGAGGATCG		
qacE and/or *qacEΔ1*	qacEall-F	CGCATTTATTTTCTTTCTCTGGTT	60 °C	Jechalke et al., 2014
	qacEall-R	CCCGACCAGACTGCATAAGC		
	qacEall-P	TGAAATCCATCCCTGTCGGTGT		
sul1	q-sul_1 653f	CCGTTGGCCTTCCTGTAAAG	60 °C	Heuer and Smalla, 2007
	q-sul_1 719r	TTGCCGATCGCGTGAAGT		
	tp_sul1	CAGCGAGCCTTGCGGCGG		
sul2	q_sul2 595f	CGGCTGCGCTTCGATT	60 °C	Heuer and Smalla, 2007
	q_sul2 654f	CGCGCGCAGAAAGGATT		
	tp_sul2 614	CGGTGCTTCTGTCTGTTTCGCGC		
merRTΔP	merRT-P-P	GGGAGATCTAAAGCACGCTAAGGCRTA	62 °C	Osborn et al., 1993
	merRT-P	GGGGAATTCTTGACWGTGATCGGGCA		
16S rRNA gene	Bact1369F	CGGTGAATACGTTCYCGG	56 °C	Suzuki et al., 2000
	Prok1492R	GGWTACCTTGTTACGACTT		
	TM1389F_P	CTTGTACACACCGCCCGTC		

screened by Southern hybridization with probe for IncP-1ε or with a mixed IncP-1 probe targeting subgroups α, β, γ, δ, and ε. Plasmid DNA digestion was performed as described above. Digested plasmids were separated on a 1% agarose gel and then transferred to a positively charged nylon membrane (Roche, Mannheim, Germany). Southern blot hybridization was carried out with digoxigenin-labelled probe generated from PCR amplicons obtained with reference plasmids pKJK5 for IncP-1ε and R751 for IncP-1β (Bahl et al., 2009; Dealtry et al., 2014). The recipient strain and one of the plasmids negative for *korB* were used as negative controls.

2.8. Antibiotic susceptibility testing

A representative subset of transconjugants was analyzed for resistance to tetracycline, trimethoprim, doxycycline, ampicillin, amoxicillin, chloramphenicol, streptomycin, cefotaxime, ciprofloxacin, ceftriaxone, gentamycin, nalidixic acid, ceftazidime and sulfadiazine using disk diffusion method (Wolters et al., 2015). The recipient *E. coli* CV601 was included as a negative control. The minimal inhibitory concentration (MIC) of sulfamethoxazole for the recipient and transconjugants was determined using Etest strip (Biomerieux, France) according to instructions from the provider. The MICs of erythromycin and azithromycin were performed by broth microdilution as described previously (Donato et al., 2010). MIC values that were 2–3 fold increased compared with those for the recipient are designated as moderate resistance while those that were at least 4-fold increased in comparison with the recipient are designated as high resistance.

3. Results

3.1. Antibiotic pollution of sediments

The sediments taken from Sava river at effluent discharge site (DW0) were highly contaminated by macrolide antibiotics characteristic for corresponding manufacturing facility - azithromycin as a final product and erythromycin as a precursor in the synthesis (Table 2). The total concentration of these two macrolides reached a high level of around 10 mg/kg at DW0 and decreased with distance further downstream but was still high (> 1 mg/kg) at sites DW300 and DW700. Low total concentrations of macrolides (< 5 μg/kg) were detected in river sediments sampled upstream of discharge (UP).

For the analysis of sediments taken from the creek, the sulfonamide antibiotics were chosen as the unique targets because they usually dominate, together with oxytetracycline, in effluents of industrial facility 2 (Bielen et al., 2017). Oxytetracycline, a tetracycline antibiotic, was not analyzed because quantitative method for the analysis of tetracyclines in sediment compartment was not established in our lab; however, accumulation of tetracyclines is expected due to their constant input through effluent discharge and the strong sequestration to organic matter (Huang et al., 2011). The lowest total concentration of sulfonamides (sulfadiazine and sulfamethazine) was found at UP creek site (39 μg/kg) and an around 10-times higher concentration at site

Table 2
Average antibiotic concentrations in the sediment samples analyzed.

Industrial facility	Recipient water body	Sampling site	Antibiotics (μg/kg dry sediment)
			Total macrolides[a,b]
1 (AZI-synthesis)	Sava river	UP	4.60
		DW0	10,176
		DW300	2710
		DW700	1253
		DW4500	258

Industrial facility	Recipient water body	Sampling site	Antibiotics (μg/kg dry sediment)
			Total sulfonamides[c]
2 (drug-formulation)	Creek	UP	39
		DW0	453
		DW3000	1175

Sampling sites: UP, upstream of discharge; DW0, discharge; DW300, 300 m downstream of discharge; DW700, 700 m downstream; DW3000, 3000 m downstream; DW4500, 4500 m downstream.
[a] Sum of azithromycin and erythromycin.
[b] Milaković et al. (2019).
[c] Sum of sulfadiazine and sulfamethazine.

DW0. However, the largest amounts of sulfonamides were not found at site DW0 but at more distant site DW3000 (> 1 mg/kg).

3.2. Increased and transferable antibiotic resistance in industrially-impacted river and creek sediments

Culture-based analysis of resistant bacteria from Sava river sediments impacted by pollution from AZI-production (industrial facility 1) showed a significantly higher proportion of AZI-resistant bacteria in sediments at discharge (DW0) and downstream sites (DW300 - DW4500) compared with upstream (UP) site (Kruskal-Wallis test, $p < 0.05$; Fig. 2a). For analysis of culturable resistant bacteria from creek sediments, OTC rather than sulfonamide antibiotic was used because it is one of the drugs produced by industrial facility 2 and often found at increased μg/L concentrations in industrial effluents and surface water of the receiving creek (Bielen et al., 2017). Additionally, tetracycline and sulfonamide resistance is frequently linked in aquatic bacteria (Hu et al., 2008; Kim et al., 2008; Nonaka et al., 2012). Similar to river sediments, culturing showed a considerably higher proportion of OTC-resistant bacteria in effluent-receiving creek sediments, both at DW0 and downstream DW3000 site compared with UP site (Kruskal-Wallis test, $p < 0.05$; Fig. 2b). As expected, highest proportions of cultivable resistant bacteria were found in sediments at both DW0 sites (AZI-resistant bacteria 67%, OTC-resistant bacteria 31%) followed by a sharp decrease further downstream (Fig. 2a, b).

Exogenous plasmid isolations from the river bacteria in sediment samples taken along a gradient of macrolide pollution into *E. coli* CV601 were performed in order to capture transferable plasmids conferring ERY- or TET-resistance. TET was used for selection of captured plasmids because typically no intrinsic resistance is observed. ERY-resistant transconjugants were obtained from three sites, i.e. UP, DW0 and DW700 (Fig. 2c). Transfer frequencies were considerably higher for both polluted sites (DW0 and DW700) in comparison with non-polluted UP site. However, higher log transfer frequency was obtained for more

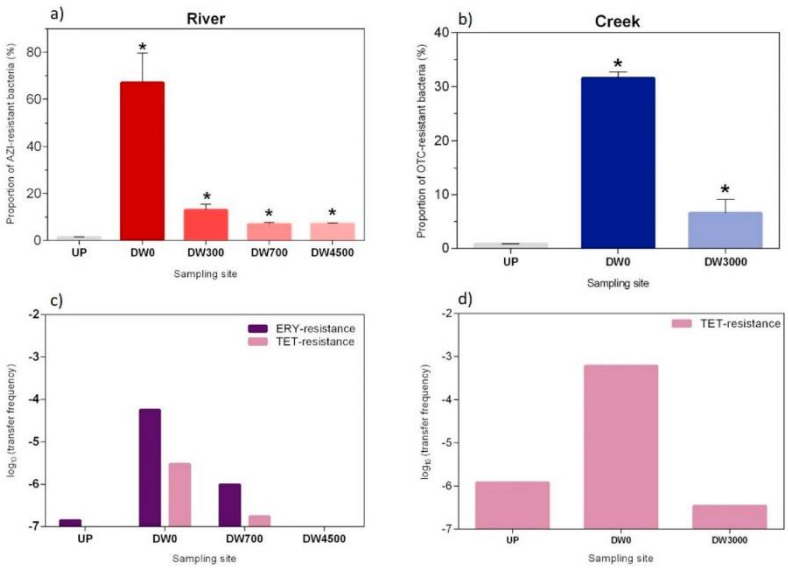

Fig. 2. The proportion of cultivable bacteria (mean ± SD) resistant to azithromycin (AZI) in Sava river sediments (a) and to oxytetracycline (OTC) in creek sediments (b). Transfer frequencies of erythromycin (ERY)- and tetracycline (TET)-resistance from Sava river sediments (c) and TET-resistance from creek sediments (d). Significant differences in the percentage of resistant bacteria were assessed by Kruskal-Wallis test ($p < 0.05$). UP - upstream site, DW0 - discharge site, DW300 - 300 m downstream of discharge, DW700 - 700 m downstream, DW3000 - 3000 m downstream, DW4500 - 4500 m downstream.

polluted site DW0 (−4.3) than for DW700 (−6.0). Further, both DW0 and DW700 sites showed considerably higher log transfer frequencies of TET-resistance plasmids (DW0, −5.5; DW700, −6.8) compared with UP or DW4500 site from which no TET-resistant transconjugants were obtained (Fig. 2c). Additionally, no transconjugants could be captured from effluent of industrial facility 1.

Besides river sediments, transferable TET-resistance plasmids could be also captured from creek sediments from sites UP, DW0 and DW3000 with log transfer frequencies of −5.9, −3.2, and −6.5 (Fig. 2d). However, only DW0 site showed higher transfer frequency in comparison with UP site. In addition to sediments, TET-resistant transconjugants were also obtained from effluent of industrial facility 2 at log transfer frequency of −4.74.

3.3. Transferable plasmids belonged to IncP-1 and IncN groups and conferred multiple resistances

A total of 153 potential transconjugants were further confirmed based on their growth on selective media, GFP fluorescence and BOX-PCR. PCR-based screening of the transconjugants for the presence of *korB*, *traI*, *trfA*, *rep*, *intI1*, *qacE/qacEΔ1*, *sul1*, *sul2*, *tet*(A) and *merRTΔP* genes revealed that the plasmids captured formed 22 different groups (Table S1).

The majority of exogenously captured plasmids (88/153) were assigned to the IncP-1 group as revealed by qPCR targeting the *korB* gene. Further characterization by RT-PCR targeting the *trfA* gene and Southern blot hybridization with primers/probes specific for IncP-1ε showed that majority of IncP-1 plasmids belonged to this subgroup. These IncP-1ε plasmids originated from both study areas including effluent of industrial facility 2 (WW2) and creek sediments from both upstream and discharge site (UP, DW0) as well as river sediments closer to discharge pipe from industrial facility 1 (DW0 and DW700) (Tables S1 and 3). Furthermore, all of these IncP-1ε plasmids, except one (#5), were tested positive for integrase gene of class 1 integrons (*intI1*), the TET-resistance gene *tet*(A), the sulfonamide resistance gene *sul1* and the quaternary ammonium compounds resistance gene *qacE/qacEΔ1* (Table 3). In addition, based on the antibiotic susceptibility testing, all plasmids of the IncP-1ε group conferred resistance to antibiotics from 2 to 4 antibiotic classes (Table 3).

Two transconjugants (Table S1) contained IncP-1β plasmids that were captured from river sediment from polluted DW700 site. This plasmid did not carry any of the tested genes but displayed resistance toward macrolides according to antibiotic susceptibility testing (#12; Table 3). One transconjugant (#6) carried an IncN plasmid and originated from creek sediment from discharge site. This transconjugant conferred resistance to multiple antibiotic classes including tetracyclines, trimethoprim, sulfonamides and beta-lactams (Table 3). The plasmids contained in the remaining transconjugants could not be assigned to any of the plasmid group tested (Tables S1 and 3). The IncN plasmid was the only one that harbored *sul2* in addition to *qacE/qacEΔ1*, *sul1* and *tet*(A) genes (Tables S1 and 3).

The number of resistances to different antibiotic classes captured

Table 3
Plasmid specific sequences and resistance genes detected via end-point PCR, Southern blot hybridization and RT-PCR in plasmid DNA of *E. coli* CV601 transconjugants and corresponding antibiotic resistance patterns as determined by antibiotic susceptibility testing.

#	Site and industrial facility[a]	Antibiotic for capturing	Inc groups	Resistance and integrase genes	Antibiotic resistance profile[b]
1	UP[1]	ERY	IncP-1	*intI1*, *qacE/qacEΔ1*, *merRTΔP*, *sul1*	SMX, AZI, ERY
2	DW0[1]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP^{M}, SMX^{M}, DOX^{M}
3	DW0[1]	ERY	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AZI, ERY, DOX^{M}
4	DW0[1]	ERY	IncP-1	*intI1*, *qacE/qacEΔ1*, *sul1*	SMX, ERX, AZI
5	DW0[1]	TET	IncP-1ε	*intI1*, *sul1*, *tet*(A)	TET, SMX, DOX^{M}
6	DW0[1]	TET	IncN	*intI1*, *qacE/qacEΔ1*, *sul1*, *sul2*, *tet*(A)	TET, TMP, SMX, AM, AMX, DOX^{M}
7	DW0[1]	ERY		*intI1*, *qacE/qacEΔ1*, *sul1*	AM, AMX, AZI, ERY, SMX
8	DW0[1]	TET			TET
9	DW0[1]	ERY	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AZI, ERY, DOX^{M}
	DW0[1], DW700[1]	TET			
10	DW700[1] DW0[1,2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, DOX, ERY
11	DW700[1]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX
12	DW700[1]	ERY	IncP-1β		ERY, AZI^{M}
13	DW700[1]	ERY		*sul2*	SMX, AZI, ERY
14	DW700[1]	ERY		*sul2*	SMX, AZI, ERY
15	DW700[1]	TET			TET, DOX
16	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX^{M}
17	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, DOX
18	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TMP, SMX, TET^{M}, DOX^{M}
19	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX
20	UP[2]	TET		*tet*(A)	TET
21	UP[2]	TET		*tet*(A)	TET
22	UP[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	DOX, SMX, TET^{M}
23	UP, DW0[2]	TET		*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX^{M}
24	DW0[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AM, AMX, DOX
25	DW0[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, TMP, SMX, AM, AMX, DOX
26	DW0[2]	TET		*tet*(A)	TET, DOX^{M}
27	DW3000[2]	TET		*merRTΔP*, *sul2*	AM, AMX, DOX, CHL
28	DW3000[2]	TET		*sul2*	AM, AMX, DOX, CHL
29	DW3000[2]	TET		*sul2*	AM, AMX, DOX, CHL
30	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	SMX, TET^{M}, DOX^{M}
31	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX^{M}
32	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, SMX, DOX^{M}
33	WW[2]	TET	IncP-1ε	*intI1*, *qacE/qacEΔ1*, *sul1*, *tet*(A)	TET, DOX, SMX, ERY^{M}

[a] UP, upstream; DW0, discharge; WW2, effluent from drug-formulation facility; DW3000, 3000 m downstream of discharge; DW700, 700 m downstream of discharge; 1, industrial facility 1 - AZI-synthesis; 2, industrial facility 2 - drug-formulation.

[b] TET: tetracycline; ERY: erythromycin; AZI: azithromycin; TMP: trimethoprim; DOX: doxycycline; AM: ampicillin; AMX: amoxicillin; CHL: chloramphenicol; S: streptomycin; SMX: sulfamethoxazole; M - moderate antibiotic resistance.

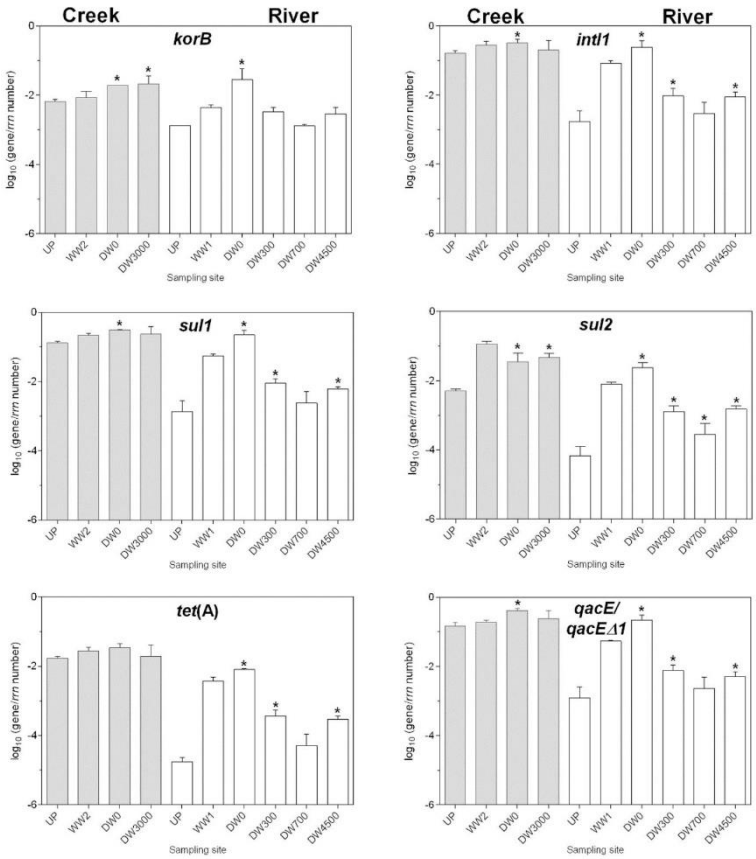

Fig. 3. Relative abundance of antibiotic- and disinfectant-resistance genes [*sul1*, *sul2*, *tet*(A), *qacE/qacEΔ1*], class 1 integron-integrase gene (*intI1*) and IncP-1 plasmids (*korB*) in industrial effluents (WW1 and WW2) and sediments of receiving river and creek from different sites. WW1 - effluent from azithromycin-synthesis facility. WW2 - effluent from drug-formulation facility. Each value is the mean ± SD of four replicates. Sediment sampling locations: UP, upstream; DW0 - discharge; DW300 - 300 m downstream of discharge; DW700 - 700 m downstream; DW3000 - 3000 m downstream and DW4500 - 4500 m downstream. Asterisks represent significant difference ($p < 0.05$; Kruskal-Wallis) between each DW site and reference UP site.

tended to be higher for transconjugants from most polluted sites (Sava river, DW0 site; creek, DW3000 site) in comparison with reference UP sites (Tables 1 and S1). Strikingly, all plasmids captured from the creek at the site situated 3 km downstream of discharge (DW3000), displayed the most pronounced multi-resistance phenotypes as they conferred resistance against five antibiotic classes including tetracyclines, trimethoprim, sulfonamides, beta-lactams and chloramphenicol.

3.4. Effects of industrial effluent discharges on the relative abundance of target resistance genes and MGEs in receiving sediments

We quantified the abundance of MGEs such as IncP-1 plasmids (*korB*) and class 1 integrons (*intI1*), and their typically associated resistance genes [*sul1*, *qacE/qacEΔ1* and *tet*(A)] as well as the sulfonamide resistance gene *sul2* in TC-DNA from two industrial effluent samples as well as sediment samples of the receiving river and creek by using qPCR (Fig. 3).

TC-DNA from effluent of industrial facility 1 (WW1) contained high relative abundances of resistance genes [*sul1*, *sul2*, *tet*(A) and *qacE/qacEΔ1*] with around −1 to −2 log gene copies/*rrn* copies (Fig. 3). In addition to resistance genes, TC-DNA from WW1 effluent contained high levels of MGE genes, *intI1* (−1 log gene copies/*rrn* copies) and *korB* (−2 log gene copies/*rrn* copies). The discharge of these effluents significantly increased the relative abundances of *sul1, sul2, tet*(A) and *qacE/qacEΔ1* genes in sediments from the receiving river at nearly all downstream (DW) sites compared with the UP site ($p < 0.05$; Kruskal-Wallis; Fig. 3). The highest relative abundances of the above genes occurred at discharge DW0 site (−0.6 to −2 log gene copies/*rrn* copies), with the highest relative abundance for *qacE/qacEΔ1* and *sul1*. At all downstream sites (DW300 - DW4500) the relative abundances of target resistance genes were around −2 to −3 log gene copies/*rrn* copies (Fig. 3). Similarly, the relative abundance of the *intI1* gene was also significantly increased in river sediments downstream vs upstream and its relative abundance was similar to that of *qacE/qacEΔ1* and *sul1*. However, the relative abundance of *korB* was higher only at DW0 site in comparison with UP site.

In effluent of industrial facility 2, all analyzed resistance and MGE genes (*korB* and *intI1*) were also detected in high levels (up to −0.6 log gene copies/*rrn* copies; Fig. 3). However, the discharge of these effluents only slightly, but significantly, increased the relative abundances of all target genes, except *tet*(A), in receiving creek sediments compared to sediment from the UP site ($p < 0.05$; Kruskal-Wallis; Fig. 3). The abundances of *intI1*, *sul1* and *qacE/qacEΔ1* genes relative to the *rrn* copy number were significantly higher only at DW0 site, with the average −0.5 log gene copies/*rrn* copies. Compared to UP site, the relative abundances of *korB* and *sul*2 genes were significantly higher at both DW0 and DW3000 sites with −1.4 log units (*korB*) and − 1.7 log units (*sul2*), respectively. However, strikingly, compared to river sediment from UP site, relative abundances of all target genes were up to 3 orders of magnitude higher in the creek sediment samples from UP site, indicating a higher background level of ARGs in the creek than in the river.

4. Discussion

Discharges from antibiotic manufacturing facilities have repeatedly been shown to provide conditions for the selection, spread and persistence of AR in the receiving aquatic environment (Larsson, 2014; Flach et al., 2015). Our previous study demonstrated that effluents from two Croatian antibiotic manufacturing industries studied here contained high levels of organic compounds, nutrients and antibiotics (mg/L range) as well as a high proportion of ARB (Bielen et al., 2017). The present study provided further evidence that as a result of effluent discharges from these industrial facilities the proportion of ARB in effluent-receiving sediments was significantly increased compared to those in non-exposed sediment samples. This indicated either propagation of indigenous sediment bacteria due to antibiotic selection pressure or survival of ARB released with the effluent. In addition, a combination of these contributors may take place as well. Our findings are in accordance with previously published studies demonstrating increased levels of ARB in aquatic environments exposed to discharges from antibiotic manufacturing (Li et al., 2010; Flach et al., 2015; González Plaza et al., 2018).

Such increased abundance of ARB in the environment might contribute to increased levels of AR in human and animal pathogens by HGT. We have, therefore, applied exogenous plasmid capture using sediment bacteria as donor cells and GFP-marked *E. coli* CV601 recipient to study transferability of ARGs. The experiments showed that effluent discharges had an impact on the transfer of resistance, but differences between two investigated study areas were observed. In Sava river sediments, the higher transfer frequency was observed for sediments taken from macrolide-polluted sites (DW0, DW700) compared to those from least-polluted (UP) or less-polluted sites (DW4500). These observations suggest that polluted sites, particularly site DW0, may be hot-spots for plasmid-mediated transfer of ERY- and TET-resistance among the sediment bacterial population. This is likely due to the high number of bacteria as well as high nutrient and macrolide levels in sediments from these sites (Milaković et al., 2019). This is in accordance with a study by Flach et al. (2015) who showed that Indian lake sediments polluted by extreme fluoroquinolone levels through manufacturing discharges displayed higher transfer frequencies of AR plasmids compared with non-polluted sediments. They reported transfer frequencies in the range from 10^{-4} to 10^{-6} which were comparable to those obtained in this study (range from 10^{-3} to 10^{-6}). In contrast to river sediments, transfer frequency was only increased in creek sediments taken from more sulfonamide-polluted site DW0, but not from the most polluted site DW3000 in comparison with UP site. However, selection of acquired resistances was done for TET rather than for sulfonamides to avoid false positives due to high levels of intrinsic sulfonamide resistance. In addition to sediments, transconjugants were also obtained from effluent of drug-formulation facility, indicating that effluent is a source of transferable AR. We have previously reported that this effluent contained 17 μg/L of oxytetracycline, an analogue of tetracycline (Bielen et al., 2017). Additionally, previous studies have demonstrated that TET concentration as low as 10 μg/L promoted HGT (Kim et al., 2014; Jutkina et al., 2016). Together, this suggests that OTC in studied effluent could stimulate plasmid-mediated HGT among effluent bacteria. To the best of our knowledge, this is one of the first reports on the transferability of ARGs harbored in bacteria resident in effluents from pharmaceutical industries. In contrast to drug-formulation effluent, no transfer of ERY- or TET-resistance to *E. coli* CV601 was obtained from effluent of AZI-synthesis facility. This is likely a consequence of very high concentrations of macrolides found in that effluent sample (6.5 mg/L total) (Bielen et al., 2017) which could have inhibited the recipient strain. Indeed, a previous study showed that the macrolide antibiotic ERY inhibited plasmid-mediated resistance transfer at 1 mg/L and even more at 10 mg/L (Jutkina et al., 2018). In contrast to AZI-production effluent, sediment bacteria from both macrolide-polluted sites (DW0 and DW700) with up to 10 mg macrolides/kg sediment successfully transferred resistance plasmids to *E. coli* CV601; however, due to sorption of macrolides to sediment, their bioavailability might be lower than the total concentration measured in sediments.

A large proportion (84/153) of the plasmids captured during this study belonged to the IncP-1ε subgroup (Table S1). The majority of them (68/84) originated from antibiotic-polluted matrices (drug-formulation effluent and creek sediment from DW0 site; river sediments from DW0 and DW700 sites). This study suggests that IncP-1ε plasmids might substantially contribute to the local adaptation and survival of the bacterial communities in response to strong pollution from antibiotic manufacturing facilities. Although IncP-1 plasmids were originally discovered in clinical isolates (Datta et al., 1971), they were later observed in a wide range of different habitats including mercury-polluted river sediment, sewage, sludge and rivers (Haines et al., 2006; Smalla et al., 2006; Bahl et al., 2009; Moura et al., 2010; Heuer et al., 2012; Oliveira et al., 2012), and a correlation of IncP-1 plasmid abundance and pollution was suggested (Smalla et al., 2006). Further, in the present study, a few captured plasmids from macrolide-polluted river sediments could be assigned to the IncP-1β subgroup and IncN incompatibility group. The latter is in agreement with the study of Flach et al. (2015), who captured IncN plasmids from Indian lake impacted by extreme fluoroquinolone pollution from manufacturing sites. Considering the BHR of both IncP-1 and IncN plasmids (Musovic et al., 2006; Klümper et al., 2015; Shintani et al., 2015; Matsumura et al., 2018), we suggest that captured plasmids might be widely shared among sediment bacteria in the studied river and creek. An increased and high relative abundance of the promiscuous IncP-1 plasmids in sediments from both discharge sites (Fig. 3) further indicated that these sites are hot-spots of bacterial populations carrying this plasmid type. This is the first report on IncP-1 plasmids in antibiotic manufacturing effluents and receiving freshwater sediments. These plasmids persisted in downstream sediments as shown by *korB* qPCR data (Fig. 3) which may allow further environmental spread of ARGs localized on these plasmids. We therefore screened plasmids and investigated sediments for the presence of various resistance genes, including tetracycline [*tet*(A)], quaternary ammonium compound (*qacE/qacEΔ1*) and sulfonamide resistance genes (*sul1*, *sul2*). These resistance genes were selected for three reasons: i) all of them, except *qacE/qacEΔ1*, were previously identified in this study area by functional metagenomics (González-Plaza et al., 2018); ii) association of these genes with MGEs - the *sul1* and *qacE/qacEΔ1* genes are often found on class 1 integrons localized on IncP-1 plasmids (Wolters et al., 2015; Jechalke et al., 2014), while the *sul2* and *tet*(A) genes are often located on various transferable plasmids belonging to different incompatibility groups (Heuer et al., 2009; Flach et al., 2015; Blau et al., 2018); iii) TaqMan probe-based RT-PCR method targeting these genes has been established during our previous work. We showed that IncP-1ε plasmids captured from effluent and receiving sediments were associated with *qacE/qacEΔ1*, *sul1* and *tet*(A) resistance genes as well as class 1 integrons (*intI1*) as also shown in other studies (Jechalke et al., 2014; Wolters et al., 2015). In addition to these resistance genes, IncP-1ε and IncN plasmids characterized in this study often conferred phenotypic resistance to other clinically relevant antibiotic classes such as macrolides, trimethoprim or beta-lactams. Such multi-resistance phenotypes seemed to be the most pronounced for plasmids captured from the most polluted sediments (Sava river, site DW0; creek, site DW3000), indicating that industrial discharges might select for bacterial populations that carry multi-resistance plasmids. Earlier studies have demonstrated that the discharge of antibiotic manufacturing effluents contributes to the multidrug resistance among exposed environmental isolates (Li et al., 2009, 2010).

In addition to increased abundance of IncP-1 plasmids, the relative abundance of plasmid-associated resistance genes, i.e. *sul1*, *sul2*, *tet*(A) and *qacE/qacEΔ1* was also significantly elevated in river sediments impacted by AZI-synthesis effluents compared to upstream sediment. This implied that industrial discharges enriched the receiving river with

bacteria carrying these resistance genes likely due to deposition of effluent-associated bacteria or the propagation of indigenous sediment bacteria that are intrinsically resistant or acquired ARGs via plasmid-mediated transfer from effluent bacteria under selection pressure from antibiotics. Although only macrolide antibiotics (AZI, ERY) were detected in high concentrations in effluents (mg/L range) (Bielen et al., 2017) and in receiving sediments, the high levels of *sul1*, *sul2*, *tet*(A) and *qacE/qacEΔ1* genes could be due to co-selection via increasing HGT in response to exposure to macrolides. Indeed, these resistance genes have commonly been found on plasmids that often carry macrolide resistance genes (Nonaka et al., 2012; Dolejska et al., 2014; Rahube et al., 2014), and thus, could be selected due to the presence of macrolides in analyzed sediment samples. In addition, the trends of increased relative abundances of the *intI1*-associated class 1 integrons reflected the trends of the ARGs, which were found elevated in effluent-receiving sediments, except *tet*(A) in creek sediments. The class 1 integron-integrase gene, *intI1*, was previously proposed as a proxy for anthropogenic pollution (Gillings et al., 2015) and its plasmid localization would facilitate potential for HGT in these sediments.

Compared with river sediments, all resistance genes targeted and class 1 integrons were found considerably increased in creek sediments already at the upstream site by up to 3 orders of magnitude. This could be explained by, for example, input from activities such as agricultural runoff and discharges of household sewage into the creek which flows through an area without sanitation infrastructure. This could also be a possible explanation for a moderate pollution of upstream creek sediments by sulfonamide antibiotics. Nevertheless, direct discharge of partially treated effluents from drug-formulation facility affected slightly, but significantly, sediment levels of IncP-1 plasmids and some resistance genes targeted. These effluents have earlier been shown to contain high concentrations of sulfonamide antibiotics (up to 230 μg/L) (Bielen et al., 2017) and consequently, what is shown here, effluent discharges contributed to the accumulation of sulfonamides in receiving creek sediments. In addition to antibiotics, we showed in the present study that effluent also introduced the *sul*, *tet* and *qac* genes as well as MGEs such as class 1 integrons and IncP-1 plasmids in relatively high amounts into the creek ($> 10^{-2}$ gene copies/*rrn*, Fig. 3). Therefore, the introduction of these ARGs and MGEs, together with antibiotics present in effluents contributed to elevated levels of almost all target resistance genes and MGEs in sediment at the discharge site. However, contrary to the *intI1*, *sul1* and *qacE/qacEΔ1* genes, the relative abundance of *sul2*- and the IncP-1 plasmid-bearing bacteria were still maintained elevated at the site located 3 km downstream of discharge (DW3000) compared to UP site. Although *sul2* genes were often found on IncQ or the LowGC plasmids (Sköld, 2000; Smalla et al., 2000; Heuer et al., 2009), its increased abundance might point to a response of their hosts to sulfonamides, which were found in higher levels in sediments at DW3000 (> 1 mg/kg) compared to UP and DW0 sites (Table 2). This accumulation of sulfonamides might be the result of the slower flow rate of the creek at DW3000 than at DW0 site, which in turn might accelerate the sedimentation of antibiotics in the sediment. Interestingly, three plasmids exogenously captured from this DW3000 site could not be assigned to any incompatibility group tested, but carried *sul2* gene and displayed phenotypic resistance to five antibiotic classes, widely used in clinical settings for human therapy. Therefore, these plasmids replicating in *E. coli* may be important vectors for accumulating and spreading multiple and clinically relevant resistance genes among environmental bacteria, including pathogens.

In conclusion, this study has shown that industrial effluents from two Croatian antibiotic manufacturing facilities are significant sources of MGEs associated with ARGs, contributing to their increased levels in the sediments of receiving water bodies. Although effluents from both industries contained similar levels of targeted ARGs and MGEs, much more pronounced effects of effluent discharge on the resistance gene levels were observed for river sediments rather than for creek sediments due to much higher background levels of investigated genes in the creek. Effluent discharge sites might be considered as hot-spots of plasmid-mediated HGT, fostering the adaptation of sediment bacteria to stresses such as exposure to high concentrations of antibiotics and other toxic agents from pharmaceutical production. Plasmids of the IncP-1ε subgroup might have contributed to further dissemination of multiple ARGs to other bacteria in downstream environment. Further sequencing studies should be done to assign those plasmids which were not assigned by the RT-PCR systems used in the present study and to provide better insight into the entire accessory gene content of these plasmids as well as to reveal whether the same plasmids are present in clinically important pathogens from the surrounding areas.

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Effects of industrial effluents containing moderate levels of antibiotic mixtures on the abundance of antibiotic resistance genes and bacterial community composition in exposed creek sediments

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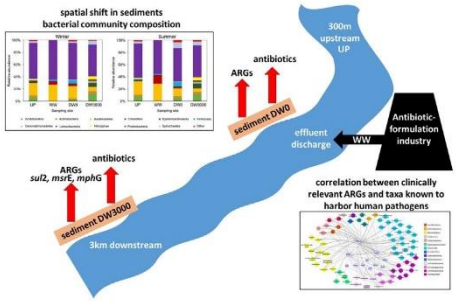
HIGHLIGHTS

- Antibiotic pollution of creek sediments receiving drug-formulation effluents
- Increased relative abundance of most target ARGs in sediments from discharge site
- Three ARGs had increased relative abundance 3 km downstream of the discharge site.
- Spatial shifts of bacterial community composition in exposed sediments
- Associations between increasing ARGs and potential bacterial hosts

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental discharges of very high (mg/L) antibiotic levels from pharmaceutical production contributed to the selection, spread and persistence of antibiotic resistance. However, the effects of less antibiotic-polluted effluents (μg/L) from drug-formulation on exposed aquatic microbial communities are still scarce. Here we analyzed formulation effluents and sediments from the receiving creek collected at the discharge site (DW0), upstream (UP) and 3000 m downstream of discharge (DW3000) during winter and summer season. Chemical analyses indicated the largest amounts of trimethoprim (up to 5.08 mg/kg) and azithromycin (up to 0.39 mg/kg) at DW0, but sulfonamides accumulated at DW3000 (total up to 1.17 mg/kg). Quantitative PCR revealed significantly increased relative abundance of various antibiotic resistance genes (ARGs) against β-lactams, macrolides, sulfonamides, trimethoprim and tetracyclines in sediments from DW0, despite relatively high background levels of some ARGs already at UP site. However, only sulfonamide (*sul*2) and macrolide ARG subtypes (*mph*G and *msr*E) were still elevated at DW3000 compared to UP. Sequencing of 16S rRNA genes revealed pronounced changes in the sediment bacterial community composition from both DW sites compared to UP site, regardless of the season. Numerous taxa with increased relative abundance at DW0 decreased to background levels at DW3000, suggesting die-off or lack of transport of effluent-originating bacteria. In contrast, various taxa that

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were more abundant in sediments than in effluents increased in relative abundance at DW3000 but not at DW0, possibly due to selection imposed by high sulfonamide levels. Network analysis revealed strong correlation between some clinically relevant ARGs (e.g. bla_{GES}, bla_{OXA}, *erm*B, *tet*39, *sul*2) and taxa with elevated abundance at DW sites, and known to harbour opportunistic pathogens, such as *Acinetobacter*, *Arcobacter*, *Aeromonas* and *Shewanella*. Our results demonstrate the necessity for improved management of pharmaceutical and rural waste disposal for mitigating the increasing problems with antibiotic resistance.

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1. Introduction

The rise in antibiotic resistance (AR) represents a serious and growing threat for human health worldwide (O'Neill, 2016). Highly similar or even identical antibiotic resistance genes (ARGs) have been found in both environmental and pathogenic bacteria (Poirel et al., 2005; Forsberg et al., 2012), emphasizing a potentially shared resistome. Under a selection pressure from antibiotics or from a combination of antibiotics and other co-selective agents (metals, biocides), e.g. caused by discharges from antibiotic production facilities, the environmental resistome becomes enriched with antibiotic-resistant bacteria (ARB) and ARGs they carry (Milaković et al., 2019; Lübbert et al., 2017; Šimatović and Udiković-Kolić, 2019). This increase in ARG abundance was invariably accompanied by the increased occurrences of mobile genetic elements (MGEs) associated with ARG transfer (González-Plaza et al., 2019; Kristiansson et al., 2011; Flach et al., 2015), and a recent study showed that a significantly larger fraction of ARGs are indeed potentially mobilized after selective pressure from antibiotics (Sáenz et al., 2019). Consequently, environments polluted by discharges from antibiotic manufacturing have been identified as 'high risk' environments for AR selection and dissemination into human or animal pathogenic bacteria. It is, therefore, of urgent concern to investigate such contaminated areas for determining the abundance of AR and identifying the critical control points to reduce its emergence and spread (Šimatović and Udiković-Kolić, 2019).

Large environmental pollution from the antibiotic manufacturing sector was reported to be a problem mostly in Asian countries, such as India, China, Korea and Pakistan, but also, to a lesser extent, in Europe (Larsson, 2014; Bielen et al., 2017; Šimatović and Udiković-Kolić, 2019; Sidrach-Cardona et al., 2014). Very high, mg/L-levels of antibiotics have been detected in effluents from antibiotic production facilities in above-mentioned countries, which led to high antibiotic pollution as well as the selection, maintenance and spread of AR in the receiving aquatic environment (Flach et al., 2015; González-Plaza et al., 2019; Larsson, 2014; Milaković et al., 2019; Šimatović and Udiković-Kolić, 2019). Additionally, the exposure to these effluents introduced various toxic effects in fish and other aquatic organisms as well as pronounced changes in exposed aquatic bacterial communities (Bielen et al., 2017; Milaković et al., 2019; Kristiansson et al., 2011; Carlsson et al., 2009). Despite these detrimental environmental effects, there are still no established limits for releases of antibiotics from companies producing and formulating antibiotics nor for the content of antibiotics in the environment.

In contrast to high antibiotic loads in effluents from antibiotic production companies, effluents from companies involved in the formulation of drugs contain much more modest antibiotic levels (typically <100 μg/L), however still being selective for AR (Šimatović and Udiković-Kolić, 2019; Bielen et al., 2017). The levels are still about one to two orders of magnitude higher than levels commonly detected in municipal effluents (<10 μg/L) (Michael et al., 2013; Zhou et al., 2019), which were also shown to increase the abundance, diversity and potential spread of ARGs in recipient water bodies (Osinska et al., 2017; Lekunberri et al., 2018; Corno et al., 2019). Further, often combinations of various antibiotics have been detected at sites from drug-formulation companies (Khan et al., 2013; Bielen et al., 2017), however, the effects of combined exposures of moderate levels of various antibiotics on environmental biota associated are far less explored.

In our previous study (Bielen et al., 2017), we showed that effluents from Croatian drug-formulation industry contained a range of antibiotics, including sulfonamides, tetracyclines and trimethoprim, in concentrations up to approximately 250 μg/L. More recently (González-Plaza et al., 2018, 2019), we also demonstrated that these effluents were sources of diverse ARGs and significant amounts of culturable ARB, ARGs and MGEs such as broad host range IncP-1 plasmids and class 1 integrons. The aim of this study was to investigate the effects of these formulation discharges on exposed creek sediments during the warm (summer) and the cold (winter) sampling conditions. We used chemical analyses of selected antibiotics, metals and nutrients to explore the pollution levels in the receiving creek sediments. The relative abundance of 15 ARG subtypes against 5 major antibiotic classes (sulfonamides, diaminopyridines, tetracyclines, β-lactams and macrolides) was determined by quantitative PCR. Illumina-based 16S rRNA amplicon sequencing was applied to assess the impact on sediment bacterial community structure and network analysis was used to infer about potential bacterial hosts of increasing ARGs.

2. Materials and methods

2.1. Study area, sample collection and DNA extraction

For this study samples were obtained from Kalinovica creek located in rural area in the northwest of Croatia, near the city of Zagreb, where the local drug-formulation facility discharges its wastewaters (Bielen et al., 2017; González-Plaza et al., 2018, 2019). This facility formulates various plant protection products and a wide range of drugs for human and veterinary use, including antibiotics mainly from sulfonamide, tetracycline, β-lactam, diaminopyridine and macrolide classes.

Sediment samples were collected from 3 sites along the recipient creek over two sampling campaigns performed in winter (January, monthly average 0.8 °C) and summer (July, monthly average 22.4 °C) of 2016. The sampling sites included reference site (300 m upstream, UP), effluent discharge site (DW0) and one site downstream of the discharge (3000 m downstream, DW3000) (González-Plaza et al., 2019). The sites UP and DW0 are located in agricultural area, while site DW3000 is located in forest area where no agriculture is taking place and the flow rate is slower than at DW0 site. From each site, four replicates (approximately 500 g each) were collected within approximately 1–2 m apart from the surface of the sediment (0–5 cm) using a plastic core tube and immediately transported to the laboratory on ice. Subsamples from each of the four replicate sediment samples (approximately 2 g) were stored at −80 °C for DNA extraction, while the rest of the subsamples were composited (10 g of each subsample used) and air-dried at ambient temperature for physico-chemical analyses.

In addition to sediments, we used and analyzed the same wastewater samples of the industry as described recently (Bielen et al., 2017; González-Plaza et al., 2018, 2019). Aliquots of wastewater samples (50–100 mL), collected in two sampling campaigns (winter and summer), were vacuum-filtered through a 0.22 μm pore-size membrane (GE Healthcare Life Sciences, PA, USA) and filters were stored at −80 °C until DNA extraction. DNA was extracted from filters and sediment cores using the Power Soil DNA isolation kit (MoBio, CA, USA.) Non-template extraction control was set up using DNA-free water. Extracted DNA was stored at −20 °C until use.

2.2. Physico-chemical analyses of sediments

Dry composite sediment samples were coarse grounded to <2 mm. Physico-chemical properties, including pH, total organic carbon (TOC), total carbon (TC), total nitrogen (TN), total phosphorus (TP), nitrate, nitrite and ammonia nitrogen were determined using ISO standardized methods (Milaković et al., 2019). Quantification of antibiotics (sulfadiazine, SDZ; sulfamethazine, SMZ; trimethoprim, TMP; and azithromycin, AZI) in sediments was performed following the analytical methodology and protocols previously described (Senta et al., 2008, 2013). The contents of heavy metals (Cd, Cr, Cu, Pb, Ni, Zn, K, Na, Li, Mg, Fe, Mn, Cs, Rb, Al, Sr, Ba, Be) were measured by inductively coupled plasma mass spectrometry as described previously (Dautović et al., 2014).

2.3. Quantification of ARGs and 16S rRNA genes

ARGs conferring resistance to tetracyclines (*tet*C and *tet*39), β-lactams (bla_{GES}, bla_{VEB}, bla_{OXA-1} and bla_{OXA-2}), trimethoprim (*dfr*A14 and *fol*A), sulfonamides (*sul*1 and *sul*2) and macrolides (*mph*G, *mph*E, *msr*E, *mef*C and *erm*B) were quantified in effluent and sediment samples using SYBR-Green quantitative real-time PCR (qPCR). The 16S rRNA gene (*rrn*) was also analyzed for normalization of the data. All qPCR assays were performed using ABI 7300 Real-time PCR system (Applied Biosystems, CA, USA) and the reaction setup as described previously (Milaković et al., 2019). Specific primer sets, annealing temperatures, amplification accuracies and efficiencies are listed in Table S1. Standard curves were prepared by cloning target genes (amplified using gene-positive bacteria) into JM109 competent cells using a pGEM-T Easy vector cloning kit (Promega, France) (Milaković et al., 2019). Efficiency and accuracy values (Table S1) were determined from six points of the serial dilutions of plasmid carrying ARG. For all but three ARGs (i.e. bla_{OXA-1}, bla_{OXA-2} and *erm*B) the following program was used: 95 °C for 15 min, 30 cycles at 95 °C for 15 s, annealing at corresponding temperature (Table S1) for 30 s, and 72 °C for 30 s. A previously described qPCR conditions were used for quantification of bla_{OXA-1} and bla_{OXA-2} (Zhai et al., 2016), *rrn* (López-Gutiérrez et al., 2004) and *erm*B (Chen et al., 2007). The quantification limit for all target ARGs was 10^2 gene copies per reaction. To minimize the variance in bacterial concentration or amplification efficiency between samples, the relative gene abundance was expressed as the ratio of ARG copy number per 16S rRNA gene copy number.

2.4. Bacterial community analyses

The bacterial community composition was analyzed by 16S rRNA gene amplicon sequencing as described previously (Milaković et al., 2019). Briefly, amplicon libraries of the V1-V2 hypervariable region of the bacterial 16S rRNA gene were prepared from each extracted DNA sample and barcode sequenced using the Miseq platform (Illumina, Chesterford, United Kingdom). The QIIME 2 v2018.2.0 (https://qiime2.org) was used for bioinformatic analysis and amplicon sequencing variants (ASVs) were taxonomically classified using the SILVA v132 database. Alpha diversity was estimated based on calculated Shannon diversity indices and generated rarefaction curves. Beta diversity between samples was examined using Bray-Curtis dissimilarity and ordinated using non-metric multidimensional scaling (NMDS).

2.5. Statistical analyses

All statistical analyses, except Kruskal-Wallis test, were performed in R studio (v1.1.383). Shapiro Wilk's test was performed to test for normal distribution of $\log_{10}$ transformed qPCR data with "fitdistrplus" and "stats" packages. Kruskal-Wallis test (performed in GraphPad Prism v6.01) and the package DESeq2 (v1.22.1) were used to test statistically significant differences ($p < 0.05$) of the relative abundance of ARGs and bacterial phyla/genera between each DW site and UP site (Milaković et al., 2019). Co-occurrence patterns of ARGs and the bacterial genera were revealed by a network analysis (Li et al., 2015), based on the Spearman's correlation. Networks were visualized using Cytoscape v3.7.0. (Shannon et al., 2003). A correlation between two nodes was statistically significant if $\rho > 0.7$ and the p-value was <0.01. To avoid false-positive correlations, the p-values were adjusted by using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

3. Results

3.1. Physical and chemical properties of sediments

As summarized in Table S2, the sediment samples were slightly acidic to alkaline (pH 6.81–7.98) with a silty-sand texture (silt 53–67%, sand 27–43%) (Wenthworth, 1922). The sediments from DW0 site had the lowest TOC (max 2.13%), TC (max 3.34%) and TN (max 0.17%) values over both seasons. In contrast, the maximum value of NO_3^- (24 mg/kg) was observed at this site during summer, and NH_4^+ (16 mg/kg) during winter.

All target antibiotics (sulfadiazine, sulfamethazine, trimethoprim and azithromycin) were detected in sediments from all sampling sites, being present in the lowest levels (≤0.04 mg/kg) at UP during both seasons (Table 1). In contrast, the highest levels of TMP (up to 5.08 mg/kg) and AZI (up to 0.39 mg/kg) were detected at DW0 during both seasons, particularly during summer. Despite the decrease in levels of these compounds at the more distant site (DW3000), the antibiotics were still present in up to one order of magnitude higher amounts at DW3000 compared to UP. For SDZ and SMZ, the highest amounts were not found at site DW0 but at site DW3000 (total 1.17 mg/kg - winter and 0.56 mg/kg - summer, Table 1).

For the analysis of heavy metals, Cd, Cr, Cu, Pb, Ni and Zn were chosen as primary targets because they have previously been reported to promote antibiotic resistance (Seiler and Berendonk, 2012). The concentration of Cr, Pb and Ni was slightly higher at DW0 compared to UP only during winter (Table S3). Surprisingly, highest concentrations of both Cu and Zn were measured in sediments from UP during both seasons, especially of Zn (475 mg/kg two-season average), with a decrease of approximately 2 times (Zn) or 3 times (Cu) at DW0. Higher concentrations of both of these metals were found at DW3000 compared to DW0. It is important to emphasize that concentrations of both Cu and Zn at all sites were above the minimum co-selective concentrations, i.e. concentration needed to co-select for metal and antibiotic resistance (Seiler and Berendonk, 2012). In addition to the above-mentioned metals, we also measured other metals not associated with antibiotic resistance, such as K, Na, Li, Mg, Fe, Mn, Cs, Rb, Al, Sr, Ba, Be, and their concentrations were generally lower in DW sediments compared with UP or equal among all sampling sites (data not shown).

3.2. Target ARGs in industrial effluents and creek sediments

We estimated the relative abundances of 15 ARGs in effluent and sediment samples over two seasons by using qPCR (Fig. 1). Among the analyzed ARGs in effluent samples, the most abundant genes were *sul*1, *sul*2, *mph*G, *msr*E, *tet*C, *tet*39, *dfr*A14 and bla_{OXA-2}, with an average values of the two seasons mainly in the range of −1 to −2 log gene copies/*rrn* copies (Table S4; Fig. 1). However, the relative abundances of bla_{GES}, bla_{VEB}, bla_{OXA-1}, *mef*C and *erm*B were in most cases 10-times lower (approximately −3 log units), while the relative abundance of *fol*A and *mph*E subtypes was approximately 100-times lower (around −4 log units) (Table S4).

In creek sediments at UP site, the *sul*1, *dfrA*14, *tet*C and bla_{VEB} genes were detected at relatively high abundances of approximately −2 log units (two-season average), while the relative abundances for *tet*39, bla_{OXA-1}, bla_{OXA-2}, *sul*2 and *fol*A were around −3 log units (Fig. 1, Table S4). Genes *mph*G and *msr*E were detected only during winter (around −4 log units) while *mph*E and *mef*C were detected only during

Table 1
Quantification of antibiotics belonging to three different classes over winter and summer season in creek sediments receiving drug-formulation effluents.

Antibiotic class	Antibiotic	Antibiotic abbreviation	Season	Sampling sites (mg/kg dry sediment)		
				UP	DW0	DW3000
Sulfonamides	Sulfadiazine	SDZ	Winter	0.007	0.26	0.69
			Summer	0.02	0.04	0.16
	Sulfamethazine	SMZ	Winter	0.03	0.19	0.48
			Summer	0.03	0.09	0.40
Diaminopyridines	Trimethoprim	TMP	Winter	0.04	0.37	0.28
			Summer	0.04	5.08	0.30
Macrolides	Azithromycin	AZI	Winter	0.01	0.15	0.07
			Summer	0.01	0.39	0.35
Total antibiotics, $\sum$			Winter	0.09	0.97	1.52
			Summer	0.10	5.60	1.21

UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

summer (around −2.5 log units). In contrast, the genes bla_{GES} and *ermB* were below quantification limit in UP sediment in both seasons (Table S4).

The discharge of industrial effluents differently affected the relative abundance of targeted ARGs in the receiving creek sediments (Fig. 1). During both seasons, the relative abundances of ARGs to β-lactams, TMP, macrolides and sulfonamides significantly increased in sediments from DW0 compared to UP ($p < 0.05$; Kruskal-Wallis), with increases varying from only about one half to four orders of magnitude (Fig. 1, Table S4). Also, seasonal differences in the relative abundance of ARG subtypes were observed. Specifically, among target β-lactam ARGs, relative abundances of both bla_{GES} and bla_{OXA-1} subtypes increased by more than one order of magnitude at DW0 during both seasons, while bla_{OXA-2} subtype was about 0.8 log units higher at DW0 compared to UP only during summer (Fig. 1 and Table S4). Regarding TMP resistance, only the difference in abundance of *dfr*A14 between DW0 and UP was significantly increased (about 0.8 log units) during summer. Considering macrolide ARGs, *erm*B subtype significantly increased by more than two orders of magnitude at DW0 compared to UP during both seasons, while *mph*E and *mef*C were found elevated only in winter (Fig. 1 and Table S4). During summer, *mph*G and *msr*E subtypes significantly increased in relative abundance (up to three orders of magnitude) not only at DW0, but also at DW3000 compared to UP ($p < 0.05$; Kruskal-Wallis; Fig. 1 and Table S4). Similar to this, the sulfonamide resistance gene *sul*2 increased in relative abundance by around one order of magnitude at both DW0 and DW3000 compared to UP in both seasons, whereas *sul*1 increased only at DW0 compared to UP. In contrast, tetracycline ARGs were found significantly elevated during summer only at DW0 site, with increases of around one order of magnitude (Fig. 1, Table S4).

3.3. Impact of formulation effluents on sediment bacterial communities

A total of 2,265,504 high-quality reads from effluent and sediment samples were obtained after quality-filtering. Those were assigned to a 13,461 ASVs at 99% similarity level, which were used for all downstream analyses. Rarefaction analysis showed that the sequencing depth of 27 datasets was sufficient to detect the most of the ASVs in the analyzed samples (Fig. S1).

Discharge of pharmaceutical effluents had no significant effect ($p > 0.05$, Kruskal-Wallis) on overall bacterial diversity in sediments from both DW0 and DW3000 sites in comparison with UP site during both seasons, as indicated by Shannon-Wiener diversity index (Fig. S2). However, the structure of sediment bacterial communities was found to be significantly altered according to NMDS analysis based on Bray-Curtis dissimilarity. Sediment samples from three studied sites (UP, DW0 and DW3000) clustered separately (Adonis $R^2 = 0.8254$, $p < 0.05$), independent from sampling season (Fig. 2).

At the phylum level, *Proteobacteria* and *Bacteroidetes* were the most abundant in all sediment and effluent samples during both seasons (Fig. S3). Other abundant phyla were *Acidobacteria* in all sediment samples, and *Firmicutes* and *Epsilonbacteraeota* in both effluents and DW0 sediments. In addition, *Spirochaetes* and *Chloroflexi* dominated in DW3000 sediments (Fig. S3). Effluent discharge resulted in significant changes ($p < 0.05$) in the relative abundance of different phyla at DW0 and DW3000 compared to UP site as shown by DESeq2 analysis

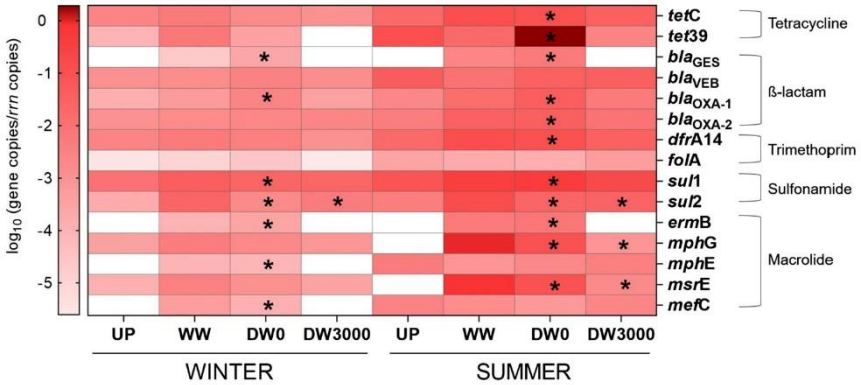

Fig. 1. Heat map of relative abundances of 15 targeted ARGs in effluent (WW) and sediment samples taken from three sampling sites (UP, DW0, DW3000) over winter and summer season. Plotted values represent the natural logarithm-transformed the relative abundance of each ARG target (per 16S rRNA gene copy numbers). Asterisks represent statistically significant difference ($p < 0.05$, Kruskal-Wallis) between each DW and UP site. UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge.

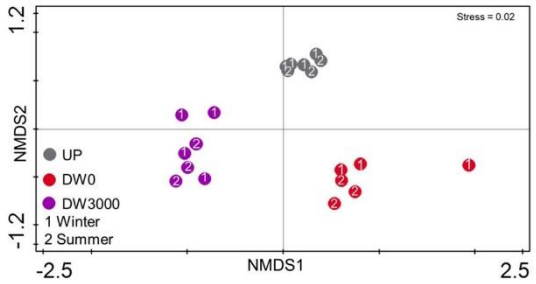

Fig. 2. NMDS analysis based on Bray-Curtis dissimilarity showing the spatial changes in sediment community composition across three to four replicates of each of the three sites along the creek. The replicate samples from the same site were marked with the same color, and from the same season with the same number. Sampling sites: UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. S4). Phyla such as *Firmicutes* and *Epsilonbacteraeota* showed the most significant increase in the relative abundance at DW0 compared to UP during both seasons (increased 5.2% and 2%, respectively; two-season average), but decreased to background levels (*Firmicutes*) or significantly below background levels (*Epsilonbacteraeota*) at DW3000. Several phyla significantly increased at DW3000 versus UP over both seasons, including *Acidobacteria* (5.6% two-season average), *α-Proteobacteria* (5.4%), *Chloroflexi* (4.9%), *Nitrospirae* (3.4%), *Spirochaetes* (2.9%), *Gemmatimonadetes* (2.6%) and *Latescibacteria* (2.1%) (Fig. S4).

At the family or genus level, some bacteria that were highly abundant (>1%) in sediments from UP site in both seasons, such as *Prolixibacteraceae* (BSV13, *Prolixibacter*, WCHB1-32), *Cyclobacteraceae*, *Ignavibacterium*, *Geobacter*, *Anaeromyxobacter*, *Steroidobacteraceae* and *Sphaerochaeta*, significantly decreased in sediments from both DW sites (Tables S5 and S6). In contrast, genera that were highly abundant (≥1%) in effluents in both seasons, such as *Acidovorax*, *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Roseimarinus* and *Arcobacter* (Fig. 3), significantly increased in relative abundance in sediments from DW0 compared to UP, but not at DW3000 in both seasons (Fig. 3, Tables S7 and S8). Besides, various other genera with low abundance in effluents (<0.1%) and UP sediment (≤0.8%) also showed significantly increased abundance in DW0 (>1%) compared to UP, with differences between seasons. These, for instance, included *Sideroxydans* (1.2%) and *Luteimonas* (1.9%) in winter, and *Solobacterium* (1.2%), *Treponema2* (1%) and *Smithella* (1.4%) in summer (Fig. 3, Tables S7 and S8). In addition, some psychrophilic genera such as *Sulfurospirillum*, *Pseudoalteromonas*, *Massilia* and *Polaromonas* increased in abundance at DW0 compared to UP only in winter, but their proportion was generally low (≤0.6%). Further, some genera were significantly increased in relative abundance at DW0 in both seasons, including those specific to sediment (*Desulfobulbus* – 2%) or effluent (*Thauera* – 0.9%). Among all above-mentioned genera with enhanced relative abundance at DW0, only the relative abundance of *Sideroxydans* (1.6% two-season average) and *Smithella* (1.4% summer) was still significantly increased at DW3000 compared to UP (Fig. 3, Tables S7 and S8). In addition, unassigned members of the family *Sphingomonadaceae* were found in significantly increased relative abundance at both DW0 and DW3000 sites compared to UP during both seasons. There were few other taxa

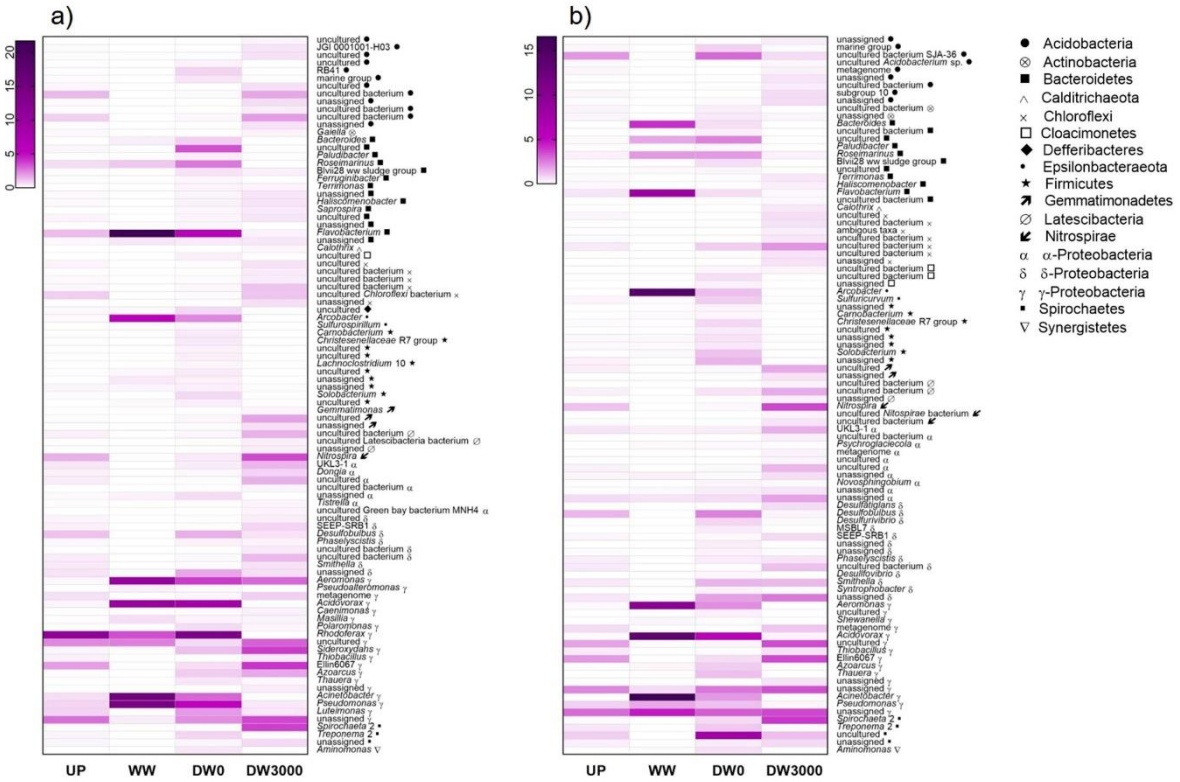

Fig. 3. Heat maps of the relative abundance of genera (%) that were significantly increased at DW sites compared to UP site ($p < 0.05$, DESeq2) during a) winter and b) summer season. UP, upstream of discharge; DW0, discharge site; DW3000, 3000 m downstream of discharge; WW, effluent.

which were significantly increased in relative abundance at both DW sites compared to UP, but their proportion was <0.5%. Exceptions are *Azoarcus* (winter) and *Acidovorax* (summer) which had relative abundance of 1% and 5%, respectively at DW0 site (Fig. 3, Tables S7 and S8). Finally, the majority of genera that were significantly elevated and dominated (>3%) at downstream DW3000 site during both seasons, such as *Spirochaeta* 2, Ellin6067 group and *Nitrospira*, were present in low relative abundance (<0.7%) in DW0 sediment and originated mostly from UP sediments (Fig. 3, Tables S7 and S8). Notably, many of the taxonomic groups with significantly higher abundances at DW3000 compared to UP could not be classified to the genus level.

3.4. Co-occurrence between target ARGs and bacterial taxa

Network analysis was performed to identify potential bacterial taxa that might be associated with the analyzed ARGs (Fig. 4). The entire network, consisting of 83 nodes and 155 edges, had a modular structure with a modularity index of 0.722 (Newman, 2006). Out of 15 ARGs targeted in this study, 11 of them (bla_{GES}, bla_{OXA-1}, bla_{OXA-2}, *sul*1, *sul*2, *tet*39, *tet*C, *dfr*A14, *mph*G, *msr*E and *erm*B) were significantly positively correlated with bacterial genera. In total, targeted ARGs had 72 potential bacterial hosts which mainly belonged to the *Firmicutes* (22), *Proteobacteria* [α- (6), δ- (4), γ- (9)], *Bacteroidetes* (12), and *Epsilonbacteraeota* (4) (Fig. 4). Regarding single ARG-host correlations, the gene bla_{GES} had the highest number of potential bacterial hosts (18), including *Azoarcus*, *Aeromonas* and members of uncultured family *Barnesiellaceae*, which were found to be highly abundant (≥1%) and increased at DW0 compared to UP (Fig. 4, Tables S7 and S8). The gene *sul*2 was the only one with increased abundance at both DW sites which showed significant correlations with ASVs of the family *Sphingomonadaceae* (*α-Proteobacteria*). However, among the multi ARGs-host correlations (at least 2 ARGs in individual host), the three ARGs, i.e. bla_{GES}, *tet*39 and *erm*B co-occurred in the highest number of potential hosts (24), including those with significantly increased relative abundance at DW0 in both seasons, i.e. *Arcobacter*, *Thauera* and *Aminomonas* (Fig. 4, Tables S7 and S8). Besides *erm*B, the genes bla_{GES} and *tet*39 co-occurred with bla_{OXA-2} in genera *Acinetobacter* and *Roseimarinus* which were increased and highly abundant (>1%) at DW0 but not at DW3000 in both seasons. In addition, the co-occurrence of four ARGs was found for *Shewanella* (bla_{GES}, *tet*39, *erm*B and bla_{OXA-1}) and *Desulfovibrio* (*tet*39, *tet*C, bla_{OXA-2} and *dfr*A14), both significantly higher in abundance in summer at DW0 compared with UP (Fig. 4, Tables S8).

4. Discussion

The present study provides a comprehensive dataset on the effects of discharges of partially-treated effluents from Croatian drug-formulation pharmaceutical industry on the sediments from the receiving creek.

4.1. Contribution of industrial waste to antibiotic, metal and nutrient pollution of the receiving creek sediments

We showed that industrial discharges contributed to antibiotic accumulation in creek sediments, with levels typically highest at DW0 site for both trimethoprim (up to 5.08 mg/kg) and azithromycin (up to 0.39 mg/kg), whereas the total concentration of two sulfonamides was the highest at the site located 3 km downstream (up to 1.17 mg/kg). The latter could be the consequence of the slower flow rate of the creek at DW3000 compared to DW0, which might accelerate the deposition of antibiotics into the sediment. Additionally, sulfonamides are liable to degradation by sunlight (Baena-Nogueras et al., 2017) and the forest around DW3000 may have protected them from potential photodegradation resulting in their greater persistence in sediments at DW3000 versus DW0. Further, total antibiotic levels measured in the present study (up to 5 mg/kg at DW0 and 1.5 mg/kg at DW3000) were lower than what is generally found in sediments impacted by discharges from antibiotic production (tens of mg/kg) (Gothwal and Shashidhar, 2017; Kristiansson et al., 2011; Milaković et al., 2019), but higher than levels found in sediments exposed to treated effluents from municipal wastewater treatment plants (WWTPs) (up to 0.6 mg/kg total) (Li et al., 2019; Guang et al., 2019; Marti et al., 2014). In addition to antibiotics, we found that formulation effluents also

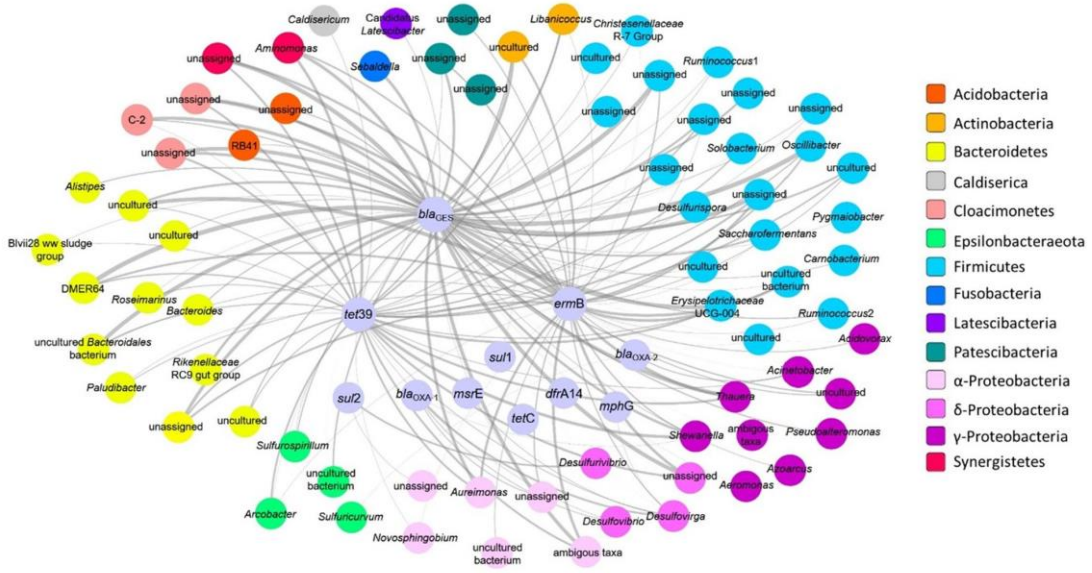

Fig. 4. Network analysis revealing co-occurrence patterns among analyzed ARGs and their potential bacterial hosts. The nodes were colored according to the phylum affiliation. A connection represents strong (Spearman's correlation coefficient (ρ) > 0.7) and significant ($p < 0.01$) correlation. Node size was weighted according to the number of connections (i.e. degree) and edges were weighted according to the correlation coefficient. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contributed to a slight accumulation of nutrients, especially N compounds at DW0 which may affect the composition of bacterial communities (Ibekwe et al., 2016). Some metals were also found to be elevated at DW0 compared to UP; however, surprisingly, the majority of targeted metals, especially Cu and Zn, were found in higher concentrations at UP than at both DW sites, suggesting other sources of contamination at UP. Importantly, Cu and Zn levels at UP and DW sites could co-select for AR (Seiler and Berendonk, 2012). Besides metals, there were also relatively low levels of antibiotics in UP sediments (up to 0.04 mg/kg). Given that studied creek flows through rural area without sewage treatment infrastructure, we speculate that untreated household waste disposal and agricultural runoff might be sources of pollution of UP sediments with antibiotics and metals.

4.2. Effects on antibiotic resistance genes in exposed sediments

Besides introducing antibiotics (Bielen et al., 2017), we showed in this study that formulation effluents in both seasons also introduced relatively high amounts (≥−2.5 log gene copies/*rrn*) of the ARGs conferring resistance to sulfonamides (*sul*1, *sul*2), tetracyclines (*tet*39, *tet*C), macrolides (*mph*G, *msr*E), β-lactams (bla_{OXA-2}), and trimethoprim (*dfr*A14). The relative abundance of almost all these ARG subtypes significantly increased in the sediment at DW0 compared to UP during summer, but not during winter (except the *sul* ARGs). This seasonal difference may be linked to the warmer temperatures which may promote the survival of effluent-associated bacteria carrying ARGs or horizontal gene transfer (HGT) in sediments (González-Plaza et al., 2019), and thus lead to an increased relative abundance of ARGs, despite the relatively high ARGs abundance already present in the background sediments (UP site). In addition to the above-mentioned highly abundant ARGs, effluents also contained moderate amounts (on average −3 to −4 log ARG copies/*rrn*) of various ARG subtypes encoding resistance to β-lactams (bla_{GES}, bla_{VEB}, bla_{OXA-1}), trimethoprim (*fol*A) and macrolides (*erm*B, *mph*E, *mef*C). Most of these ARGs showed different dynamics during winter and summer sampling, but were always higher in relative abundance at DW0 compared to UP, with the exception of *fol*A, *erm*B and bla_{GES}. These differences between seasons may be explained by variations in background sediment levels of analyzed ARGs.

*erm*B and bla_{GES} ARGs, which are of high relevance in clinical settings (Guo et al., 2018; Wibberg et al., 2018) were below quantification limit in UP sediment, but found elevated at DW0 site during both seasons, suggesting deposition from incoming industrial effluents. Indeed, both genes were measured in analyzed industrial effluents in concentrations of −3 to −4 log gene copies/*rrn* copies which is comparable to or even lower than concentrations previously found in municipal effluents (−2 to −3 log gene copies/*rrn* copies) (Rodríguez-Mozaz et al., 2015; Rafraf et al., 2016; Yang et al., 2016). As a consequence, the increase in abundance of these genes was also reported in sediments exposed to treated (*erm*B gene; Sabri et al., 2018) or untreated municipal effluents (bla_{GES}; Marathe et al., 2017). The *erm*B gene was also reported to be enriched in sediments exposed to drug-formulation effluents in Pakistan (Khan et al., 2013).

However, most of ARGs with increased abundance in sediments at DW0 did dissipate to background levels at the more distant DW3000 site. These results indicate either limited transport/death of bacterial hosts (Milaković et al., 2019), degradation of extracellular DNA containing ARGs (Nnadzoie and Odume, 2019), binding of ARGs to sediment (Calero-Cáceres et al., 2017) or a combination. Nevertheless, three gene subtypes, i.e. *sul*2, *mph*G and *msr*E, were detected significantly elevated above background also at DW3000 site. This might be due to growth of their hosts as a result of selection pressure from residual antibiotics (particularly sulfonamides) or expansion of hosts due to HGT, rather than transport of fecal bacteria from DW0. The latter cannot be entirely excluded, although the relative abundance of taxa from the orders of *Bacteroidales* and *Clostridiales*, typically associated with fecal contamination (Halliday et al., 2014; McLellan et al., 2010), was low at DW3000 site. In contrast, the hypothesis for HGT is further supported by a previous study reporting the selection of *sul*2-carrying population in soil via HGT already at SDZ concentrations of 0.15 mg/kg (Heuer et al., 2008), which is lower than 0.16 mg SDZ/kg (summer) and 0.69 mg SDZ/kg (winter), measured in DW3000 sediments in this study. In addition, the selective concentrations of macrolides in the sediment are currently unknown, and thus, it is difficult to estimate whether sediment levels of AZI measured at DW3000 site (0.35 mg/kg) were selective for bacteria carrying *mph*G and *msr*E genes or increased HGT for these genes. Alternatively, increased relative *mph*G and *msr*E abundance at DW3000 may be a result of co-selection by sulfonamides. Co-localization of *mph*G and *sul*2 ARGs on the same genetic element further supports this assumption (González-Plaza et al., 2018; Nonaka et al., 2012).

4.3. Effects on bacterial communities in creek sediments

The community analysis revealed small difference in the number of taxa between the UP and DW sediment samples, suggesting that a wide range of bacteria are able to survive at high concentrations of antibiotic mixtures. A similar conclusion was reached in other studies investigating community changes in response to high antibiotic selection pressure (Milaković et al., 2019; Bengtsson-Palme et al., 2019; Kristiansson et al., 2011). However, we observed clear effects of effluent discharge on the sediment bacterial community composition as the NMDS analysis revealed separate groups for UP, DW0 and DW3000 sediments, regardless of the season. Interestingly, DW0 where the industrial effluent is mixed with the creek water seemed to be taxonomically (phylum-level) more similar to effluent rather than to UP or DW3000 sediments. The relative abundance of *Firmicutes* and *Epsilonbacteraeota*, which were among the most abundant phyla in the analyzed effluents, but also in pharmaceutical effluents described previously (Li et al., 2010; Marathe et al., 2013; Milaković et al., 2019), were significantly increased at DW0 site in both seasons, suggesting a deposition of effluent-associated bacteria in sediments close to the effluent outfall. At the downstream DW3000 site, these phyla were significantly reduced in proportion (<1%), likely due to die-off or lack of transport of effluent-originating bacteria. In contrast, phyla that were more abundant in sediments than in effluents, such as *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, *Gemmatimonadetes*, *Latescibacteria* and *α-Proteobacteria*, increased in relative abundance at DW3000, but not at DW0 compared to UP site. Such distinct community composition at DW3000 site compared to both UP and DW0 sites may potentially be due to selection imposed by high concentration of antibiotics (total >1.2 mg/kg in both seasons) and other co-existing pollutants including heavy metals. However, other environmental factors including nutrients and habitat alterations can contribute as well.

Since the bacterial community has been identified as one of the key drivers that shape the ARG profiles in antibiotic-rich environments (Forsberg et al., 2014; Su et al., 2015), we performed network analysis in order to link variation of analyzed ARGs with the dynamic of the bacterial community. We found an association between some clinically relevant ARGs and ASVs more abundant at effluent-receiving sediments. For instance, *Azoarcus* and *Aeromonas* were found to host clinically relevant β-lactam bla_{GES} subtype, while *Sulfuricurvum* mainly carried bla_{OXA-2}. Previous studies reported localization of bla_{GES} on plasmid in *Aeromonas* spp. isolated from rivers (Girlich et al., 2011) and from urban WWTP (Piotrowska et al., 2017), suggesting that waterborne *Aeromonas* species can be important reservoirs and vehicles for dissemination of extended-spectrum β-lactamases in the environment (Harnisz and Korzeniewska, 2018). Some taxa took along 3–4 ARGs including *Arcobacter* (bla_{GES}, *tet*39, *erm*B), *Acinetobacter* (bla_{GES}, bla_{OXA-2}, *tet*39) or *Shewanella* (bla_{GES}, bla_{OXA-1}, *tet*39, *erm*B). The last three genera together with *Aeromonas* had been considered as the opportunistic human and/or animal pathogenic bacteria (Janda and Abbott, 2010; Janda, 2014; Ferreira et al., 2015; Wong et al., 2017), suggesting that industrial effluent discharge increased the prevalence of pathogenic bacteria carrying multiple ARGs of clinical

relevance in the receiving creek sediments. This may increase the risk of direct transmission of these multi-resistant pathogens to humans. However, for ARGs with higher prevalence at DW3000 versus UP site (*sul*2, *mph*G and *msr*E), we found an association of only *sul*2 with ASVs of the family *Sphingomonadaceae* which were more abundant at both DW sites than at UP site. This family has already been linked with sulfonamide resistance (Narciso-da-Rocha et al., 2014; Vaz-Moreira et al., 2011) and assumed for being prone for acquiring *sul* genes (Narciso-da-Rocha et al., 2014).

5. Conclusions

The present study revealed that effluent discharges from local drug-formulation facility contributed to pollution of the receiving creek sediments with antibiotics and ARGs despite relatively high background levels of the investigated genes in the creek. In addition, effluent discharge caused pronounced changes in sediment bacterial communities from both downstream sites compared to upstream, but the overall taxonomic diversity was not affected. In contrast to effluent discharge site where increased levels of analyzed ARGs are likely a consequence of deposition of effluent-associated bacteria, the accumulated levels of sulfonamides at more distant downstream site could play a role in shifting community composition and increasing some sulfonamide and macrolide ARGs. Our results demonstrate the necessity for implementing/improving infrastructure for the treatment of sewage and industrial waste in the analyzed region in order to limit environmental transmission of antibiotic residues and antibiotic resistance determinants.

Data accessibility

The 16S rRNA gene sequences that support the findings of this study have been deposited in GenBank within the BioProject with the accession code PRJNA588393.

CRediT authorship contribution statement

Milena Milaković: Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Gisle Vestergaard:** Software, Formal analysis, Data curation, Visualization, Writing - review & editing. **Juan Jose González-Plaza:** Methodology, Investigation. **Ines Petrić:** Methodology, Validation, Data curation. **Josipa Kosić-Vukšić:** Methodology, Validation, Formal analysis. **Ivan Senta:** Methodology, Validation, Formal analysis. **Susanne Kublik:** Methodology, Validation. **Michael Schloter:** Methodology, Resources, Writing - review & editing. **Nikolina Udiković-Kolić:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.136001.

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Characterization of macrolide resistance in clinical and environmental isolates from macrolide-polluted and unpolluted river sediments (working title)

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ABSTRACT

Environments polluted with high levels of antibiotics released from manufacturing sites can act as a source of transferable antibiotic resistance (AR) genes to human commensal bacteria and pathogens. The aim of this study was to evaluate AR of bacteria isolated from Sava river sediments (Croatia) at the discharge site of effluents from azithromycin production, and compared to those from upstream site and a clinical setting in Croatia. A total of 228 environmental strains were isolated, with 124 from the discharge site and 104 from the upstream site. In addition, a total of 90 clinical streptococcal and staphyloccocal isolates obtained from the Croatian Reference Center for Antibiotic Resistance Surveillance were analyzed. Sequencing of 16S rRNA gene of environmental isolates showed that the genus *Microbacterium* dominated at both river sites, followed by *Bacillus* at upstream site and *Cellulosimicrobium, Brevundimonas, Enterococcus, Streptococcus* and *Chryseobacterium* at the discharge site. Screening of all isolates by PCR for the presence of 11 relevant macrolide resistance genes (MRGs) showed that 46.8 % of discharge isolates, 16.3 % of the upstream strains and nearly 60% of clinical isolates carried at least one MRG. Discharge isolates demonstrated greater detection frequencies for 4 gene targets (*erm*B, *msr*E, *mph*E and *erm*F) compared to upstream isolates. Among clinical isolates, the most frequently detected gene was *erm*B, followed by *msr*D, *mef*A/E and *mef*C. Discharge site demonstrated a greater abundance of isolates with co-occurence of two different MRGs (predominantly *msrE-mphE*) than upstream site, but lower abundance than clinical sources (most commonly *msr*D-*mef*E). The simultaneous presence of three or even four MRGs was specific for discharge and clinical isolates, but not for upstream isolates. When MRG results were sorted by gene mechanism, the ribosomal methylation (*erm*) and protection genes (*msr)* were the most frequently detected among both discharge and clinical isolates. Additionally, the efflux *mef* genes dominating among clinical isolates were poorly represented among discharge isolates. Following sequencing, high nucleotide sequence similarity (> 98 %) was observed between *erm*B in discharge isolates (mainly streptococci and enterococci) and clinical streptococcal isolates, suggesting the possible transfer of the *erm*B gene between bacteria of clinical and environmental origin. Our study highlights the importance of environmental bacterial populations as reservoirs for AR genes.

53 Keywords: antibiotic resistance; manufacturing; sediment; macrolide resistance genes;
54 environment, bacteria

INTRODUCTION

Overuse and misuse of antibiotics in human and veterinary medicine is one of the key drivers of the emergence and spread of antibiotic resistance (AR), which is a serious threat to public health and clinical medicine worldwide (UNEP, 2017; WHO, 2014). Antibiotic resistant bacteria and their AR genes, however, are not limited to the clinic; rather, they are already prevalent in the natural environment. Pollution of the environment with antibiotics further increased the amount of such resistance determinants (Bengtsson-Palme et al., 2014; Cacace et al., 2019) or dissemination of AR through horizontal gene transfer (HGT) (Heuer and Smalla 2007, González-Plaza et al., 2019). Several studies have documented similar or even identical AR genes between environmental bacteria and clinical isolates (Forsberg et al., 2012; Humeniuk et al., 2002; Poirel et al., 2005), suggesting the movement of AR genes between the environment and the clinic. Of particluar concern are environments impacted by discharges from antibiotic manufacturing facilities as such environments often contain very high levels of antibiotics contributing to the selection and/or induction of AR in environmental bacteria (through mutations or HGT) (Kristiansson et al., 2011, Milaković et al., 2019, Flach et al., 2015, González-Plaza et al., 2019, Bengtsson-Palme et al., 2019). These environments with both high abundance and diversity of AR genes were, thus, considered as 'hotspots' for resistance development, maintenance and/or transmission (Bengtsson-Palme et al., 2018). In order to recognize and evaluate the full risks associated with AR in the environment and its relation to human health risk scenarios, the role of such 'hotspots' as a point source and route of dissemination of resistant bacteria cannot be ignored.

Macrolides such as azithromycin (AZM) are one of the most successful and widely prescribed antibiotics in the world. In addition, they have been recently classified as the highest priority critically important antibiotics for human medicine (WHO, 2017). AZM and other macrolides have been largely used to treat infections caused by Gram-positive bacteria such as *Streptococus*, *Staphylococcus* or *Enterococcus*, and as a substitution for β-lactams in patients with penicillin allergies (Golkar et al., 2018). Besides, they do have good activity against various Gram-negative bacteria like *Campylobacter* spp., *Haemophilus influenzae* or *Neisseria gonorrhoeae*, and other organisms such as *Mycoplasma*, *Chlamydia*, *Bordetella* or *Moraxella* species (McKenna and Evans, 2001). These antibiotics inhibit bacterial protein synthesis through binding to the 23S rRNA in the large ribosomal subunit (50S) (Dinos, 2017; Fyfe et al., 2016). However, macrolide therapy is lately becoming ineffective due to increasing macrolide resistance among clinically relevant pathogens including group A streptococci and

invasive *S. pneumoniae* strains in Croatia as well as in Norway and Hungary (ECDC, 2019; Tambić Andrašević et al., 2019). Different macrolide resistance genes (MRGs) have been described, leading to resistance through various mechanisms such as ribosome modifications by rRNA methyltransferases (*erm* genes), macrolide efflux pumps (*mef* genes), ribosome protection by ABC-F type proteins and GTPase proteins (*msr* genes and *hfl*X) or macrolide-inactivation mediated by phosphotransferases (*mph* genes) or esterases (*ere* genes) (Dinos, 2017; Duval et al., 2018; Fyfe et al., 2016). Additionally, different plasmid-mediated MRGs have been identified, such as *erm*B, *erm*C, *mph*G, *msr*E, *mef*C and *mph*E (Blackwell et al., 2017; Bonnin et al., 2013; de Vries et al., 2012; McCarthy and Lindsay, 2012; Poirel et al., 2011; Sugimoto et al., 2017). Therefore, in order to preserve effectivenes of these valuable antibiotics, it is important to understand resistance mechanisms in both clinical pathogens and environmental bacteria exposed to high levels of antibiotics. However, to our best knowledge, no previous research has investigated what kind of bacteria are harboring the resistance determinants in heavily macrolide-polluted environment.

This study was carried out on the two sites along the Sava river, one directly at the discharge point of effluents from AZM-production industry and a second site upstream of discharge (reference site) (Bielen et al., 2017; Milaković et al., 2019). Results from our previous studies demonstrated high concentrations of macrolides, particularly AZM (up to 10 mg/kg), increased levels of MRGs (*mph*E, *mph*G, *msr*E, *mef*C and *erm*B) and MGEs (IncP-1 plasmids and class 1 integrons) as well as increased rate of plasmid-mediated transfer of MRGs in sediments from the discharge *vs* reference site (González-Plaza et al., 2018, 2019; Milaković et al., 2019). These sediments may thus act as a reservoir from which resistance genes could be transferred to pathogens or to the normal commensal bacteria that coexist within and on humans (Vikesland et al., 2017). The present study thus aimed to investigate the prevalence of MRGs in sediment isolates from the discharge and upstream site as well as in clinical isolates of relevant pathogens from Croatian nearby hospitals. We used both culture-dependent (plating and turbidity-based methods) and independent methods (16S rRNA gene sequencing, PCR of MRGs and sequencing) in order to link specific MRGs to their bacterial hosts in environmental settings, which would help to target appropriate control and mitigation strategies. Moreover, selected MRGs were compared among environmental and clinical isolates in order to make a potential link between the environment with high selection pressure from macrolides and MRGs in bacteria found in Croatian clinics.

MATERIALS AND METHODS

Antimicrobial agens and media

The antibiotics azithromycin (AZM), erythromicin (ERY), clarithromycin (CLA), tetracycline (TET), ampicillin (AMP) and ciprofloxacin (CIP) as well as the antimycotic cycloheximide (CHX) used in this study were purchased from Sigma-Aldrich (Taufkirchen, Germany). Culture media included Mueller-Hinton broth (MHB), purchased from Merck (Darmstadt, Germany), R2A broth, purchased from Lab M Ltd. (Heywood, UK), and Columbia agar, purchased from MAST Group Ltd. (Bootle, UK). Mueller-Hinton agar (MHA) and R2A agar plates were made by amending MHB or R2A broth with 1.5 % agar (BioLife Italiana). Defibrinated horse blood and yeast extract were obtained from Biognost (Zagreb, Croatia) and BioLife Italiana (Milano, Italy), respectively. Staph/Strep selective supplement (S/S) was purchased from MAST Group Ltd. (Bootle, UK).

Study site and sampling of river sediment

Sediment samples were collected from the Sava river, located in the north-west of Zagreb (Croatia) on February 2016. This river receives the wastewater effluents from the pharmaceutical industry producing antibiotic azithromycin and was previously shown to be heavily polluted with macrolide antibiotics, especially at the site of effluent discharge (Bielen et al., 2017; Milaković et al., 2019). Surface sediment samples (0-10 cm) were collected from this discharge site (DW0) and reference site located 7.5 km upstream of discharge (UP) as previously described (González-Plaza et al., 2018). Briefly, four sediment sub-samples were collected with a plastic core tube from each site, transported to the laboratory on ice and composited into one sediment sample per site. A subsample of that composite was removed for culturing of macrolide-resistant bacteria and processed within 6h. Another subsample was frozen at -80 °C for later DNA extraction.

Isolation of macrolide-resistant bacteria from river sediments

Macrolide-resistant bacterial strains were isolated from DW0 and UP sediment samples by the spread plate method. One g of each sediment sample (wet weight) was vortexed in 9 mL of physiological saline (0.85 % NaCl). The heavy particles were allowed to settle for 1 min and 1 mL of the supernatant was used to prepare the dilutions. Several 10-fold dilutions (0.1 mL) were plated on different culture media supplemented with AZM at a final concentration of 15

mg/L. This concentration was selected according to the Clinical and Laboratory Standards Institute (CLSI) quidelines (2015) where most of isolates categorized as macrolide-resistant exhibited MIC values ≥ 8. For isolation of diverse bacterial species from sediments, three different culture media were used: R2A agar supplemented with yeast extract (5 g/L) to select for heterotrophs and MHA with S/S as well as Columbia agar with S/S and defibrinated horse blood (5 %) to target Gram-positive cocci, particularly streptococci and staphylococci. All media were supplemented with CHX (100 mg/L) to inhibit fungal growth. All plates were incubacted aerobically at 28 °C for 5 to 7 days, except for Columbia agar plates, which were incubated at 35°C for 2 days. After incubation, morphologically different colonies were re-streaked on the same media to obtain pure cultures. The isolated strains were stored at -80 °C in MHB containing AZM (4 mg/L) and 20 % glycerol.

Clinical isolates

This study was conducted on a collection of streptococcal and staphylococcal clinical isolates obtained from the Croatian Reference Center for Antibiotic Resistance Surveillance. A total of 90 macrolide-resistant clinical isolates were collected in a period from April 2014 to August 2018 from various clinical specimens including throat, pharynx, nasopharynx, tonsils, ear canals, ulcer and skin wounds.

Identification of environmental and clinical isolates

Environmental and clinical AZM-resistant isolates were identified by 16S rRNA gene sequencing. Extractions of the total DNA of the isolates were performed using a proteinase K protocol (Chèneby et al., 1998) and the 16S rDNA was amplified by PCR using primers 27F and 1492R (Lane, 1991) with the following program: denaturation for 5 min at 98 °C followed by 35 cycles consisting of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min 30 s, final extension at 72 °C for 5 min. Purification of PCR products and their unidirectional sequencing using the 27F or 1492 primer was carried out by Macrogen DNA Sequencing Service (Amsterdam, Netherlands). The obtained sequences were edited using the Chromas Lite v2.6.6. (Technelysium, Australia) and compared against the sequences available in the National Center for Biotechnology Information (NCBI) database using the BLASTn program (Altschul et al., 1990). The 16S rRNA sequences were deposited in the NCBI Sequence Read Archives (SRA) under accession numbers MN923288-MN923515 (environmental isolates) and MN922958-MN923046 (clinical isolates). Taxonomic diversity of environmental isolates was assessed

using the Shannon-Wiener diversity index (H), calculated as described previously (Udiković-Kolić et al., 2010). $H = - \sum_{i=1}^{S} p_i * \ln p_i$ where „p“ is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N) and „s“ is the number of species.

Macrolide-resistance gene detection

All AZM-resistant isolates (environmental and clinical) were screened by end-point PCR for the presence of 11 relevant macrolide-resistance genes (MRGs) according to the methods described previously (Table 1). Target MRGs comprized of 4 ribosomal methylation genes (*erm*), 2 macrolide efflux genes (*mef*), 3 ribosomal protection genes (*msr*), 1 gene conferring resistance through enzymatic modification of macrolides (*mph*E) and 1 gene coding for GTPase binding proteins (*hfl*X). Each PCR reaction was carried out in a total volume of 25 µL containing 15 µL of EmeraldAmpMax PCR Master Mix (Takara Biotechnology, China), 0.4 µM of each primer (Table 1), 25 ng of DNA and 8 µL of DNA-free water. Negative and positive controls (bacteria carrying the MRG included in the study) were used to validate the results. The amplification conditions were as follows: 10 s at 94 °C, X cycles (Table 1) for 10 s at 98 °C, 30 s at gene-specific annealing temperature (Table 1), elongation at 72 °C for duration specific for each gene (Table 1), and final elongation at 72 °C for 7 min. Amplification of the expected fragment sizes were verified by gel electrophoresis on 1% (w/v) agarose gels (Sigma-Aldrich, Germany).

All positive *erm*B, *mef*A/E, *mph*E and *msr*D PCR products were purified and bi-directionally sequenced by Macrogen using the same primers that were used for PCR screening. In order to discriminate between *mef*A and *mef*E, sequence analysis was based on the presence of BamHI and DraI restriction sites as previously suggested (Grivea et al., 2012).The resulting sequences were edited as mentioned above and compared with reference sequences in the NCBI database using the online BLASTX search. Nucleotide sequences were aligned and compared using ClustalX software (http://www.clustal.org/clustal2/). Phylogenetic trees were inferred using Neighbor-Joining (NJ) analysis with the software MEGA-X (Tamura et al., 2013). Bootstrap values were calculated based on 1,000 replications. Phylogenetic trees were visualized with Interactive Tree of Life (ITOL) software (https://itol.embl.de/). The nucleotide sequences of *erm*B, *mef*A/E, *mph*E and *msr*D genes were deposited in the NCBI under accession numbers MT337696-MT337738, MT345159-MT345176, MT317310-MT317331, MT321146-MT321166 and MT330117.

Table 1. Primers and PCR conditions used for detection of macrolide-resistance genes.

Target gene	Amplicon size/bp	Primer sequence (5'–3')	Tm*/ °C	Elongation time	Number of cycles	Reference
***erm*A**	645	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	53	1 min	35	Mišić et al., 2017
***erm*B**	639	F: GAAAAGGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTAC	51	1 min	30	Gygax et al., 2006
***erm*C**	642	F: TCAAAACATAATATAGATAAA R: GCTAATATTGTTTAAATCGTCAAT	51	45 sec	35	Mišić et al., 2017
***erm*F**	466	F: CGGGTCAGCACTTTACTATTG R: GGACCTACCTCATAGACAAG	59	45 sec	35	Roberts et al., 1999
***mef*A/E**	1759	F: GCGTTTAAGATAAGCTGGCA R: CCTGCACCATTTGCTCCTAC	55	2 min	35	Grivea et al., 2012
***mef*C**	1156	F: GGCTGGACAGTTTGCTTC R: CATTAAGTTGCGAGTGCTAAAC	52	2 min	30	This study**
***msr*A**	940	F: GGCACAATAAGAGTGTTTAAAGG R: AAGTTATATCATGAATAGATTGTCCTGTT	56	1 min	35	Mišić et al., 2017
***msr*E**	1352	F: GCGAGAAACATACGCTTG R: GGTCAAGATAGTTTCCTGGTTC	53	2 min	30	This study**
***msr*D**	1202	F: TGCCTATATTCCCCAGTTG R: GTAATCGTTTATCGTGGGTG	57	1.5 min	35	Amezaga et al., 2002
***mph*E**	855	F: CACTTGCTGAAGCACACG R: CTCCCAACTGAGCTTTTGC	53	1 min	30	This study**
***hfl*X**	1191	F: CTGAAGACCGAAGAGCCATC R: CTTCGACCAGTGTACCTTCTTCC	58	2 min	30	This study**

* annealing temperature

** primers were constructed based on sequences of previously identified macrolide-resistance genes (González-Plaza et al., 2018) using DNASTAR Lasergene software package (v14.0) software

Antibiotic susceptibility testing

All MRG-positive isolates (environmental and clinical) were evaluated for resistance to six antibiotics from four classes: (i) macrolides: AZM (0.125-128 μg/mL), ERY (0.125-128 μg/mL), and CLA (0.125-128 μg/mL), (ii) tetracyclines: TET (2-128 μg/mL), (iii) β-lactams: AMP (0.125-128 μg/mL), and (iv) fluoroquinolones: CIP (0.25-16 μg/mL).

Resistance was estimated by determination of minimum inhibitory concentrations (MICs) using a 96-well broth dilution method according to the Clinical Laboratory Standards Institute (CLSI) Guidelines M100-S24 (CLSI 2020) and M45 (CLSI 2015). The wells contained 0.09 mL of MHB or MHB supplemented with 5% lysed horse blood (genera *Streptococcus*, *Arthrobacter*, *Microbacterium*, *Cellulosimicrobium*, *Aerococcus* and *Lactococcus*) and serially diluted target antibiotics. Bacterial culture suspensions grown to log phase were adjusted at a 0.5 McFarland turbidity and an aliquot of 0.01 mL of each bacterial suspension was inoculated into each well. Microplates were incubated for 18-24h at 35-37 °C in air (environmental isolates exept *Aerococcus* sp.) or in air with 5 % CO_2 (clinical isolates and *Aerococcus* sp.). The MIC was identified as the minimum concentration at which visible growth was inhibited (compared to a positive growth control). Susceptibility results were interpreted according to the CLSI breakpoints (CLSI 2015, 2020).

RESULTS AND DISCUSSION

Evidences show that some of the resistance genes that we see in clinical pathogens originate from non-pathogenic environmental bacteria (Peterson and Kaur, 2018). There are concerns that heavy environmental pollution with antibiotics from manufacturing sites may increase the recruitment of resistance genes to clinically relevant pathogens. However, the role of antibiotic pollution in this context is still not clear. We used a combination of standard culture and molecular methods (PCR and sequencing) to isolate and characterize AZM-resistant bacterial strains from macrolide-polluted and unpolluted river sediments as well as from Croatian hospitals in order to compare macrolide-resistance mechanisms between environmentally- and clinically-acquired isolates.

Diversity and phenotypic resistance of bacterial isolates from river sediments

Culture on three different media supplemented with AZM allowed us to isolate a total of 228 bacterial strains from the Sava river sediments, with 104 from the UP site and 124 from the DW0 site. Although standard culturing is known to underestimate the actual microbial diversity, it still remains important method for detailed characterization of individual bacterial strains allowing linkage of antibiotic resistance phenotypes to the genetic basis of these phenotypes. Analysis of 16S rRNA gene sequences and calculation of the Shannon-Wiener diversity (H) showed that isolates from DW0 site were more diverse (H=2.73) than isolates from the UP site (H=1.43). Higher diversity at DW0 site may be explained either by deposition of AZM-resistant bacteria via discharge of AZM-production effluents (Bielen et al., 2017) and/or by proliferation of AZM-resistant bacteria resident to sediment as a response to increased concentration of macrolides (up to 24 mg/kg) and nutrients at DW0 compared to UP (Milaković et al., 2019).

Actinobacteria were found to be most prevalent among isolates from both sites (UP, 57 %; DW0, 52 %) (Fig. 1A). However, there were considerable differences in the prevalence of isolates belonging to phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* between the two sites. The *Firmicutes* isolates were more frequently isolated from UP site than from DW0 (UP, 42 %; DW0, 20 %), which is in contrast with our previous findings obtained by Illumina-based 16S rRNA gene sequencing (Milaković et al., 2019). This difference in findings may be due to unoptimized culture conditions to grow and isolate *Firmicutes* members. In contrast, *Proteobacteria* isolates were more frequently isolated from DW0 site than from UP site (DW0,

22 %; UP, 1 %), while *Bacteroidetes* isolates were isolated only from the DW0 site (Fig. 1A). The higher proportion of *Proteobacteria* and *Bacteroidetes* at DW0 vs UP is consistent with our previous Illumina-based findings (Milaković et al., 2019), but also with several other studies conducted on antibiotic-polluted aquatic bacterial communities (Kristiansson et al., 2011; Li et al., 2010).

The most dominant genus at both DW0 and UP sites was *Microbacterium*, representing 42 % and 30 % of the total isolates from DW0 and UP, respectively (Table S1 Fig. 1B,) and displaying resistance to all three tested macrolides ($MIC_{AZI, ERY, CLA}$ > 128 μg/mL) (Table S2). This genus is known to be ubiquitous in aquatic environments including river sediments (Iyer et al., 2017; Pashang et al., 2019), but it has also recently emerged as an opportunistic human pathogen with acquired resistance to macrolides (Gneiding et al., 2008; Sharma et al., 2013). *Bacillus* and *Cellulosimicrobium* were the two other dominant genera among the isolated strains, with *Bacillus licheniformis* dominating among UP isolates (33.6 %) and *Cellulosimicrobium* sp. among DW0 isolates (7.3 %). All *Bacillus* isolates exhibited resistance to all three tested macrolides ($MIC_{AZI, ERY, CLA}$ > 128 μg/mL) (Table S2), as consistent with previous reports in *Bacillus* isolates from environmental samples (Barbosa et al., 2014; Song et al., 2019). The *Cellulosimicrobium* isolates from the DW0 site were resistant to macrolides (ERY, AZM, CLA) and intermediate resistant to fluoroqionolones (CIP) (Table S2). This genus has previoulsy been isolated from heavy metal-polluted wastewaters and exhibited resistance to both heavy metals and various antibiotics including macrolides and fluroquinolones (Bharagava and Mishra, 2018). Therefore, pollution of the DW0 sediments with heavy metals, especially Cu and Zn (Milaković et al., 2019), may favor proliferation of *Cellulosimicrobium* at DW0 site. Furthermore, other most frequently isolated strains from DW0 site included *Brevundimonas* (7.3 %), *Enterococcus* (5.6 %), *Streptococcus* (4.8 %) and *Chryseobacterium* (4.8 %) (Fig. 1B, Table S1). Members of *Enterococcus* and *Streptococcus* spp. are clinically relevant. *Enterococcus* isolates were mainly shown to be resistant to ≥ 3 antibiotic classes, including β-lactams (AMP) which could be problem for the treatment of enterococcal infections (Arias and Murray, 2012; Gagetti et al., 2019). *Streptococcus* isolates were resistant to both macrolides ($MIC_{AZI, ERY, CLA}$ > 128 μg/mL) and β-lactams (AMP) (Table S2). Given that macrolides and penicillins are the basis of first-line therapy for respiratory tract infections caused by streptococcci (Fernandes et al., 2013; Fyfe et al., 2016), infection caused by macrolide- and penicilin-resistant streptococci may lead to treatment failure.

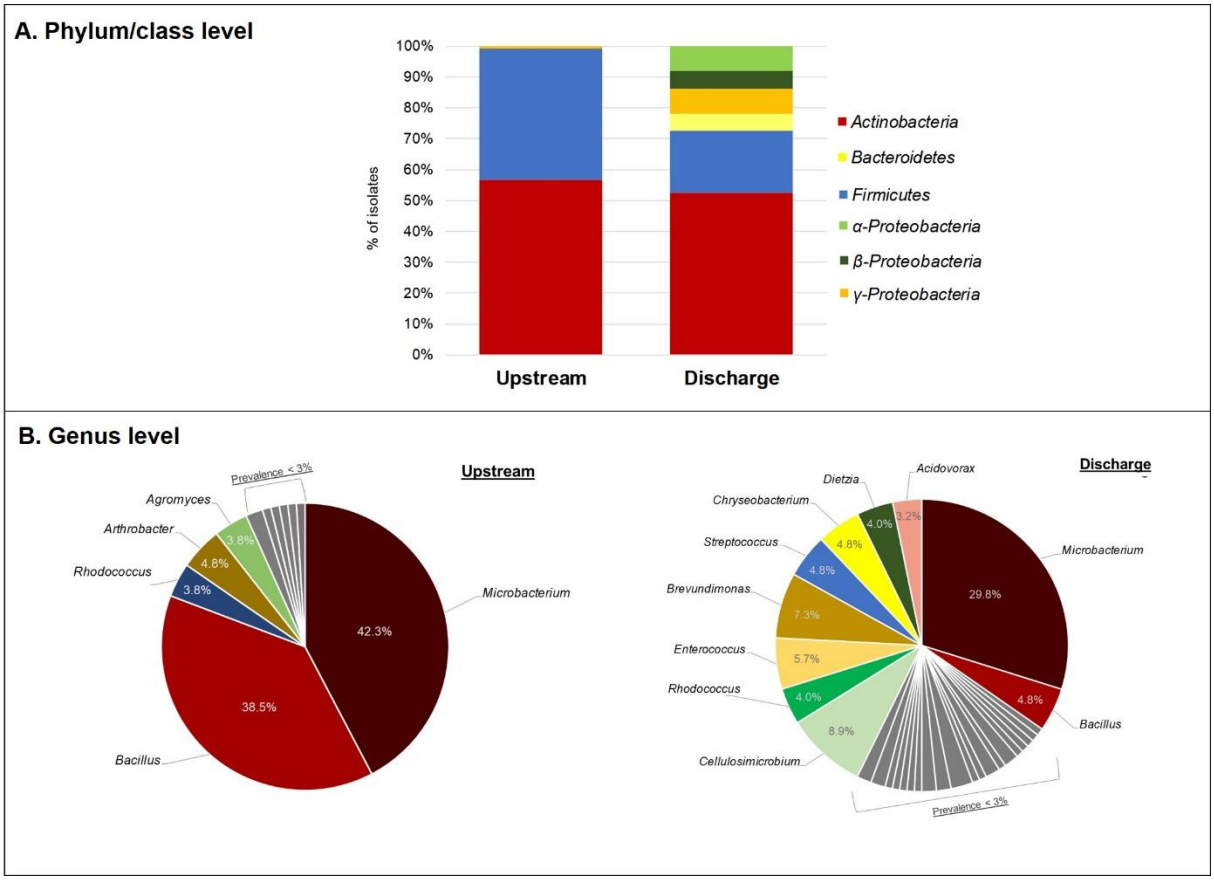

Figure 1. Percentage of isolates from sediments at two sites (upstream and discharge) at the (A) phylum/class and (B) genus levels. Taxa with prevalence below 3% in all samples were presented as a low prevalent genera.

Prevalence of macrolide resistance genes and mechanisms among environmental isolates

All environmental bacterial isolates were further analyzed for the presence of 11 MRGs that were previously identified in the studied area by functional or shotgun metagenomics (*erm*B, *erm*F, *msr*E, *msr*D, *mef*C, mphE and *hfl*X) (González-Plaza et al., 2018; Bengtsson-Palme et al 2019) or recognized as clinically relevant (*erm*A, *erm*C, *msr*A and *mef*A/E). Target MRGs were more prevalent among isolates from DW0 site (n=58, 46.8 %) than among those deriving from UP site (n=17, 16.3 %), suggesting that sediment at the discharge site is a reservoir for MRGs possibly originating from industrial sources. As shown in Fig. 2A, 7 out of 11 target MRGs were detected in upstream isolates (*erm*F, *msr*A, *msr*D, *msr*E, *mef*E, *mph*E and *hfl*X). This indicates the persistance of various MRGs in sediment bacteria even when macrolide concentration was very low (0.04 mg/kg) or absent (Milaković et al., 2019). The most prevalent MRGs among upstream isolates were *hfl*X (3.8 % of isolates), detected in *Bacillus* isolates only, and *erm*F (3.8 %), detected in *Bacillus*, *Microbacterium* and *Arthrobacter* isolates. All these isolates exhibited resistance to all three macrolides (Table S2). To our knowledge this is the first report of *hfl*X or *erm*F genes in these genera (http://faculty.washington.edu/marilynr/ermweb4.pdf), suggesting their potential role in the intrinsic macrolide resistance in these isolates from UP site. Although the mechanism conferred by HflX-like protein, which rescues stalled ribosomes by separating their sub-units, is relatively new, it is believed to be shared by numerous environmental bacteria, particularly among the members of the phylum *Firmicutes* (Duval et al., 2018). The remaining MRGs investigated (*erm*A, *erm*B, *erm*C and *mef*C) were not found in any isolate from UP site.

Approximately 50 % isolates from DW0 site were positive for et least one MRG, and 9 of 11 target MRGs were detectable among them (Fig. 2A). Genes *erm*A and *erm*C were not detected in any of the discharge isolates. This indicates that the AZM-resistant discharge isolates could also harbor other MRGs not investigated in this study or genes for non-specific, multi-drug efflux pumps responsible for macrolide resistance (Delmar et al., 2014; Sun et al., 2014). The most frequently detected gene was *erm*B (18.6 % of isolates), followed by *msr*E (13.7%), *mph*E (13.7%) and *erm*F (9.7%) (Fig. 2A). The *erm*B was found primarily in *Enterococcus* and *Streptococcus* species (Table S2), as consistent with previous frequent detection of this gene in enterococci and streptococci from environmental and clinical samples (Harimaya et al., 2007; Okitsu et al., 2005). The *erm*B is typically associated with conjugative transposons (Okitsu et al., 2005; Roberts and Mullany, 2011) and plasmids (Lim et al., 2006; Poirel et al., 2011; Teuber et al., 2003), which facilitates its dissemination across species.

Streptococcal species have been shown to exchange conjugative transposons via transformation in addition to conjugation (Chancey et al., 2015), and exposure to antibiotics stimulates transformation in these species (Prudhomme et al., 2006). All of this suggests that strong macrolide pollution as previously observed at DW0 site (Milaković et al., 2019) may contribute to the expansion of *erm*B-mediated macrolide resistance among environmental streptococci. This may be of clinical concern as macrolides are often used in the treatment of respiratory infections caused by streptococci. Besides *erm*B, *erm*F gene was found in various Gram-positive and Gram-negative genera at DW0 site (Table S2), some of which have not been previously reported as hosts of *erm*F gene, such as *Arthrobacter*, *Bacillus*, *Microbacterium*, *Lysinibacillus*, *Chryseobacterium*, *Trichococcus* and *Brevundimonas* (http://faculty.washington.edu/marilynr/ermweb4.pdf). The *erm*F was previously found to be particularly responsive to high levels of macrolides among activated sludge community (Bengtsson-Palme et al., 2019). When MRG data were sorted by gene mechanism, the three resistance mechanisms were present in considerably greater frequency in discharge isolates than the upstream isolates. These included the ribosomal methylation (*erm*B and *erm*F genes, total - 28.2 % of discharge isolates) and protection (*msr*E, *msr*D and *msr*A; total 16.9 %) as well as macrolide enzymatic inactivation (*mph*E, 13.7 %) (Fig. 2A). This hints at the possibility that these mechanisms may represent the most effective means of decreasing exposure of sediment bacteria at DW0 site to very high concentrations of macrolides (24 mg/kg) (Milaković et al., 2019). Efflux pumps (*mef*A/E, *mef*C) and a ribosome-associated GTPase (*hfl*X) were the least prevalent mechanisms, represented in 2.1 % and 3.2 % discharge isolates, respectively.

Further analysis showed that majority of MRG-positive isolates from both UP (88.2 % of isolates) and DW0 sites (72.4 %) harbored only one mechanism of resistance to macrolides (one MRG) (Fig. 2B). However, when comparing the percentage of isolates harboring two or more MRGs between the two sites, higher proportion of isolates with two different macrolide resistance mechanisms was detected at DW0 site (24.1 % vs 11.8 %). The most frequently found dual mechanism in these isolates involved a ribosomal protection gene (*msr*E) and a macrolide inactivation gene (*mph*E). This gene cluster was detected in various Gram-negative proteobacterial genera (*Citrobacter*, *Acinetobacter*, *Comamonas*, *Rhodobacter* and *Stenotrophomonas*) (Table S2), likely originated from effluent-associated bacteria as demonstrated by our recent study (González-Plaza et al., 2018). However, we also detected *mph*E-*msr*E genes in *Acinetobacter* isolate from UP site (Table S2), indicating that it can be also present in resident sediment bacteria. Although macrolide resistance in above mentioned

Gram-negative species is not clinically important, MRGs might be mobilized under macrolide selective pressure and horizontally transferred to other bacteria, including relevant pathogens inside or outside the environment. This mobility is further supported by the fact that *mph*E-*msr*E is typically located on conjugative plasmids as observed in our recent study (unpublished data) and other studies (Blackwell and Hall, 2017; Kadlec et al., 2011; Zhang et al., 2013). Interestingly, isolates with the simultaneous presence of three or four macrolide resistance mechanisms were detected only at DW0 site (3.4 %) but not at UP site (Fig. 2B). Triple mechanisms conferred by *erm*F-*msr*E-*mph*E genes were detected in *Citrobacter freundii*, whereas quadruple mechanisms encoded by *mef*A-*msr*E-*mph*E-*hfl*X genes were found in *Acidovorax* sp. (Fig. 2B, Table S2). These observations suggest the importance of acquiring multiple macrolide-resistance mechanisms in the adaptation of bacteria to environmental conditions in which the environment is full of antibiotics.

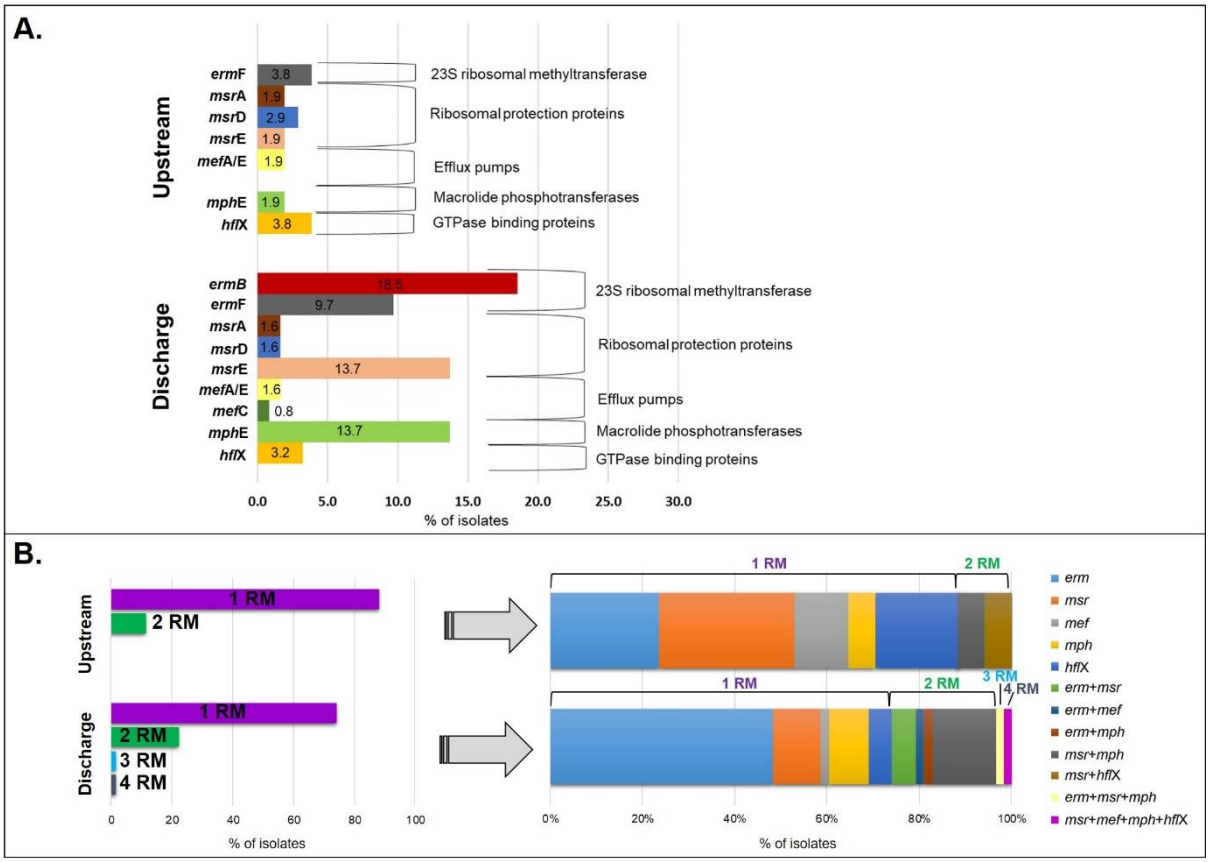

Figure 2. Distribution of single macrolide-resistance genes (A) and macrolide-resistance mechanisms (B) in environmental isolates from sediments at upstream and at effluent discharge site. Graph A shows % of isolates with single macrolide resistance gene in relation to the total number of isolates from a particular site, while graph B shows % of isolates with one or more

390 resistance mechanisms (RM) in relation to the number of isolates harbouring at least one
391 macrolide-resistance gene.
392

Diversity of clinical isolates and distribution of macrolide-resistance genes

Sequencing of 16S rRNA genes of 90 clinical isolates analyzed in the present study revealed that majority of them (84 isolates, 93.3 %) belonged to *Streptococcus* genus, with the most abundant species *Streptococcus pyogenes* (56; 62.2 %) and *Streptococcus dysgalactiae* (17; 18.9 %) (Table 2). Both these species are an important pathogens that cause throat, skin and soft tissue infections, but also life-threating infections such as sepsis and toxic shock syndrome (Barnett et al., 2019; Laabei and Ermert, 2018). These streptococcal isolates were mostly resistant (MIC > 1 μg/mL) to all three macrolides (AZM, ERY, CLA) and tetracyclines (TET), whereas only one *S. pyogenes* isolate showed additional intermediate resistance to fluoroqinolones (CIP) (Table 2). A smaller proportion of clinical isolates (6 isolates, 6.7 %) belonged to *Staphylococcus* genus and included *S. aureus*, *S. epidermidis* and *S. warneri* (Table 2). These species are known to cause a wide range of human infections, particularly skin and soft tissue infections (Legius et al., 2012; Otto, 2009; Tong et al., 2015). The isolates of *Staphylococcus* genus were mostly resistant to ERY and AMP, while only *S. epidermidis* showed additional resistance to TET (Table 2).

Table 2. Phenotypic and genotypic characteristics of the studied clinical isolates.

Genus	Species (n)	N	MRGs	Antibiotic resistance profile
Streptococcus	*S. pyogenes* (56)	1	*msr*D	ERY; AZM; CLA; TET
		6	ermB	
		1	*erm*B+*erm*C	
		2	*mef*C	
		2	*mef*A+*msr*D	
		1	*mef*E+*msr*D	
		1	*erm*B	ERY; AZM; CLA; TET; CIP
		5	*erm*B	ERY; AZM; CLA
	S. dysgalactiae (17)	1	*mph*E	ERY; AZM; CLA; TET
		1	*erm*B+*msr*D	
		1	*mef*C+*mph*E	
		1	*mef*E+*msr*D	
		1	*msr*D	ERY; AZM; CLA
		4	*mef*E+*msr*D	
		1	*mph*E	
		1	*erm*B+*mph*E	
		1	*erm*B+*msr*A	
		1	*mef*C	
	S. salivarius (5)	2	*mef*E+*msr*D	ERY; AZM; CLA; AMP
		2	*mef*E+*erm*B+*msr*D	
	S. anginosus (2)	1	*mef*C	ERY; AZM; CLA; AMP
		1	*mef*E+*msr*D	

	S. pneumoniae (2)	1	*mef*E+*erm*B+*msr*D	ERY; AZM; CLA; TET; CIP
		1	*erm*B+*msr*D+*mph*E	ERY; AZM; CLA; TET
	S. agalactiae (2)	1	*erm*B	ERY; AZM; CLA; TET; CIP
		1	*erm*F	ERY; AZM; CLA
Staphylococcus	*S. aureus* (2)	1	*mef*E	ERY; AMP
	S. epidermidis (2)	1	*erm*C	ERY; AZM; CLA; TET; AMP
		1	*mef*C+*msr*A	ERY; AZM; CLA; AMP
	S. warneri (2)	0	ND	NA

n - the total number of isolates belonging to the same species. N – number of MRG-positive isolates; MRG – macrolide resistance genes. ERY – erythromycin; AZM – azithromycin; CLA – clarithromycin; TET – tetracycline; AMP – ampicillin; CIP – ciprofloxacin. ND – not detected; NA – not analyzed.

PCR screening of 90 clinical isolates for 11 MRG targets showed that 51 of them (57%) carried at least one of the 8 targets (Fig. 3A). The most frequently detected gene was *erm*B (24% of isolates), followed by *msr*D (21%), *mef*A/E (16,7%) and *mef*C (12,2%). Genes *erm*A, *msr*E and *hfl*X were not detected in any of the clinical isolates. Genes *erm*B and *mef*C were the most frequently detected in *Streptococcus pyogenes,* as consistent with previous reports. On the other hand, *msr*D and *mef*E were the most commonly detected in *Streptococcus dysgalacticae* (Table 2). The other MRGs (*mph*E, *erm*C, *msr*A and *erm*F) were less prevalent and found in a total of 10 isolates (*Streptococcus dysgalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Staphylococcus epidermidis*,). When the MRG data were sorted by gene mechanism of action, the efflux genes (*mef*A/E and *mef*C genes; total 28.9 %) were the most prevalent, in stark contrast to environmental isolates from the discharge site where these *mef* genes were poorly represented (2.1 %) (Figs 2 and 3). The other two dominant mechanisms of action among assayed clinical isolates were the ribosomal methylation (*erm*B, *erm*C and *erm*F; total 27.7 %) and protection genes (*msr*D, and *msr*A; total 23.3 %), similar to observations among discharge isolates. Nevertheless, notable difference between clinical and discharge isolates was in gene variants responsible for ribosomal protection – clinical isolates contained predominantly *msr*D, while environmental isolates carried mainly *msr*E. (Figs. 2A and 3A).

Besides single MRG per isolate, the co-existence of two or three MRGs was detected in 17/51 (33%) and 4/51 (8%) MRG-positive clinical isolates, respectively (Fig. 3B). The most frequent detected combination of MRGs among these isolates was *msr*D-*mef*E, a dual efflux pump responsible for high-level macrolide resistance which has been reported previously for streptococcal isolates (Ambrose et al., 2005; Fyfe et al., 2016; Schroeder et al., 2019; Wierzbowski et al., 2005). This dual efflux pump has been often associated with Mega, Macrolide Efflux Genetic Assembly and derivative transposons Tn*2009* and Tn*2010* (Fyfe et

al., 2016). Combinations of three MRGs including *erm*B-*msr*D-*mef*E was detected in *Streptococcus pneumoniae* and *Streptococcus salivarius*, while *erm*B-*msr*D-*mph*E was detected only in *S. pneumoniae* (Table S3). The *erm*B-*msr*D-*mef*E cluster has been detected in an increasing number of *S. pneumoniae* isolates worldwide (Bowers et al., 2012; Farrell et al., 2008; Schroeder and Stephens, 2016), probably due to association with the transposon Tn*2010* containing both *erm*B gene and the Mega element (Del Grosso et al., 2006; Schroeder and Stephens, 2016).

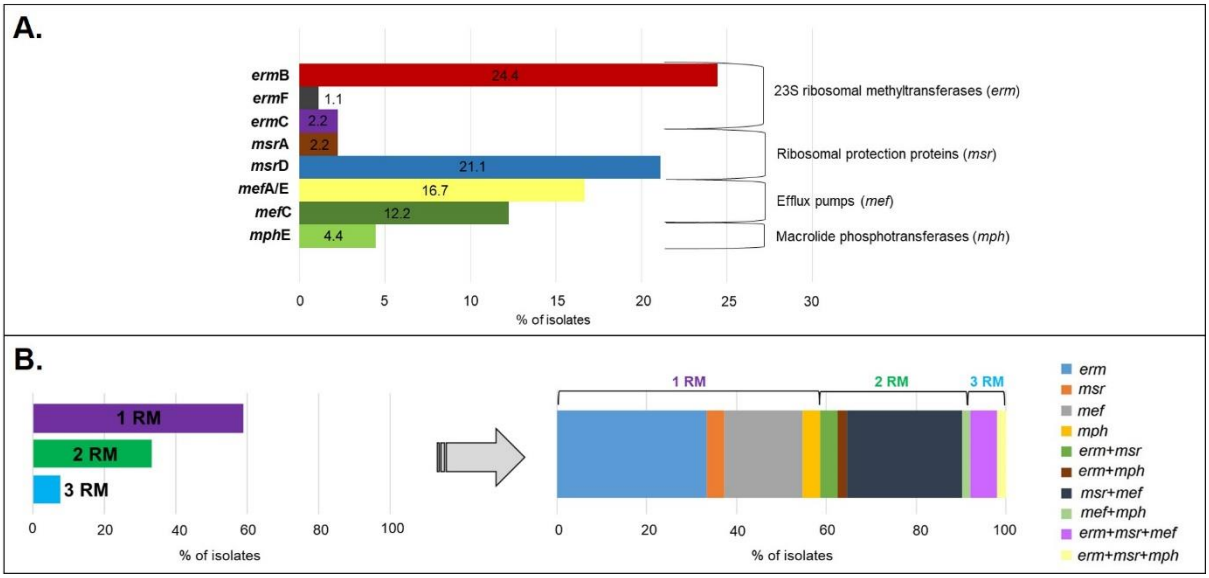

Figure 3. Percentage of single macrolide-resistance genes (A) and macrolide-resistance mechanisms (B) identified in clinical isolates. Graph A shows % of isolates with single resistance gene in relation to the total number of isolates tested, while graph B shows % of isolates with one or more resistance mechanisms (RM) in relation to the number of isolates harbouring at least one macrolide-resistance gene..

Comparison of selected MRGs from clinical and environmental isolates

Four MRGs representing the most dominant mechanisms of action were selected for further sequence analysis: (i) *erm*B, the most commonly detected gene in both environmental and clinical isolates, (ii) *mef*A/E and *msr*D genes, more frequently found in clinical isolates, and (iii) *mph*E, more frequently identified in environmental isolates. The nucleotide sequences of all four analyzed MRGs were highly similar (≥ 98 %) to the nucleotide sequences deposited in the NCBI database.

Phylogenetic analysis of the nucleotiode sequences of the *erm*B gene found in both sediment isolates from the discharge site and clinical isolates revealed two different genotypes separated in two distinct clusters (1 and 2). Although there is high *erm*B sequence homology (> 98 %) between these two sub-clusters, variability in the sequence may indicate the origin of *erm* genes found in isolated strains. The genotype associated with cluster 1 has been mostly found in environmental isolates from DW0 site (20/25) (the genera *Lysinibacillus*, *Streptococcus*, *Aerococcus*, *Microbacterium*, *Bacillus*, *Enterococcus*, *Cellulosimicrobium*, *Lactococcus* and *Trichococcus*) and in 5 clinical streptococci characterized in this study (Fig. 4). In contrast, the genotype associated with cluster 2 has been mainly identified in clinical streptococcal isolates assayed in this study (15/18), but also in three discharge isolates, including the genera *Brevundimonas* and *Enterococcus*. Collectively, these observations suggest the possible transfer of the *erm*B gene between bacteria of clinical and environmental origin.

Phylogenetic analyses of *mef*A/E and *msr*D genes (Figs. S1 and S2), which were mostly associated with clinical streptococcal isolates, showed no clear grouping between clinical and environmental isolates from either UP or DW0 site due to high overall homology between sequenced genes. Interestingly, phylogenetic analysis of the *mef*A/E gene revealed two sub-groups (Fig. S1), despite previously reported high nucleotide sequence homology of *mef*A and *mef*E genes (Roberts et al., 1999). The *mef*A sub-group consisted of only 2 clinical isolates of *S. pyogenes* and 1 discharge isolate of *Acidovorax* sp., suggesting that these three *mefA* efflux pumps are highly similar at their nucleotide sequence level. On the other hand, the *mef*E sub-group consisted mostly of clinical streptococcal isolates and 2 *Microbacterium* isolates from both UP and DW0 site. This indicates high *mef*E homology between clinical streptococci and environmental *Microbacterium* isolates, regardless of macrolide pollution. Similar findings were observed for the *msr*D gene (Fig. S2), whose nucleotide sequences in studied clinical

485 streptococci were highly homologous to those in evironmental isolates of *Microbacterium* and
486 *Bacillus* from UP site as well as *Microbacterium* and *Chryeobacterium* from DW site.

487 Regarding the phylogenetic analysis of *mph*E gene, which was mostly found in Gram-
488 negative environmental isolates, we found high nucleotide sequence similarity between *mph*E
489 in clinical streptococcal isolates (*S. pneumoniae* and *S. dysgalactiae*) and environmental
490 isolates from either UP (2 isolates) or DW0 site (14 isolates) (Fig. S3). These observations
491 suggest that macrolide phosphotransferase MphE found in pathogenic streptococci from
492 clinical samples are closely related to those in environmental bacteria, irrespective of whether
493 sediment samples were collected from macrolide-polluted or unpolluted sediment.
494 anthropogenic influences. However, despite the high similarity between analyzed genes from
495 clinical pathogens and environmental isolates, the potential transfer mechanisms of target
496 MRGs between their genomes remains unknown and should be further explored.
497

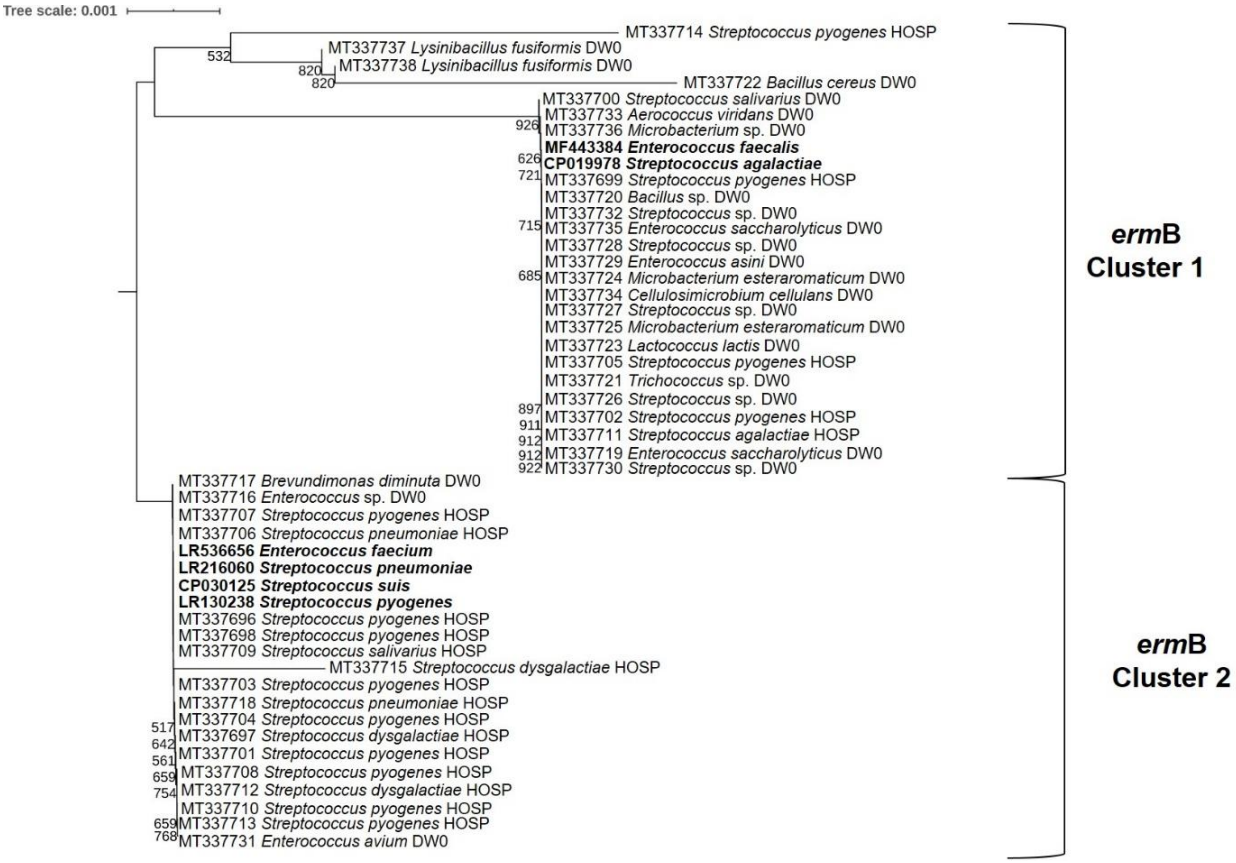

Figure 4. Neighbor-joining phylogenetic tree generated by analysing near complete *erm*B gene sequences of environmental isolates from the effluent discharge site (DW0) as well as clinical isolates (HOSP) and strains of its closest relatives retrieved from NCBI database (in bold). Accession numbers are given in front of isolate name. Bootstrap values (>500 only) calculated from 1000 re-samplings are shown. Scale bar = 0.001 changes/site.

CONCLUSIONS

This study demonstrates the importance of river sediment exposed to very high levels of macrolides discharged from azithromycin-producing factory as a reservoir of diverse macrolide-resistant bacteria, including opportunistic pathogens. Besides single MRGs, isolates from the effluent discharge site demonstrated a greater incidence of the co-occurence of multiple MRGs compared to isolates from the upstream site, suggesting the importance of acquiring multiple macrolide resistance mechanisms in the adaptation of bacteria to high macrolide levels. Discharge isolates also showed a greater incidence of the ribosomal methylation and protection mechanisms compared to upstream ones, suggesting that these mechanisms have an important role in defence of bacteria against high concentration of macrolides. Although the same mechanisms were among the most frequently detected among clinical isolates, a different variants of these genes were primarily found between environmental and clinical sources, with the exception of the *erm*B gene. The fact that this gene was the dominant resistance gene in both the discharge and clinical streptococcal isolates, together with high *erm*B nucleotide sequence homology between these isolates suggested a possible exchange between bacteria of clinical and environmental origin. However, further studies, focusing on the mobility potential of identified environmental genes and clonal relations between environmental and clinical isolates, are needed to better elucidate the impact of environmental pollution with macrolides on the promotion of macrolide resistance in clinically relevant pathogens in Croatia.

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RASPRAVA

4. RASPRAVA

Rastuća pojava sve većeg broja bakterija otpornih na antibiotike predstavlja jedan od najvećih javnozdravstvenih problema na globalnoj razini, a potaknuta je prekomjernom potrošnjom antibiotika u ljudi i životinja te njihovim kontinuiranim ispuštanjem u okoliš. Otpadne vode farmaceutske industrije mogu predstavljati značajne točkaste izvore antibiotika te stoga pridonose onečišćenju recipijentnog vodenog okoliša ovim dragocjenim lijekovima (Fick i sur., 2009.; Kristiansson i sur., 2011.; Larsson, 2014a.; Šimatović i Udiković-Kolić, 2019.). Kako se radi o biološki aktivnim spojevima, prisutnost antibiotika u povišenim koncentracijama u okolišu može izazvati akutne toksične učinke na vodene organizme, što za posljedicu ima narušavanje osjetljive ravnoteže ekosustava (Carlsson i sur., 2009.; Larsson i sur., 2007.). Osim toga, izloženost povišenim koncentracijama antibiotika može dovesti i do dugotrajnih učinaka poput stjecanja otpornosti na antibiotike među okolišnim bakterijama, čime se indirektno ugrožava zdravlje ljudi i životinja (Bengtsson-Palme i sur., 2014.; Flach i sur., 2015.; Kristiansson i sur., 2011.; Li i sur., 2009., 2010.; Rutgersson i sur., 2014.; Sidrach-Cardona i sur., 2014.). Do danas se relativno malo zna o utjecaju otpadnih voda farmaceutskih industrija na stupanj onečišćenja okoliša i stjecanje otpornosti na antibiotike, a dostupne spoznaje potječu uglavnom iz azijskih država poput Indije i Kine, dok u ostalim dijelovima svijeta, uključujući i Hrvatsku, ovo važno pitanje ostaje nerazjašnjeno. Iz tog je razloga u ovom radu obuhvaćena sjeverozapadna Hrvatska, i to dva studijska područja u okolici grada Zagreba gdje su smješteni proizvodni pogoni farmaceutskih industrija. Jedna od tih industrija (Industrija 1) ima dugu tradiciju u proizvodnji makrolidnog antibiotika azitromicina, dok je druga industrija (Industrija 2) uglavnom uključena u formulaciju veterinarskih lijekova koji kao aktivne tvari sadrže antibiotike iz različitih skupina. Glavni je cilj ovog rada bio razjasniti učinke ispusta tih industrijskih otpadnih voda na taksonomski sastav i rezistom prirodnih bakterijskih zajednica primjenom naprednih analitičkih metoda u kombinaciji sa standardnim mikrobiološkim metodama te inovativnim metagenomskim pristupima, što će biti iscrpno izneseno dalje u raspravi.

4.1. Utjecaj industrijskih ispusta na onečišćenje recipijentnog vodenog okoliša

Prvi dio istraživanja obuhvaća fizikalnu, kemijsku i mikrobiološku analizu industrijskih otpadnih voda, a potom i procjenu onečišćenja recipijentnih prirodnih voda, i to rijeke Save u koju se ulijevaju otpadne vode Industrije 1 te potoka Kalinovica u koji se ispuštaju otpadne vode Industrije 2. Rezultati ovog dijela istraživanja objavljeni su pod rednim brojevima 1-5 u popisu znanstvenih radova (str. 40-92).

4.1.1. Procjena kakvoće industrijskih otpadnih voda

Industrijske otpadne vode prikupljene su tijekom četiri sezone 2016. godine: zime (siječanj/veljača), proljeća (travanj/svibanj), ljeta (srpanj/kolovoz) i jeseni (listopad/studeni). Kemijska analiza otpadne vode Industrije 1 provedena je mjerenjem koncentracija osnovnih sastojaka koji bi mogli potjecati iz proizvodnog pogona te industrije, eritromicina kao ishodišne tvari u sintezi (ERY), N-desmetil azitromicina (N-DMA) kao jednog od međuprodukata sinteze te samog konačnog proizvoda, azitromicina (AZI). Sva tri spomenuta makrolida detektirana su u razmjerno visokim koncentracijama reda veličine mg L^{-1}, posebice tijekom zime (ukupno do 6,5 mg L^{-1}) i proljeća (ukupno do 10,5 mg L^{-1}), što je vjerojatno posljedica šaržne proizvodnje azitromicina tijekom tih zimskih i proljetnih mjeseci. Nažalost, propisi koji reguliraju koncentracije antibiotika u otpadnim vodama ne postoje pa se stoga ne može načiniti na propisima utemeljena procjena kakvoće. Međutim, činjenica da su te koncentracije bile čak do 3 reda veličine više od koncentracija uobičajenih za bolničke i komunalne otpadne vode (< 10 μg L^{-1}) ipak upućuje na značajnu razinu onečišćenja industrijskih otpadnih voda makrolidima (Michael i sur., 2013.; Verlicchi i sur., 2012.; Zhou i sur., 2019.). Ipak, zanimljivo je primijetiti da su maksimalne koncentracije makrolida, zabilježene tijekom zime i proljeća, bile općenito niže od koncentracija antibiotika u otpadnim vodama farmaceutskih industrija u azijskim zemljama (do 44 mg L^{-1}) (Larsson i sur., 2007.; Li i sur., 2008a, b.; Sim i sur., 2011.). Nadalje, analiza fizikalno-kemijskih pokazatelja propisanih Pravilnikom o graničnim vrijednostima emisija otpadnih voda NN 80/13 (43/14, 27/15, 3/16) pokazala je da su vrijednosti organskih pokazatelja poput KPK, BPK_5 i TOC te anorganskih pokazatelja poput ukupnog fosfora i nitrita često premašivale granične vrijednosti dane u pravilniku, što upućuje na nezadovoljavajuću kakvoću tih otpadnih voda za ispust u okoliš. Ti podaci također ukazuju na neučinkovito pročišćavanje otpadnih voda iz pogona za proizvodnju azitromicina u membranskom biološkom reaktoru u krugu te tvornice. Važno je napomenuti da, iako maksimalno dopuštene koncentracije makrolidnih antibiotika nisu definirane zakonskim

propisima, azitromicin i eritromicin su nedavno uvršteni u listu za praćenje, tzv. „*watch listu*“ u okviru EU Okvirne direktive o vodama zbog svojih karakteristika poput toksičnosti, bioakumulativnosti i postojanosti u okolišu (Direktiva 2015/495/EU od 20. ožujka 2015.) (Barbosa i sur., 2016.).

Kemijska analiza otpadnih voda Industrije 2 provedena je mjerenjem koncentracija antibiotika karakterističnih za tu industriju, uključujući antibiotike iz skupina sulfonamida (sulfadiazin, SDZ; sulfametazin, SMZ), 2,4-diaminopirimidina (trimetoprim, TMP), fluorokinolona (enrofloksacin, ENR) i tetraciklina (oksitetraciklin, OTC). Koncentracije tih pojedinačnih antibiotika općenito su bile znatno niže od mg L^{-1} koncentracija makrolida u otpadnim vodama Industrije 1 i kretale su se u rasponu od nekoliko μg L^{-1} do maksimalno 231 μg L^{-1}. Ipak, potrebno je napomenuti da su te koncentracije i dalje često bile i za čitav red veličine više od uobičajenih koncentracija antibiotika u komunalnim otpadnim vodama (Michael i sur., 2013.; Zhou i sur., 2019.). Nadalje je zapaženo da u otpadnim vodama ove industrije, slično kao u otpadnim vodama Industrije 1, postoje značajne sezonske varijacije u koncentracijama antibiotika, što je naročito razvidno u slučaju sulfametazina čije su koncentracije varirale u rasponu od 7 μg L^{-1} do 231 μg L^{-1}. Takve oscilacije u koncentracijama antibiotika mogu biti posljedica višestrukih razloga uključujući razlike u ciklusima proizvodnje antibiotika te ispiranju reakcijskih/formulacijskih spremnika (Larsson, 2014a.; Pruden i sur., 2013.). Analiza fizikalno-kemijskih pokazatelja propisanih Pravilnikom NN 80/13 (43/14, 27/15, 3/16) nadalje je pokazala da su otpadne vode Industrije 2 uglavnom bile zadovoljavajuće kakvoće za ispust u okoliš, budući da su vrijednosti ispitivanih pokazatelja bile ispod propisanih dopuštenih maksimuma.

Najveća zabrinutost vezana uz ispuštanje antibiotika iz proizvodnih pogona je razvoj bakterija otpornih na te antibiotike, problem koji nije ograničen na zemljopisno područje ili državu u kojoj nastaje nego predstavlja potencijalnu prijetnju zdravlju ljudi širom svijeta. Neki su autori analizirajući vodeni okoliš izložen onečišćenju antibioticima iz farmaceutske proizvodnje uočili obogaćivanje bakterija otpornih na antibiotike i njihovih gena za otpornost (Flach i sur., 2015.; Li i sur., 2009., 2010., 2011.; Lübbert i sur., 2017.; Rutgersson i sur., 2015.; Sidrach-Cardona i sur., 2014.). Pored toga, istraživanja su pokazala da čak i niske, subinhibitorne koncentracije antibiotika, slične onima nađenim u različitim vodenim sustavima, mogu promicati otpornost na antibiotike (Andersson i Hughes, 2014.; Berendonk i sur., 2015.; Gullberg i sur., 2011.). Međutim, iako ne postoje ni domaći ni inozemni zakonski propisi koji bi definirali maksimalno dopuštene koncentracije antibiotika u okolišu, koncentracije

azitromicina izmjerene u otpadnim vodama Industrije 1 bile su čak do 4 reda veličine više od njegove PNEC vrijednosti (250 ng L^{-1}), pa je za očekivati da će ispust tih otpadnih voda potaknuti razvoj bakterijske otpornosti na makrolide u rijeci Savi. Što se tiče otpadnih voda Industrije 2, koncentracije pojedinačnih antibiotika, iako znatno niže u usporedbi sa koncentracijama u otpadnim vodama Industrije 1, također su premašivale odgovarajuće PNEC vrijednosti, ukazujući na potencijalni rizik za poticanje razvoja bakterijske otpornosti (Bengtsson-Palme i Larsson, 2016.).

Kemijskom analizom je nadalje utvrđeno da otpadne vode obiju industrija, pored antibiotika, sadrže i metale (As, Cd, Cr, Cu, Pb, Ni i Zn); međutim, njihove su koncentracije tijekom obje sezone uglavnom bile niže od maksimalno dopuštenih koncentracija prema važećem Pravilniku NN 80/13 (43/14, 27/15, 3/16). Ipak, valja naglasiti da su izmjerene koncentracije tih metala bile čak do 85 puta više od graničnih koncentracija iznad kojih se može očekivati ko-selekcija otpornosti na metale i antibiotike (Seiler i Berendonk, 2012.). Ti podaci ukazuju na doprinos metala selekciji bakterija otpornih na antibiotike, barem u otpadnim vodama. Među analiziranim metalima posebno se ističu Cu i Zn koji su imali najvišu koncentraciju u otpadnim vodama obiju industrija u odnosu na druge metale te je njihova povezanost sa poticanjem nastanka bakterija otpornih na antibiotike poznata od ranije (Becerra-Castro i sur., 2015.; Poole, 2017.).

Mikrobiološka analiza otpadnih voda iz obje istraživane industrije provedena je nacjepljivanjem uzoraka otpadnih voda na selektivne krute hranjive podloge sa antibioticima, azitromicinom (Industrija 1) te sulfametazinom ili oksitetraciklinom (Industrija 2). Dobiveni rezultati ukazali su na visok udio bakterija otpornih na azitromicin (do 83 %) u ukupnoj kultivabilnoj bakterijskoj populaciji otpadne vode Industrije 1, napose tijekom zime i proljeća kada su u tim uzorcima bile izmjerene i visoke koncentracije azitromicina. Ti podaci ukazuju na moguće namnožavanje bakterija u otpadnoj vodi kao odgovor na visoki selekcijski pritisak azitromicina. Slično tome, u otpadnoj vodi Industrije 2 uočen je visok udio bakterija otpornih na sulfametazin (do 91 %) i oksitetraciklin (do 50 %). Pritom je zamijećena pozitivna korelacija između udjela bakterija otpornih na oksitetraciklin i koncentracije oksitetraciklina, što upućuje na zaključak da oksitetraciklin vjerojatno potiče selekciju otpornih bakterija u otpadnoj vodi.

Sažeto rečeno, iz dosadašnje rasprave proizlazi da se ispustom otpadnih voda iz lokalnih farmaceutskih industrija unose ne samo toksične i selektivne tvari poput antibiotika i metala

nego i bakterije otporne na antibiotike u recipijentne prirodne vode. Takve otpadne vode, stoga, predstavljaju potencijalnu opasnost za zdravlje okoliša i ljudi.

4.1.2. Kemijsko i mikrobiološko onečišćenje vodenog okoliša

Prema dostupnoj literaturi ispusti otpadnih voda farmaceutskih industrija dovode do značajnog onečišćenja recipijentnih prirodnih voda antibioticima i bakterijama otpornim na te antibiotike (Fick i sur., 2009.; Flach i sur., 2015.; Kristiansson i sur., 2011.; Li i sur., 2009., 2010.; Lübbert i sur., 2017.; Sidrach-Cardona i sur., 2014.). Budući da industrijske otpadne vode istraživane u ovom radu karakterizira razmjerno visoka razina kemijskog i mikrobiološkog onečišćenja, može se očekivati onečišćenje rijeke Save odnosno potoka Kalinovica u koje se te otpadne vode ulijevaju. Stoga je cilj ovog dijela istraživanja bio istražiti u kojoj je mjeri došlo do onečišćenja spomenutih prirodnih voda.

Kemijskom analizom površinske vode rijeke Save utvrđene su povišene koncentracije ciljanih makrolida (AZI, ERY i N-DMA) na dvije lokacije 700 m (DW700) i 4,5 km nizvodno od ispusta (DW4500) u odnosu na uzvodnu lokaciju. Iako su te koncentracije varirale tijekom sezona, najviše koncentracije izmjerene su tijekom zime (ukupno oko 20 $\mu g\ L^{-1}$) i proljeća (ukupno do 30 $\mu g\ L^{-1}$) na nizvodnim lokacijama kada su i industrijske otpadne vode bile najviše opterećene makrolidima. Nadalje, koncentracije makrolida u rijeci Savi uglavnom su se smanjivale udaljavanjem od lokacije ispusta, vjerojatno uslijed razrjeđenja riječnom vodom te prirodnih eliminacijskih procesa u samoj rijeci. Međutim, povremeno su više koncentracije makrolida izmjerene na udaljenijoj lokaciji DW4500 u odnosu na lokaciju bliže ispustu (DW700), što upućuje na to da industrijske otpadne vode nisu jedini točkasti izvor onečišćenja Save tim spojevima. Ta činjenica ne iznenađuje ako se uzme u obzir podatak da se između navedenih nizvodnih lokacija, neposredno ispod lokacije DW700, nalazi utok rijeke Krapine u Savu. Naime, rijeka Krapina je recipijent ispusta iz uređaja za pročišćavanje otpadnih voda grada Zaprešića na kojem se dijelom obrađuju i otpadne vode Industrije 1, tako da postoji mogućnost da utok rijeke Krapine manjim dijelom pridonosi onečišćenju rijeke Save ovim antibioticima. Važno je također napomenuti da je uzvodna lokacija UP3500 (3,5 km uzvodno od ispusta otpadnih voda Industrije 1) smještena netom poslije utoka nekadašnjeg potoka Gorjak u Savu. Taj je potok, koji je sada potpuno presušen, služio kao dugogodišnji recipijent nepročišćenih otpadnih voda (sanitarnih i tehnoloških) tvrtki PLIVA i KVASAC sve do 2007. godine, pa su prijašnje analize pokazale da je sediment tog potoka jako opterećen nizom organskih spojeva, posebice makrolidima, i to azitromicinom (Izvještaj, 2008.). Prema tome,

koncentracije makrolida određene u zimskim mjesecima (ukupno do 3,9 μg L^{-1}) na toj lokaciji mogu se objasniti migracijom makrolidnog opterećenja iz suhog korita potoka u Savu tijekom visokih vodostaja Save uslijed intenzivnih oborina u zimskim mjesecima. Zato su tijekom ponovljenog zimskog uzorkovanja u veljači 2017. godine, kao i daljnih uzorkovanja tijekom preostalih sezona, uzeti uzorci sedimenta na dodatnoj lokaciji kod Otoka Samoborskog (UP7500; 7,5 km uzvodno od industrijskog ispusta), na ulazu rijeke Save u Hrvatsku. Ta je lokacija ujedno predstavljala referentnu lokaciju za sve daljne analize provedene na uzorcima prikupljenim iz rijeke Save. Važno je također napomenuti da su koncentracije azitromicina u vodi rijeke Save, izmjerene na lokaciji UP3500 tijekom zime te nizvodnim lokacijama DW700 i DW4500 tijekom zime i proljeća, premašivale njegove PNEC vrijednosti, što ukazuje na potencijalni rizik za selekciju makrolidne otpornosti u samoj rijeci (Bengtsson-Palme i Larsson, 2016.).

Osim onečišćenja površinske vode rijeke Save, industrijski ispusti iz pogona za proizvodnju azitromicina pridonijeli su također i onečišćenju sedimenta te rijeke antibioticima. Iako su koncentracije makrolida, posebice azitromicina, varirale u sedimentima u širokom rasponu, izraziti maksimum zabilježen je na lokaciji ispusta (ukupno do 24 mg kg^{-1}). Također, razmjerno visoke koncentracije (> 1 mg kg^{-1}) nađene su i na lokaciji 700 m nizvodno od ispusta, što ukazuje na značajan doprinos farmaceutske industrije povećanom sadržaju makrolida u riječnom sedimentu. Međutim, povišene razine azitromicina, oko 1 mg kg^{-1}, detektirane su i u sedimentu na uzvodnoj lokaciji UP3500 (nije prikazano), iz čega se može zaključiti da potok Gorjak također pridonosi onečišćenju sedimenta rijeke Save ovim antibiotikom i to zbog jakog onečišćenja potoka u prošlosti te nepovoljnih hidroloških prilika (visoki vodostaji). Nadalje, usprkos tome što su koncentracije makrolida u otpadnoj i riječnoj vodi bile više tijekom zimske negoli ljetne sezone, obrnuta situacija je zapažena za uzorke sedimenata. Razlog tome bi mogao biti veći protok rijeke Save tijekom zime (1194 m^3/h) u odnosu na ljeto (421 m^3/h), što je moglo dovesti do resuspenzije onečišćenog sedimenta i prijenosa makrolida u vodenu matricu iznad sedimenta te, posljedično, smanjenja njihovih koncentracija u zimskim uzorcima tih sedimenata. Nadalje, razmjerno visoke razine makrolida u sedimentima, barem na najviše onečišćenoj lokaciji ispusta, mogle bi potaknuti selekciju otpornosti na makrolide među bakterijama sedimenta. Iako je teško procijeniti stupanj bioraspoloživosti detektiranih makrolida u sedimentima, prijašnje istraživanje na primjeru tilozina je pokazalo da makrolidi zadržavaju antimikrobnu aktivnost čak i kad su čvrsto vezani na čestice sedimenta (Chander i sur., 2005.). Usporedbom razina makrolida izmjerenih u

sedimentima rijeke Save u ovom radu i razina fluorokinolona u sličnim istraživanjima u Indiji utvrđeno je da su razine makrolida bile puno niže od razina fluorokinolona u riječnom sedimentu (54 mg kg^{-1}) u regiji Patancheru (Kristiansson i sur., 2011.), ali ipak više od razina fluorokinolona u sedimentu rijeke Musi (do 3 mg kg^{-1}) (Gothwal i Shashidhar, 2017.). Osim antibiotika, ispusti otpadnih voda Industrije 1 rezultirali su akumulacijom metala, posebice Cu i Zn, u riječnom sedimentu, što ukazuje da ti metali ulaze u rijeku Savu industrijskim otpadnim vodama. Budući da je izmjerena koncentracija tih metala premašivala njihovu teorijsku minimalnu ko-selektivnu koncentraciju (Seiler i Berendonk, 2012.), može se očekivati doprinos Cu i Zn razvoju otpornosti na antibiotike u sedimentima putem ko-selekcije. Navedena pretpostavka je u skladu s ranijim istraživanjima koja su pokazala da uslijed izloženosti metalima dolazi do indirektne selekcije otpornosti na antibiotike zbog zajedničke lokalizacije gena za otpornost na metale i antibiotike na istim genetičkim elementima (Di Cesare i sur., 2016.; Guo i sur., 2018b.; Pal i sur., 2016a.; Song i sur., 2017.). Tako je primjerice u nekih bakterija već potvrđena istovremena otpornost na Cu i na makrolidne antibiotike (Amachawadi i sur., 2011.; Hasman i Aarestrup, 2002.). Povrh antibiotika i metala, značajne količine organskog materijala, fosforovih i dušikovih spojeva također su unešene u rijeku Savu putem industrijskih otpadnih voda, što je rezultiralo akumulacijom tih spojeva u sedimentima, posebice na lokaciji ispusta.

Slično uočenom kemijskom onečišćenju sedimenta rijeke Save, značajno viši udjeli kultivabilnih bakterija otpornih na azitromicin zamijećeni su u izloženim sedimentima, posebice na mjestu ispusta (oko 67 % otpornih bakterija). To može biti posljedica: a) unosa otpornih bakterija otpadnom vodom i njihovog preživljavanja u sedimentu te prijenosa do nizvodnih lokacija, b) namnožavanja autohtone bakterijske populacije sedimenta urođeno otporne na makrolide, c) selekcije novih bakterijskih populacija koje su stekle otpornost mutacijama ili, vjerojatnije, prijenosom gena za otpornost iz bakterija prisutnih u sedimentu ili otpadnoj vodi. Kombinacije ovih mogućnosti također su vrlo vjerojatne. Dobiveni su rezultati u skladu s povećanim razvojem otpornosti među kultivabilnim bakterijama koja se opisuje u vodenom okolišu koji je pod utjecajem ispusta iz proizvodnje antibiotika (Flach i sur., 2015.; Li i sur., 2009., 2010.). Bitno je također napomenuti da usprkos makrolidnom opterećenju sedimenta na lokaciji UP3500 u blizini utoka nekadašnjeg potoka Gorjak, udjeli kultivabilnih bakterija otpornih na azitromicin (prosječno 5,4 %; nije prikazano) bili su ipak niži od udjela u nizvodnim sedimentima (od 7 % do 27 %), ali opet viši od udjela u referentnom sedimentu (prosječno 0,9 %). Iz toga se može zaključiti da, iako mikrobiološko opterećenje sedimenta

rijeke Save ovisi dominantno o ispustima iz pogona Industrije 1, suho korito potoka Gorjak također manjim dijelom pridonosi tom onečišćenju.

Kemijskom analizom površinske vode potoka Kalinovica detektirani su antibiotici iz skupina sulfonamida (SDZ i SMZ), 2,4-diaminopirimidina (TMP), fluorokinolona (ENR) i tetraciklina (OTC) u koncentracijama koje uglavnom nisu premašivale 10 μg L^{-1} u blizini lokacije ispusta, dok su na lokaciji 3 km nizvodno od ispusta te koncentracije bile znantno niže, uglavnom oko 1 μg L^{-1} ili niže. Međutim, važno je napomenuti da su te koncentracije bile i do 30-tak puta više od onih izmjerenih na uzvodnoj lokaciji, što ukazuje na doprinos Industrije 2 onečišćenju potoka Kalinovica ovim antibioticima. Pored toga, koncentracije pojedinačnih antibiotika u potočnoj vodi bile su više od njihovih PNEC vrijednosti, što ukazuje na mogućnost selekcije otpornosti na antibiotike u samom potoku (Bengtsson-Palme i Larsson, 2016.). Nadalje, analiza antibiotika u sedimentu potoka ukazala je da je na mjestu ispusta sediment najviše onečišćen antibioticima, i to trimetoprimom (oko 5 mg kg^{-1}) i azitromicinom (do 0,4 mg kg^{-1}). Suprotno tome, najviša koncentracija sulfonamida, SDZ i SMZ, bila je na lokaciji udaljenoj 3 km nizvodno od ispusta (do 1,2 mg kg^{-1}). Razlog tome može biti sporiji protok vode na toj lokaciji i, posljedično, nagomilavanje antibiotika u sedimentu. Pored toga, šumovito područje koje okružuje tu lokaciju vjerojatno je omogućilo zaštitu sulfonamida od fotorazgradnje i time produljilo njihovu postojanost u sedimentu (Baena-Nogueras i sur., 2017.). Naposljetku, ukupne koncentracije antibiotika u sedimentu potoka (ispust, oko 5 mg kg^{-1}; nizvodno, do 1,5 mg kg^{-1}) općenito su bile niže od koncentracija izmjerenih u sedimentima rijeke Save te riječnim sedimentima u sličnim istraživanjima u Indiji (Gothwal i Shashidhar, 2015.; Kristiansson i sur., 2011.). Ipak, važno je istaknuti da su utvrđene koncentracije i dalje bile više od koncentracija u sedimentima izloženim komunalnim otpadnim vodama (ukupno do 0,6 mg kg^{-1}) (Guan i sur., 2018.; Li i sur., 2019.; Marti i sur., 2014.). Što se tiče analiziranih metala, posebice Cu i Zn, njihove koncentracije su na svim lokacijama duž potoka prelazile minimalne ko-selektivne koncentracije (Seiler i Berendonk, 2012.), što govori u prilog potencijalnom doprinosu tih metala razvoju otpornosti na antibiotike u potočnom sedimentu. Međutim, iznenađujuće je da su navedeni metali bili prisutni na uzvodnoj lokaciji u koncentraciji i do 3 puta višoj u odnosu na lokaciju ispusta i nizvodnu lokaciju, što ukazuje na doprinos nekih drugih izvora tih metala na istraživanom području. Budući da je uzvodna lokacija smještena u ruralnom području bez uređene kanalizacijske infrastrukture, unošenje metala, ali i antibiotika (do 0,04 mg kg^{-1}) u potok na toj lokaciji može biti povezano s difuznim izvorima, prije svega ispiranjem s okolnih polja koja su tretirana stajskim gnojivom te ilegalnim

ispustima kanalizacije iz kućanstava. Osim antibiotika i metala, sediment potoka na lokaciji ispusta opterećen je povišenim koncentracijama dušikovih spojeva u usporedbi s uzvodnim sedimentom, što potvrđuje da ti spojevi potječu iz industrijskih otpadnih voda. Mikrobiološkom analizom je nadalje potvrđen povećani udio kultivabilnih bakterija otpornih na sulfonamide (SMZ) i tetracikline (OTC) u sedimentu potoka na ispustu i nizvodno od ispusta u odnosu na uzvodnu lokaciju. To je vjerojatno posljedica unosa otpornih bakterija otpadnom vodom, ali i selekcije otpornih populacija kao odgovor na povišene koncentracije antibiotika u sedimentu.

Zaključno se može reći da je na temelju ovog dijela rezultata potvrđena prva hipoteza doktorskog rada (H1) „Otpadne vode iz lokalnih farmaceutskih industrija onečišćuju recipijentni vodeni okoliš antibioticima, metalima i bakterijama otpornima na antibiotike“.

4.2. Sastav rezistoma industrijskih otpadnih voda i slatkovodnih sedimenata

Provedene kemijske i mikrobiološke analize nedvojbeno su pokazale da su industrijski ispusti doveli do značajnog onečišćenja istraživanih riječnih i potočnih sedimenata, posebice antibioticima, stoga postoji mogućnost da je među bakterijskom populacijom sedimenta došlo do obogaćenja poznatih, ali i nastanka novih gena za otpornost na antibiotike uslijed dugotrajne izloženosti relativno visokim razinama antibiotika. Ovo zapažanje se često navodi u literaturi za područje Azije gdje je okolišno onečišćenje antibioticima putem ispusta iz proizvodnih pogona najizraženije (Bengtsson-Palme i sur., 2014.; Kristiansson i sur., 2011.; Marathe i sur., 2018.). Zato je cilj ovog dijela istraživanja bio identificirati gene za otpornost na antibiotike u istraživanim industrijskim otpadnim vodama i izloženim sedimentima u usporedbi sa neizloženim sedimentima (uzvodne lokacije) pomoću inovativnog pristupa funkcionalne metagenomike. Prema našim saznanjima, rezultati ovog istraživanja su ujedno i prvi podaci o rezistomu industrijskih otpadnih voda i sedimenata onečišćenih visokim koncentracijama antibiotika dobiveni funkcionalnom metagenomikom, a objavljeni su pod rednim brojem 2 u popisu znanstvenih radova (str. 49-61).

Funkcionalnom analizom metagenomskih knjižnica porijeklom iz otpadne vode Industrije 1 i recipijentnih riječnih sedimenata otkriveno je 16 jedinstvenih (engl. *Non-Copy*) gena za otpornost na makrolide, pri čemu je većina tih gena bila slična (≥ 99 %) već poznatim genima kao što su *msr*E (zaštitni proteini ribosoma), *mph*E i *mph*G (makrolidne fosfotransferaze) te *mef*C (efluks pumpe). Unatoč jakom selekcijskom pritisku uzrokovanom prisutnošću makrolida, otkriven je samo jedan potencijalno novi gen (≤ 80 % sličnosti proteinskih sekvenci sa sekvencama u bazi podataka NCBI). To je bio gen *erm* koji je pokazao

67 %-tnu sličnost proteinske sekvence sa sekvencom enzima 23S rRNA metiltransferaze iz vrste *Clostridium* sp. Ti podaci ukazuju da se vjerojatno radi o novom proteinu iz porodice metiltransferaza Erm koji ispoljava visok stupanj otpornosti na eritromicin zbog metilacije ribosoma (minimalna inhibitorna koncentracija, MIK, iznosila je 1536 mg L^{-1}). Nadalje, pokazalo se da su klonovi koji su ispoljavali visoku otpornost na makrolide ($MIK_{ERI} \geq 1024$ mg L^{-1}; $MIK_{AZI} \geq 64$ mg L^{-1}) sadržavali gene za dva različita mehanizma makrolidne otpornosti kao što su *msr*E-*mph*E i *mph*G-*mef*C. Ove genske kazete pronađene su samo u knjižnicama porijeklom iz otpadne vode i sedimenta s lokacije ispusta, što upućuje na otpadne vode Industrije 1 kao važan izvor tih gena u riječnom sedimentu. Daljnom analizom metagenomskih knjižnica porijeklom iz neonečišćenih, uzvodnih sedimenata identificiran je jedan potencijalno novi mehanizam otpornosti na makrolide kojeg kodira gen *hfl*X. Taj gen eksprimira enzim GTP-azu HflX koja je pokazala nizak stupanj sličnosti proteinske sekvence (≤ 63 %) s onom iz bakterije *Emergencia timonensis*. Smatra se da ta GTP-aza HflX reagira s molekulom makrolida vezanom za ribosom čime ju izbacuje iz veznog mjesta i na taj način omogućuje neometanu sintezu staničnih proteina na ribosomu (Duval i sur., 2018.; Lau i sur., 2017.). Premda su na referentnoj lokaciji (UP7500) detektirane vrlo niske razine azitromicina (4,6 μg kg^{-1}), otpornost na makrolide posredovana genom *hfl*X mogla se razviti sasvim slučajno nakupljanjem mutacija u genomu bakterija uslijed dugotrajne izloženosti različitim drugim selektivnim spojevima (Knöppel i sur., 2017.). Tu pretpostavku potkrepljuju i literaturna zapažanja koja su pokazala da okoliš koji nije pod utjecajem antropogenog onečišćenja antibioticima može biti spremnik novih gena za antibiotičku otpornost (Kristiansson i sur., 2011.; Amos i sur., 2014.; Nesme i sur., 2014.). Nadalje, pokazalo se da su klonovi koji su nosili gen *hfl*X ispoljavali niži stupanj otpornosti na makrolide (MIK_{ERI} 64-128 mg L^{-1}; MIK_{AZI} < 16 mg L^{-1}) u usporedbi sa klonovima porijeklom iz onečišćenih uzoraka koji su posjedovali gene *msr*E, *mph*E, *mph*G i *mef*C (MIK_{ERI} 512-2048 mg L^{-1}; MIK_{AZI} 32-512 mg L^{-1}). Ti podaci ukazuju da je mehanizam posredovan genom *hfl*X, a prisutan u bakterijama neonečišćenog sedimenta, manje učinkovit u pružanju otpornosti na makrolide u usporedbi s mehanizmima prisutnim u onečišćenim uzorcima. To pak nadalje upućuje na to da su bakterije koje žive u sedimentu na mjestu ispusta evoluirale ili stekle veću otpornost na makrolide kao odgovor na izloženost visokom selekcijskom pritisku makrolida.

Funkcionalnom analizom metagenomskih knjižnica porijeklom iz otpadne vode Industrije 2 i potočnih sedimenata s uzvodne lokacije i lokacije ispusta identificirano je ukupno 66 jedinstvenih gena za otpornost na antibiotike iz skupina β-laktama, sulfonamida, tetraciklina

i 2,4-diaminopirimidina. Pritom je većina tih gena bila vrlo slična (≥ 94 %) već poznatim genima otpornosti na razini proteinske sekvence. Tako su, primjerice, svi identificirani geni za otpornost na sulfonamide bili već poznati tipovi gena poput *sul*1 i *sul*2 koji dovode do otpornosti posredstvom enzima DHPS. Za razliku od gena *sul*1 koji je nađen u svim knjižnicama, gen *sul*2 nađen je samo u knjižnicama porijeklom s lokacije ispusta, što upućuje na industrijsku otpadnu vodu kao izvor tog gena u sedimentu potoka. Oba navedena gena su već detektirana u sedimentima onečišćenim antibioticima (Bengtsson-Palme i sur., 2014.; Kristiansson i sur., 2011.; Luo i sur., 2010.), kao i u neonečišćenim sedimentima (Archundia i sur., 2017.; Czekalski i sur., 2015.). To potvrđuje njihovu široku rasprostranjenost u okolišu, vjerojatno uslijed česte lokalizacije na plazmidima čime je olakšano njihovo širenje među bakterijama (Hu i sur., 2016.; Johnson i sur., 2016.; Koczura i sur., 2016.). Slično genima *sul,* svi identificirani geni za otpornost na tetracikline bili su već poznati tipovi gena poput *tet*39, *tet*A i *tet*C. Ovi geni su također već ranije bili detektirani u sedimentima visoko onečišćenim fluorokinolonima pri čemu je gen *tet*39 dominirao (Bengtsson-Palme i sur., 2014.). Od navedenih gena, jedino je gen *tet*C nađen u uzvodnom sedimentu, što ukazuje na njegovu prirodnu prisutnost u istraživanom potoku. To je u skladu s literaturnim zapažanjima koja opisuju rasprostranjenost gena *tet*A i *tet*C u okolišu koji nije bio izložen antropogenom onečišćenju antibioticima (Andersen i Sandaa, 1994.; Durso i sur., 2016.; West i sur., 2011.). Druga je pak mogućnost da je gen *tet*C u istraživani potok dospio ispiranjem stočnog gnojiva koje je sadržavalo taj gen sa okolnih oranica. Što se tiče identifikacije gena za otpornost na trimetoprim, iako su većinom nađeni već poznati geni, identificiran je i značajan udio (oko 30 %) potencijalno novih gena. Činjenica da su poznati i potencijalno novi geni nađeni u knjižnicama porijeklom iz sedimenta s uzvodne lokacije i lokacije ispusta ukazuje na to da je sam sediment prirodan spremnik tih gena. Identificirani geni bili su najsličniji genima *dfr* koji kodiraju enzime DHFR ili genima *thy* koji kodiraju enzime TYMS. Ti enzimi dovode do otpornosti na trimetoprim zaobilaskom metaboličkog puta sinteze folne kiseline i timina. Potrebno je također napomenuti da su neki klonovi posjedovali dva različita gena za otpornost na trimetoprim, *dfr* i *thy*, koja su u prijašnjem istraživanju nađena zajedno na istom plazmidu (Kehrenberg i Schwarz, 2005.), što ukazuje na njihovu potencijalnu zajedničku mobilnost. Što se tiče gena za otpornost na β-laktame, u ovom radu je identificirano 15 jedinstvenih gena koji kodiraju za β-laktamaze iz skupina A-D. Većina tih gena bili su otprije poznati te su potjecali iz otpadne vode i onečišćenog sedimenta. Pritom valja istaknuti prisutnost klinički relevantnih gena poput gena $bla_{\text{GES-1}}$ i $bla_{\text{VEB-9}}$ (β-laktamaze skupine A) te $bla_{\text{MOX-9}}$ i $bla_{\text{CMY-10}}$ (plazmidne AmpC β-laktamaze skupine C) koji su pokazali najveću sličnost s genima iz patogenih bakterija

poput *K. pneumoniae*, *P. aeruginosa*, *A. baumanii* i *C. freundii* (Jacoby, 2009.; Paterson i Bonomo, 2005.). Pored visokog stupnja otpornosti na ampicilin (MIK > 1024 mg L^{-1}), navedeni geni bili su odgovorni i za visok stupanj otpornosti na cefotaksim, cefalosporin proširenog spektra (MIC 8-32 mg L^{-1}), koji je vrlo učinkovit u liječenju infekcija izazvanih Gram-negativnim bakterijama. Pored toga, poznato je da enzim kodiran genom *bla*$_{GES-1}$ hidrolizira karbapeneme, antibiotike posljednje linije obrane u liječenju infekcija izazvanih enterobakterijama otpornim na više skupina antibiotika (Stewart i sur., 2015.). Sva navedena zapažanja stoga upućuju na zaključak da bi potočni sediment izložen industrijskom ispustu mogao biti važan spremnik gena koji kodiraju klinički relevantne β-laktamaze proširenog spektra poput GES, VEB, MOX i CMY-10. Činjenica da su geni za navedene β-laktamaze nađeni jedino u sedimentu na lokaciji ispusta ukazuje na otpadnu vodu kao izvor tih gena. Ta činjenica ne iznenađuje ako se uzme u obzir podatak da otpadna voda koja se ulijeva u potok predstavlja mješavinu tehnoloških i sanitarnih otpadnih voda pa bi ti geni mogli biti humanog porijekla. U prilog tome govore i istraživanja koja su pokazala da su komunalne otpadne vode izvor gena za karbapenemaze iz porodice GES u recipijentnom riječnom sedimentu (Marathe i sur., 2017.), a bolničke otpadne vode izvor gena za β-laktamaze iz porodice MOX (Antonelli i sur., 2015.). Nasuprot tome, geni *bla*$_{OXA-198}$ i *bla*$_{OXA-10}$ koji kodiraju β-laktamaze skupine D, detektirani su u svim knjižnicama uključujući i onu iz uzvodnog sedimenta, što ukazuje da njihova prisutnost u sedimentu potoka nije isključivo uvjetovana industrijskim ispustom. Budući da potok teče kroz ruralno područje, izvor navedenih gena *bla*$_{OXA}$, pored otpadne vode, može biti i stočno gnojivo koje je ispiranjem završilo u potoku. Pored identificiranih poznatih gena za otpornost na β-laktame, identificirana su i četiri potencijalno nova gena koja kodiraju β-laktamaze iz skupina A, B i D, a bila su odgovorna za visok stupanj otpornosti na ampicilin (MIK > 1024 mg L^{-1}).

U konačnici, analiza genske okoline identificiranih gena ukazala je na vjerojatno plazmidno porijeklo mnogih gena, što je i u skladu s prijašnjim istraživanjima (Kadlec i sur., 2011.; Nonaka i sur., 2015.; Schlüter i sur., 2007.; Sugimoto i sur., 2017.; Zhang i sur., 2013.). Ta teza, kao i podatak da su neki klonovi, pored dvostrukih mehanizama za otpornost na jednu skupinu antibiotika, posjedovali i gene za otpornost na druge skupine antibiotika, ukazuje na vjerojatnost vezanog vertikalnog nasljeđivanja ili stjecanja šarolikog spektra gena otpornosti u slučaju horizontalnog prijenosa. Povrh toga, identificirani geni često su nađeni u sklopu genskih kazeta integrona skupine 1 koji su kao pokretni genetički elementi direktno ili indirektno

odgovorni za mobilizaciju gena otpornosti i njihovu ugradnju u plazmide (Cambray i sur., 2010.; Deng i sur., 2015.; Li sur., 2009., 2010.; Sultan i sur., 2018.).

Rezultati ovog dijela istraživanja dali su pregled prisutnosti gena za otpornost na antibiotike u industrijskim otpadnim vodama i sedimentima njihovih recipijenata, a čija identifikacija je omogućena primjenom pristupa funkcionalne metagenomike. Moguće je također da ovi uzorci sadrže i druge gene otpornosti koji nisu eksprimirani u *E. coli* pa stoga ne mogu biti identificirani ovom metodom. Usprkos tome, dobiveni rezultati ukazuju na to da sedimenti onečišćeni antibioticima putem otpadnih voda farmaceutskih industrija mogu biti važni spremnici klinički relevantnih gena otpornosti, kako poznatih tako i potencijalno novih. Slijedom navedenog, hipoteza H2 postavljena u ovom doktorskom radu „Otpadne vode i sedimenti recipijentnih voda su spremnici poznatih i potencijalno novih gena za otpornost na antibiotike“ je potvrđena.

4.3. Utjecaj industrijskih otpadnih voda na širenje otpornosti na antibiotike

Rezultati dosadašnjih istraživanja provedenih u ovom radu ukazali su na širok repertoar funkcionalnih gena za otpornost na antibiotike koji su pokazali potencijal horizontalnog širenja među bakterijama u sedimentima izloženim industrijskim ispustima. Tome u prilog idu i literaturna zapažanja koja također dovode u vezu okolišno onečišćenje otpadnim vodama farmaceutskih industrija i povećanu mobilnost gena otpornosti među okolišnim bakterijama (Bengtsson-Palme i sur., 2014.; Flach i sur., 2015.). Stoga su daljna istraživanja ovog rada bila usmjerena na procjenu utjecaja industrijskih ispusta na širenje otpornosti na antibiotike u sedimentima recipijentnih voda, što je provedeno pomoću tri serije analiza, a dobiveni rezultati objavljeni su pod rednim brojevima 3-6 u popisu znanstvenih radova (str. 62-124) te u Prilogu 7.7. (str. 242-246).

4.3.1. Kvantifikacija gena otpornosti u otpadnim vodama i sedimentima

Procjena širenja gena za otpornost na antibiotike započeta je kvantifikacijom odabranih gena u otpadnim vodama i sedimentima metodom qPCR-a te izračunom omjera tih gena u odnosu na ukupne 16S rRNA gene (*rrn*) (relativna zastupljenost u ukupnoj bakterijskoj zajednici) tijekom dvije sezone (zima i ljeto). Tako su u sedimentima rijeke Save kvantificirani geni za otpornost na makrolide (*mph*G, *mph*E, *msr*E, *mef*C i *erm*B), dok su u potoku Kalinovica kvantificirani geni za otpornost na sulfonamide (*sul*1 i *sul*2), tetracikline (*tet*C i *tet*39), β-laktame (bla_{GES}, bla_{VEB}, bla_{OXA-1} i bla_{OXA-2}), trimetoprim (*dfr*A14 i *fol*A) i makrolide (*mph*G, *mph*E, *msr*E, *mef*C i *erm*B). Iako su rezultati tih analiza pokazali da industrijski ispusti potiču

širenje gena za otpornost u izloženim sedimentima, uočene su bitne razlike između istraživane rijeke i potoka.

U otpadnoj vodi Industrije 1, među 5 ciljanih gena za makrolidnu otpornost, najzastupljeniji su bili tipovi gena koji kodiraju makrolidne fosfotransferaze (*mph*G), proteine za zaštitu ribosoma (*msr*E) i ribosomske metilaze (*erm*B), dok je relativna zastupljenost preostala dva gena koja kodiraju efluks pumpe (*mef*C) i fosfotransferaze (*mph*E) bila približno 10 puta niža. Ispusti tih otpadnih voda u rijeku Savu pridonijeli su značajno višoj relativnoj zastupljenosti svih 5 navedenih gena u sedimentu na lokaciji ispusta i 4 lokacije nizvodno od ispusta, čak do 11 km nizvodno, u odnosu na referentnu lokaciju (UP7500). Međutim, iznenađujuće je da nije zamijećen značajan sezonski utjecaj na raspodjelu ciljanih gena u sedimentima unatoč većoj brzini protoka Save tijekom zime negoli tijekom ljeta. Pritom je izuzetak uzvodna lokacija UP3500 vezana uz utok potoka Gorjak na kojoj je relativna zastupljenost svih 5 ciljanih gena bila značajno viša nego na referentnoj lokaciji jedino tijekom ljeta (nije prikazano), što ukazuje na potencijalnu selekciju otpornih bakterija u sedimentu uslijed izloženosti nagomilanim razinama antibiotika. Nadalje, u literaturi se navodi da je relativna zastupljenost gena za antibiotičku otpornost u okolišu jako onečišćenom antibioticima obično u rasponu od -2 do -1 log kopija gena otpornosti/*rrn* kopija (Gao i sur., 2018.), što je usporedivo s podacima dobivenim u ovom radu za otpadnu vodu i riječni sediment s lokacije ispusta. Nasuprot tome, prosječna količina najzastupljenijih gena (*mph*G, *msr*E i *erm*B) bila je približno 10 puta niža u sedimentima na nizvodnim lokacijama, što govori u prilog tezi da je područje rijeke Save 11 km nizvodno od ispusta umjereno onečišćeno genima za makrolidnu otpornost (Gao i sur., 2018.; Graham i sur., 2011.). Nadalje, te količine gena otpornosti usporedive su s količinama gena otpornosti na fluorokinolone izmjerenim u indijskim sedimentima visoko onečišćenim fluorokinolonima (Rutgersson i sur., 2014.).

Dobiveni rezultati koji ukazuju na veću relativnu zastupljenost gena za makrolidnu otpornost na nizvodnim lokacijama u odnosu na referentnu lokaciju mogli bi biti posljedica selekcije bakterija koje nose te gene uslijed izloženosti razmjerno visokim koncentracijama makrolida, barem na području 700 m nizvodno od ispusta koje je isključivo pod utjecajem industrijskog otpada i koje karakterizira visoko onečišćenje makrolidima. Alternativno, unos otpornih bakterija otpadnom vodom u sediment na lokaciji ispusta i njihov prijenos do nizvodnih lokacija, kao i horizontalni prijenos gena makrolidne otpornosti među bakterijama sedimenta također bi mogli doprinijeti pojavi veće količine tih gena na nizvodnim lokacijama. Toj tezi ide u prilog i činjenica da su mnogi geni za makrolidnu otpornost uključujući i gene

analizirane u ovom radu, nađeni na konjugacijskim plazmidima koje karakterizira potencijal širenja među bakterijama i koji obično nose i gene za otpornost na ostale skupine antibiotika (Sugimoto i sur., 2017.; Zhang i sur., 2013.). Prema tome, horizontalni prijenos plazmida bi mogao biti odgovoran za širenje i postojanost gena za makrolidnu otpornost u riječnim sedimentima nizvodno od ispusta.

Osim značajno veće količine ciljanih gena *msr*, *mph*, *erm* i *mef*, utvrđena je i značajno veća količina gena *int*I1 u uzorcima sedimenta na ispustu te nizvodno i uzvodno (UP3500) od ispusta u odnosu na sediment sa referentne lokacije, ali samo tijekom zimske sezone. Ti rezultati ukazuju da povećane količine integrona skupine 1 u sedimentu rijeke Save ne ovise samo o sadašnjim industrijskim ispustima u Zaprešiću, već se radi i o doprinosu prijašnjih ispusta Industrije 1 u potok Gorjak, posebno na lokaciji poslije utoka Gorjaka u Savu (UP3500). Spomenuti integroni (gen *int*I1) predloženi su kao genetički markeri antropogenog onečišćenja okoliša (Gillings, 2018., 2017.; Gillings i sur., 2015.), a njihova lokalizacija na plazmidima mogla bi potaknuti horizontalni prijenos više različitih gena za otpornost u tim sedimentima. Međutim, premda je relativna zastupljenost gena *int*I1 približno jednaka u nizvodnim sedimentima tijekom obje sezone, značajno povišenje u nizvodnim sedimentima u odnosu na referentni sediment nije uočeno tijekom ljeta, vjerojatno zbog puno veće količine tog gena u referentnom sedimentu tijekom ljeta u odnosu na zimu.

U otpadnoj vodi Industrije 2 detektirane su relativno visoke količine gena otpornosti ($\geq$ -2,5 log kopija gena otpornosti/*rrn* kopija) na sulfonamide (*sul*1 i *sul*2), tetracikline (*tet*39 i *tet*C), makrolide (*mph*G i *msr*E), β-laktame ($bla_{\text{OXA-2}}$) i trimetoprim (*dfr*A14), što ukazuje na to da ove otpadne vode, osim antibiotika, unose i znatne količine različitih gena otpornosti u recipijentni potok. Kao rezultat toga, relativna zastupljenost gotovo svih tih gena (izuzev gena *sul*) bila je značajno viša u sedimentu na lokaciji ispusta u odnosu na uzvodnu lokaciju tijekom ljeta, ali ne i tijekom zime. To je vjerojatno povezano s višim temperaturama koje mogu promovirati preživljavanje otpornih bakterija iz otpadne vode ili horizontalni prijenos gena u sedimentu. Međutim, potrebno je istaknuti da je relativna zastupljenost svih ciljanih gena bila iznenađujuće visoka i u sedimentu na uzvodnoj lokaciji (od -2 do -3 log kopija gena otpornosti/*rrn* kopija). Iz tog razloga je relativna zastupljenost gena otpornosti bila tek neznatno, ali ipak značajno viša na lokaciji ispusta u odnosu na uzvodnu lokaciju. Razlog tako visokoj zastupljenosti ciljanih gena u uzvodnom sedimentu može biti povezana sa selekcijskim pritiskom antibiotika i metala koji su na tu referentnu lokaciju vjerojatno dospjeli ilegalnim kanalizacijskim ispustima i/ili ispiranjem stočnog gnojiva s okolnih oranica. Nadalje, osim

razmjerno visoke količine nekih gena, otpadne vode Industrije 2 također su sadržavale razne podtipove gena za otpornost na β-laktame (*bla*$_{GES}$, *bla*$_{VEB}$ i *bla*$_{OXA-1}$), trimetoprim (*fol*A) i makrolide (*erm*B, *mph*E i *mef*C) u nešto nižim količinama (od -4 do -3 log kopija gena otpornosti/*rrn* kopija). Međutim, relativna zastupljenost svih navedenih gena bila je opet značajno viša u sedimentu na lokaciji ispusta u odnosu na uzvodnu lokaciju. Nadalje, uočene su i sezonske razlike u dinamici većine tih gena, vjerojatno zbog varijacija u njihovim pozadinskim razinama u sedimentu samog potoka (razine na uzvodnoj lokaciji). Jedino geni *erm*B i *bla*$_{GES}$ koji su od kliničkog značaja (Guo i sur., 2018c.; Wibberg i sur., 2018.) nisu bili detektirani u uzvodnom sedimentu, ali su pak detektirani u sedimentu na lokaciji ispusta tijekom obje sezone. Taj podatak, kao i činjenica da su oba gena detektirana i u otpadnoj vodi, nedvojbeno pokazuje da su njihov glavni izvor u potočnom sedimentu ispusti iz Industrije 2. Zanimljivo je primijetiti da je relativna zastupljenost tih gena u otpadnoj vodi te industrije (od -4 do -3 log kopija gena otpornosti/*rrn* kopija) usporediva ili čak niža od njihove zastupljenosti u komunalnim otpadnim vodama (od -2 do -3 log kopija gena otpornosti/*rrn* kopija) (Rafraf i sur., 2016.; Rodriguez-Mozaz i sur., 2015.). Ne čudi stoga činjenica da je obogaćivanje tih gena zabilježeno i u sedimentima pod utjecajem komunalnih otpadnih voda (Marathe i sur., 2017.; Sabri i sur., 2018.), kao i u sedimentima pod utjecajem otpadnih voda iz formulacije lijekova (Khan i sur., 2013.).

Nasuprot povišenoj količini gena otpornosti u potočnom sedimentu na lokaciji ispusta u usporedbi sa uzvodnom lokacijom, relativna zastupljenost većine tih gena pala je u sedimentu na lokaciji smještenoj 3 km nizvodno na razinu uzvodne lokacije. To može biti posljedica ograničenog prijenosa tih gena s lokacije ispusta nizvodno ili odumiranja njihovih bakterijskih domaćina, razgradnje izvanstanične DNA koja nosi te gene (Nnadozie i Odume, 2019.), sorpcije gena za sediment (Calero-Caceres i sur., 2017.) ili različitih kombinacija ovih procesa. Usprkos tome, relativna zastupljenost gena *sul*2, *mph*G i *msr*E bila je i dalje značajno povišena u nizvodnom sedimentu u odnosu na uzvodni. Ti se rezultati mogu donekle objasniti selekcijom bakterija koje nose te gene uslijed izloženosti razmjerno visokim koncentracijama sulfonamida na nizvodnoj lokaciji (do 1,2 mg kg^{-1}). Naime, Heuer i sur. (2008.) pokazali su da sulfonamidni antibiotik sulfadiazin već pri koncentraciji od 0,15 mg kg^{-1}, što je niže od koncentracije sulfadiazina izmjerene na nizvodnoj lokaciji u ovom radu (> 0,16 mg kg^{-1}), dovodi do selekcije bakterija koje nose gen *sul*2 putem horizontalnog prijenosa gena. Budući da nema literaturnih podataka o selektivnoj koncentraciji makrolida u sedimentu, teško je procijeniti jesu li razine azitromicina određene u sedimentu na nizvodnoj lokaciji (0,35 mg kg^{-1}) bile selektivne za

razvoj bakterija koje nose gene *mph*G i *msr*E. Alternativno, dugotrajna izloženost razmjerno visokim razinama sulfonamida mogla bi dovesti i do ko-selekcije gena za makrolidnu otpornost. U prilog tome govori i činjenica da su geni za otpornost na makrolide i sulfonamide često nađeni ko-lokalizirani na istim plazmidima (Dolejska i sur., 2014.; Nonaka i sur., 2012.; Rahube i sur., 2014.), što je u skladu i sa rezultatima funkcionalne metagenomike dobivenim u ovom radu. Prema tome, na temelju rezultata dobivenim ovim istraživanjem, hipoteza H3 „Industrijski ispusti potiču širenje otpornosti na antibiotike među bakterijama u izloženim sedimentima" se prihvaća.

4.3.2. Horizontalno širenje gena za otpornost na antibiotike

Daljnja analiza širenja gena za antibiotičku otpornost provedena je praćenjem plazmidnog prijenosa otpornosti iz bakterija otpadne vode i sedimenta kao donora plazmida u recipijentni soj *E. coli* CV601.

U sedimentima rijeke Save uočena je veća učestalost plazmidnog prijenosa otpornosti na makrolide i tetracikline na lokacijama ispusta i 700 m nizvodno od ispusta u usporedbi sa udaljenijom nizvodnom lokacijom (4,5 km nizvodno) ili pak referentnom lokacijom. Ta zapažanja sugeriraju da onečišćene lokacije, posebice lokacija ispusta, mogu biti žarišta za prijenos otpornosti na makrolide i tetracikline posredovan prijenosom plazmida među bakterijskom populacijom sedimenta. Ta povećana mobilnost je vjerojatno potaknuta visokim koncentracijama makrolida, ali i hranjivih tvari te otpornih bakterija u tim sedimentima. Navedeni rezultati su u skladu s rezultatima sličnog istraživanja provedenog na području Indije koje također navodi veću učestalost plazmidnog prijenosa antibiotičke otpornosti među bakterijama sedimenta visoko onečišćenog antibioticima (fluorokinolonima) (Flach i sur., 2015.). Ta učestalost plazmidnog prijenosa kretala se u rasponu od 10^{-4} do 10^{-6} što je usporedivo s podacima dobivenim u ovom istraživanju (od 10^{-3} do 10^{-6}). Za razliku od uspješno postignutog horizontalnog prijenosa gena u uzorcima sedimenta, taj je prijenos bio neuspješan u uzorku otpadne vode Industrije 1, vjerojatno zbog izrazito visoke koncentracije makrolida (ukupno 6,5 mg L^{-1}) koja je mogla inhibirati recipijentni soj. Toj tezi govore u prilog i rezultati prijašnjeg istraživanja koji navode da eritromicin inhibira prijenos plazmida pri koncentraciji od 1 mg L^{-1} te čak više pri 10 mg L^{-1} (Jutkina i sur., 2018.). Činjenica da je u ovom radu dokazan uspješan horizontalni prijenos otpornosti u uzorcima sedimenata visoko onečišćenim makrolidima (čak do 10 mg kg^{-1}) može se donekle objasniti smanjenom bioraspoloživošću makrolida uslijed adsorpcije na čestice sedimenta.

U sedimentima potoka Kalinovica praćena je učestalost plazmidnog prijenosa otpornosti na tetracikline koja je nađena povišena samo u sedimentu na lokaciji ispusta, ali ne i na nizvodnoj lokaciji u usporedbi sa uzvodnom lokacijom. Osim iz sedimenata, dobiven je uspješan horizontalni prijenos otpornosti i iz otpadne vode Industrije 2, što ukazuje da je ta otpadna voda izvor mobilnih gena za otpornost na tetracikline. Prijašnja istraživanja su pokazala da koncentracija tetraciklina od 10 μg L^{-1} potiče horizontalni prijenos gena (Jutkina i sur., 2016.; Kim i sur., 2014.). Temeljem toga može se pretpostaviti da je koncentracija oksitetraciklina (antibiotika iz skupine tetraciklina) u istraživanoj otpadnoj vodi (17 μg L^{-1}) bila dovoljna za poticanje horizontalnog širenja gena među bakterijama otpadne vode. Treba također istaknuti da su ti rezultati ujedno i prvi podaci o mobilnosti gena za otpornost na antibiotike među bakterijama koje obitavaju u otpadnim vodama farmaceutske industrije.

Rezultati karakterizacije mobilnih plazmida koji nose gene za otpornost pokazali su da je većina tih plazmida pripadala IncP-1 skupini inkompatibilnosti, i to podskupini IncP-1ε. Ti plazmidi su uglavnom bili izolirani iz uzoraka onečišćenih antibioticima poput otpadne vode Industrije 2 i potočnog sedimenta s lokacije ispusta te riječnih sedimenata s lokacije ispusta i lokacije bliže ispustu (700 m nizvodno od ispusta). Navedeni rezultati upućuju na zaključak da bi IncP-1ε plazmidi mogli bitno pridonijeti lokalnoj prilagodbi i preživljavanju bakterija u okolišu prepunom antibiotika. Iako su plazmidi IncP-1 prvotno otkriveni u kliničkim izolatima (Datta i sur., 1971.), kasnijim su istraživanjima detektirani u rijekama i riječnim sedimentima onečišćenim živom te u kanalizaciji i aktivnom mulju (Bahl i sur., 2009.; Haines i sur., 2006.; Heuer i sur., 2012.; Moura i sur., 2012.; Oliveira i sur., 2012.; Smalla i sur., 2006.). Predložena je također i povezanost više zastupljenosti plazmida IncP-1 i antropogenog onečišćenja okoliša (Smalla i sur., 2006.). Pored IncP-1ε plazmida, u ovom istraživanju izolirani su i plazmidi koji pripadaju podskupini IncP-1β te IncN skupini inkompatibilnosti, a potječu iz sedimenta rijeke Save onečišćenog makrolidima. Detekcija plazmida IncN u skladu je sa studijom Flach i sur., (2015.) u kojoj su takvi plazmidi izolirani iz sedimenta izrazito onečišćenog fluorokinolonima iz farmaceutskih pogona u Indiji. Budući da su plazmidi IncP-1 i IncN skupina inkompatibilnosti izrazito „promiskuitetni“ (Klümper i sur., 2015.; Matsumura i sur., 2018.; Musovic i sur., 2006.; Shintani i sur., 2015.), za očekivati je da imaju veliku važnost u horizontalnom širenju otpornosti na antibiotike među filogenetski bliskim i udaljenim bakterijama u istraživanoj rijeci i potoku. Nadalje, integroni skupine 1 (gen *int*I1) te geni koji su uglavnom sastavni dio tih integrona (*qac*E/*qac*EΔ1, *sul*1 i *tet*A) detektirani su na IncP-1ε plazmidima iz otpadne vode Industrije 2 te iz riječnih i potočnih sedimenata, što je u skladu sa

ranijim literaturnim opažanjima (Jechalke i sur., 2013.; Wolters i sur., 2015.). Ne čudi stoga podatak da su IncP-1ε i IncN plazmidi analizirani u ovom istraživanju, pored fenotipske otpornosti na makrolide ili tetracikline, često ispoljavali otpornost i na druge skupine antibiotika, poglavito sulfonamide i trimetoprim, ali i β-laktame. Takva fenotipska otpornost na više skupina antibiotika bila je najizraženija kod plazmida porijeklom iz sedimenata najviše onečišćenih antibioticima. To je vrlo vjerojatno posljedica povećane mobilnosti plazmida i genskih preslagivanja plazmidne DNA uzrokovanih, u velikom broju slučajeva, pokretnim genetičkim elementima. Genska pozadina fenotipske otpornosti na više skupina antibiotika rasvijetljena je sekvenciranjem cijelih odabranih konjugacijskih plazmida PacBio tehnologijom. Dobiveni rezultati potvrdili su klasifikaciju plazmida IncP-1ε i IncN te dodatno IncL i IncFII skupini inkompatibilnosti koje karakterizira veliki potencijal širenja među različitim vrstama bakterija, posebice među klinički značajnim enterobakterijama (Adamczuk i sur., 2015.; Bonnin i sur., 2012.; Carattoli, 2009.). Što se tiče distribucije gena za otpornost na plazmidima, rezultati sekvenciranja pokazali su šarolik spektar gena lokaliziranih na istom plazmidu. Tako su se, primjerice, na plazmidu IncL1 (DW0_S1), veličine 74,49 kb porijeklom iz sedimenta Save s lokacije ispusta nalazili geni za otpornost na makrolide (*msr*E i *mph*D), β-laktame ($bla_{\text{TEM-1}}$), sulfonamide (*sul*1), ali i geni otpornosti na arsen (*ars*C, *ars*B, *ars*R i *ars*I). Drugi plazmid IncP1-ε, veličine 57,86 kb s iste lokacije (DW0_S2) nosio je gene za otpornost na makrolide (*msr*E i *mph*D), tetracikline (*tet*C) i sulfonamide (*sul*1). Suprotno tome, plazmid IncFII (DW0_S3), veličine 145,94 kb s iste lokacije bio je odgovoran za širenje drugih tipova gena odgovornih za otpornost na čak 6 skupina antibiotika uključujući β-laktame ($bla_{\text{TEM-186}}$), tetracikline (*tet*59), kloramfenikol (*cat*II), trimetoprim (*dfr*), aminoglikozide (*aph*(3")-Ib i *aph*(6)-Id) i sulfonamide (*sul*1). Zanimljivo je primijetiti da su svi navedeni geni uglavnom bili lokalizirani u genskim kazetama integrona skupine 1 ili transpozona koji su odgovorni za njihovu mobilizaciju i ugradnju u plazmide, čime se značajno olakšava njihovo daljnje širenje među okolišnim bakterijama. Slično tome, na plazmidima IncP1ε i IncN izoliranima iz potočnog sedimenta s lokacije ispusta (DW0_K1-3) nalazilo se od 3 do 5 različitih gena otpornosti. Pritom je zanimljivo primijetiti da se na plazmidu IncN, veličine 50,95 kb nalazilo 6 gena za otpornost na 5 skupina antibiotika uključujući tetracikline (*tet*C), β-laktame ($bla_{\text{TEM-1}}$), sulfonamide (*sul*2), trimetoprim (*dfr*A14) i aminoglikozide (*aph*(6)-Id i *aph*(3")-Ib). Važno je također naglasiti da su ovo, prema našim saznanjima, prvi podaci o smještaju gena *sul*2 na plazmidu IncN skupine. Spomenuti gen je do sada uglavnom pronađen na plazmidima s malim brojem kopija (engl. *Low Copy Plasmids*) kao što su primjerice IncA/C, IncFIB, IncF, IncK, IncI1, IncQ te IncZ plazmidi (Blau i sur., 2018.; Flach i sur., 2015.; Heuer i sur., 2009.; Jiang i

sur., 2019.; Rozwandowicz i sur., 2019.). Činjenica da gen *sul*2 nije povezan s integronima skupine 1 na spomenutom plazmidu IncN je u skladu s literaturnim podacima koji ne dovode taj gen u nikakvu vezu s integronskim strukturama (Bean i sur., 2009.; Domínguez i sur., 2019.; Vinué i sur., 2010.).

Zaključno se može reći da ispusti otpadnih voda iz lokalnih farmaceutskih industrija potiču mobilnost gena za otpornost na antibiotike putem plazmidnog prijenosa među bakterijama, čime se pojačava doseg horizontalnog širenja tih gena i na različita geografska područja, ali i ukazuje na rizik prijenosa otpornosti izvan okoliša. Dobiveni rezultati stoga potvrđuju hipotezu H3 kojom je pretpostavljeno da „Industrijski ispusti potiču širenje otpornosti na antibiotike među bakterijama u izloženim sedimentima".

4.3.3. Usporedba makrolidne otpornosti između okolišnih i kliničkih izolata

Analiza širenja otpornosti bila je nadalje usmjerena na proučavanje mogućeg širenja otpornosti na makrolide izvan okoliša, što predstavlja rizik za zdravlje ljudi. Ta analiza provedena je na tri skupine bakterijskih izolata, i to okolišnih izolata porijeklom iz sedimenta rijeke Save izrazito onečišćenog makrolidima (lokacija ispusta otpadnih voda Industrije 1) te izolata iz neonečišćenog sedimenta (referentna lokacija), kao i iz kliničkih izolata prikupljenih u Referentnom centru za praćenje otpornosti bakterija na antibiotike Ministarstva zdravstva pri Klinici za infektivne bolesti „Dr. Fran Mihaljević". Mehanizmi otpornosti na makrolide ispitani su u navedenim skupinama izolata PCR detekcijom 11 relevantnih gena. Geni koji su dominirali među okolišnim izolatima i kliničkim izolatima (*erm*B, *mph*E, *msr*D i *mef*A/E) analizirani su sekvenciranjem kako bi se dobio uvid u potencijalnu sličnost njihovih nukleotidnih sekvenci.

Analiza bakterijske populacije okolišnih izolata s lokacije ispusta koji su ispoljavali otpornost na azitromicin (124) ukazala je da njih 46,8 % posjeduje neki od analiziranih gena za makrolidnu otpornost, dok je samo 16,3 % izolata s referentne lokacije (17/104) imalo neki od ciljanih gena. Daljnjom analizom tih pozitivnih izolata pokazalo se da je u izolata s lokacije ispusta došlo do znatnog obogaćenja tri mehanizma makrolidne otpornosti. Pritom je dominirao mehanizam posredovan djelovanjem enzima 23S metiltransferaza koje su većinom kodirali geni *erm*B i *erm*F. Ti geni nađeni su u različitim Gram-negativnim i Gram-pozitivnim bakterijama uključujući i klinički relevantne *Streptococcus* vrste. Nadalje, prema zastupljenosti uslijedio je mehanizam posredovan zaštitnim proteinima ribosoma kodiranim uglavnom genom *msr*E te potom mehanizam posredovan djelovanjem enzima makrolidnih fosfotransferaza koje je kodirao gen *mph*E. Navedeni rezultati upućuju na zaključak da bi ta tri mehanizma mogla

predstavljati najefikasnije načine kojima si bakterije na lokaciji ispusta osiguravaju otpornost na vrlo visoke koncentracije makrolida. Iako je većinu analiziranih okolišnih izolata odlikovao jedan mehanizam otpornosti na makrolide, u izolata s lokacije ispusta zabilježena je veća pojava dvojnih mehanizama otpornosti u odnosu na izolate s referentne lokacije. Tako je naučestaliji dvojni mehanizam bio posredovan genskom kazetom *mph*E-*msr*E koja je većinom detektirana u Gram-negativnim izolatima iz koljena *Proteobacteria,* i to rodovima kao što su *Acinetobacter, Comamonas, Citrobacter, Rhodobacter* i dr. To ukazuje da sama aktivnost enzima fosfotransferaza (MphE) ili proteina koji štite ribosom od vezanja makrolida (MsrE) nije dovoljna za detoksikaciju bakterijskih stanica od azitromicina, već je potrebno njihovo združeno djelovanje da se osigura brza zaštita od visokih koncentracija makrolida. Budući da literaturni podaci (Blackwell i Hall, 2017.; Nonaka i sur., 2012.), kao i rezultati ovog rada, često povezuju navedene gene s plazmidima, može se pretpostaviti da je navedeni dvojni mehanizam stečeni oblik otpornosti među Gram-negativnim proteobakterijama. Osim toga, pojava trostrukih (*erm*F-*msr*E-*mph*E) i četverostrukih mehanizama (*mef*A-*msr*E-*mph*E-*hfl*X) zamijećena je isključivo kod izolata s lokacije ispusta, što upućuje na važnost stjecanja višestrukih mehanizama otpornosti u prilagodbi bakterija na uvjete života u kojima je okoliš pun antibiotika. Iako su makrolidi visoko djelotvorni na Gram-pozitivne bakterije poput streptokoka i stafilokoka, njihov spektar djelovanja obuhvaća i klinički relevantne Gram-negativne bakterije poput bordetela, legionela, klamidija, mikoplazmi, *Campylobacter* sp. i *Helicobacter pylori* (Eraković Haber, 2011.). Iako okolišni izolati koji ispoljavaju otpornost na azitromicin, a prevladavaju na lokaciji ispusta uglavnom ne spadaju u klinički relevantne patogene, oni ipak mogu predstavljati važne spremnike za prijenos gena makrolidne otpornosti u patogene, bilo unutar ili izvan okoliša.

Skupinu kliničkih izolata činilo je 90 klinički relevantnih bakterijskih patogena koji su pokazivali smanjenu osjetljivost na azitromicin, a većinom su pripadali rodu *Streptoccoccus* te u manjem udjelu rodu *Staphylococcus*. Pokazalo se da je većina tih izolata (56,7 %) posjedovala neki od analiziranih gena za makrolidnu otpornost te da su među njima prevladavali geni *mef*E i *mef*C. To ukazuje da su efluks pumpe iz porodice Mef bile dominantan mehanizam makrolidne otpornosti među kliničkim izolatima. Slično kao kod izolata s lokacije ispusta, preostala dva dominantna mehanizma uključivala su 23S metiltransferaze (prvenstveno gen *erm*B) i zaštitne proteine ribosoma (uglavnom gen *msr*D). Navedeni rezultati su u skladu s literaturnim navodima koji opisuju tri spomenuta mehanizma kao najčešće mehanizme makrolidne otpornosti među kliničkim stafilokokima i streptokokima (Fyfe i sur., 2016.). Nadalje, slično

okolišnim izolatima s lokacije ispusta, kliničke izolate također je odlikovala pojava višestrukih mehanizama makrolidne otpornosti (do 3 različita mehanizma), što ukazuje na sličan odgovor patogenih bakterija na visoke koncentracije makrolida. Međutim, za razliku od okolišnih izolata među kojima je prevladavala genska kazeta *msr*E-*mph*E, najčešći dvojni mehanizam među kliničkim streptokokima bio je posredovan genskom kazetom *msr*D-*mef*E koja se učestalo opisuje među kliničkim patogenima i osigurava visoki stupanj otpornosti na makrolide (Fyfe i sur., 2016.; Schroeder i Stephens, 2016.; Wierzbowski i sur., 2005.).

Daljnom usporedbom pojedinačnih gena otpornosti između okolišnih i kliničkih izolata pokazano je da je gen *erm*B prevladavao među kliničkim izolatima i izolatima s lokacije ispusta, dok na referentnoj lokaciji nije bio detektiran. To ukazuje na vjerojatnost da metilacija ribosoma posredovana genom *erm*B predstavlja učinkovitiji mehanizam otpornosti na makrolide pri njihovim visokim koncentracijama u usporedbi s drugim analiziranim mehanizmima. Toj tezi idu u prilog i literaturni podaci koji navode da geni *erm* ispoljavaju viši stupanj otpornosti na makrolide u odnosu na gene *msr* (van Duijkeren i sur., 2018.), što vjerojatno opravdava višu zastupljenost gena *erm* negoli gena *msr* među kliničkim izolatima i izolatima s lokacije ispusta izloženim visokim koncentracijama makrolida. Nadalje, detekcija gena *erm*B u šarolikim bakterijskim vrstama na lokaciji ispusta uključujući i klinički relevantne *Streptococcus* vrste, u skladu je s literaturnim navodima koji opisuju široku rasprostranjenost tog gena u okolišu pa i u okolišu izloženom otpadnim vodama iz proizvodnje antibiotika (Khan i sur., 2013.; Pal i sur., 2016b.). Filogenetskom analizom nukleotidnih sekvenci gena *erm*B koji je dominirao među okolišnim izolatima s lokacije ispusta i kliničkim izolatima utvrđena je visoka razina sličnosti (> 98 %) između njihovih nukleotidnih sekvenci, što ukazuje na mogući prijenos ovog gena između bakterija kliničkog i okolišnog podrijetla. Međutim, na temelju rezultata ovog rada nije moguće donijeti zaključak o putevima širenja tih gena izvan okoliša što čini dobru osnovu za buduća istraživanja.

Sažeto rečeno, rezultati dobiveni u ovom dijelu istraživanja ukazuju da riječni sediment izložen onečišćenju makrolidima putem otpadnih voda lokalne farmaceutske industrije predstavlja spremnik bakterija otpornih na azitromicin uključujući i klinički relevantne patogene iz roda *Streptococcus.* Visoka sličnost nukleotidnih sekvenci gena *erm*B između okolišnih i kliničkih izolata izloženih visokim razinama makrolida ukazuje na mogućnost širenja tih gena izvan okoliša, što ide u prilog već prihvaćenoj hipotezi H3.

4.4. Utjecaj industrijskih otpadnih voda na bakterijske zajednice izloženih sedimenata

Kao što je ranije spomenuto, istraživanjima provedenim u ovom radu utvrđeno je da otpadne vode lokalnih farmaceutskih industrija onečišćuju sedimente recipijentne rijeke i potoka antibioticima i metalima, što bi moglo negativno utjecati na prirodne bakterijske zajednice tih sedimenata (Grenni i sur., 2018.; Ibekwe i sur., 2016.). Iz tog razloga sljedeći korak istraživanja bio je ispitati kako prirodne bakterijske zajednice iz sedimenata s različitih lokacija duž rijeke i potoka te u različitoj sezoni (zima/ljeto) odgovaraju na industrijske ispuste. Te analize provedene su sekvenciranjem amplikona 16S rRNA gena tehnologijom Illumina Miseq i bioinformatičkom obradom dobivenih podataka u programu QIIME2. Dobiveni rezultati prikazani su pod rednim brojem 3 i 5 u popisu znanstvenih radova (str. 62-72 i str. 84-92).

Taksonomskom analizom bakterijskih zajednica u sedimentima rijeke Save uzvodno i nizvodno od industrijskog ispusta uočene su iznenađujuće male varijacije u raznolikosti bakterijskih vrsta između tih sedimenata, što ukazuje da širok spektar vrsta može preživjeti u okolišu pri visokim koncentracijama makrolida. Do sličnih su spoznaja došli i Kristiansson i sur. (2011.) koji opisuju podjednaku bioraznolikost sedimenata izrazito onečišćenih fluorokinolonima i referentnih, neonečišćenih sedimenata. Nasuprot tome, utvrđene su značajne promjene u sastavu i strukturi zajednica u sedimentu na lokaciji ispusta, ali ne i na nizvodnim lokacijama u usporedbi s referentnom lokacijom (UP7500). Sastav bakterijskih zajednica u sedimentu na referentnoj lokaciji i nizvodnim lokacijama bio je tipičan za riječni sediment, uz koljena *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* i *Acidobacteria* koja su dominirala (Ibekwe i sur., 2016.; Wang i sur., 2018.; Xie i sur., 2016.). Međutim, u sedimentu na lokaciji ispusta utvrđeno je značajno povećanje udjela koljena *Epsilonbacteraeota, Bacteroidetes* i *Firmicutes* koja su dominirala zajedno s koljenom *Proteobacteria.* Sličan taksonomski sastav zamijećen je i za otpadnu vodu tijekom obje sezone. Ti su rezultati u skladu s literaturnim opažanjima koja su potvrdila dominaciju navedena četiri koljena i u sedimentima onečišćenim flurokinolonima u Indiji (Kristiansson i sur., 2011.). Nadalje, na taksonomskoj razini roda, u sedimentu na lokaciji ispusta uočeno je povećanje udjela rodova koji potječu iz otpadne vode (*Macellibacteroidetes, Sulfuricurvum* i vadinBC27 grupa) te rodova koji su nađeni i u otpadnoj vodi i u sedimentu na referentnoj lokaciji (*Trichococcus, Thauera, Arcobacter*, *Pseudomonas*, *Variovorax* i *Comamonas*). To upućuje na akumulaciju bakterija iz otpadne vode u sedimentu na lokaciji ispusta, ali također i na proliferaciju autohtonih bakterija

sedimenta, vjerojatno uslijed izloženosti većem sadržaju hranjivih tvari te makrolida i metala, posebice Cu, što je u skladu s rezultatima redundancijske analize. Pored toga, na toj lokaciji utvrđena je značajna pozitivna korelacija između gena za makrolidnu otpornost (*msr*, *mph*, *mef*) i Gram-pozitivnih (*Macellibacteroidetes, Trichococcus*) te Gram-negativnih (*Thauera, Arcobacter, Variovorax, Pseudomonas*) bakterijskih rodova, što ukazuje da bi te bakterije mogle biti domaćini navedenih gena. To može biti posljedica, s jedne strane, urođene makrolidne otpornosti nekih ili svih navedenih bakterija ili pak stjecanja otpornosti horizontalnim prijenosom navedenih gena. Bakterije iz spomenutih rodova se u znanstvenoj literaturi često vežu uz uređaje za obradu otpadnih voda (Cyzdik-Kwiatkowska i Zielińska, 2016.; Dichosa i sur., 2015.; Zhang i sur., 2017.) i riječne sedimente onečišćene otpadnim vodama iz tih uređaja (Lu i Lu, 2014.; Martínez-Santos i sur., 2018.) te antibioticima (Nakayama i sur., 2017.) ili teškim metalima (Suhadolnik i sur., 2017.; Zhao i sur., 2014.). Međutim, udjeli tih bakterijskih rodova koji su nađeni značajno povišeni na ispustu naglo su se smanjili u sedimentima na nizvodnim lokacijama do razine njihove zastupljenosti na referentnoj lokaciji (< 1 %). Ta su opažanja bila u skladu s rezultatima nemetrijskog višedimenzijskog grupiranja (NMDS) koji su također ukazali na sličnu strukturu bakterijskih zajednica u uzorcima sedimenta sa referentne i nizvodnih lokacija tijekom obje sezone. Ti rezultati upućuju na oporavak bakterijskih zajednica u nizvodnim sedimentima, što je vjerojatno posljedica toga da ne dolazi do transporta bakterija s lokacije ispusta nizvodno. Nadalje, uzimajući u obzir podatak da sediment na lokaciji ispusta sadrži mnoštvo bakterija iz otpadne vode, moguće je da, u slučaju njihovog transporta nizvodno, te bakterije ne preživljavaju u tim sedimentima. To ne iznenađuje budući da većina bakterija iz otpadne vode nije dobro prilagođena uvjetima u sedimentu. Važno je također napomenuti da je struktura bakterijske zajednice u sedimentu sa uzvodne lokacije UP3500 iza utoka potoka Gorjak u Savu slična strukturi zajednice sa referentne lokacije (nije prikazano), što ukazuje da potok Gorjak nema značajan utjecaj na prirodnu zajednicu sedimenta u rijeci Savi. Osim navedenih prostornih promjena, zapažene su i sezonske promjene u zajednicama sedimenata koje bi mogle biti posljedica sezonskih varijacija u taksonomskom sastavu otpadne vode zajedno s promjenama fizikalno-kemijskih pokazatelja sedimenata.

Međutim, unatoč uočenom oporavku bakterijskih zajednica u nizvodnim sedimentima, relativna zastupljenost gena za makrolidnu otpornost bila je i dalje značajno viša na nizvodnim lokacijama u odnosu na referentnu lokaciju. To se može objasniti prisutnošću tih gena u obliku izvanstanične DNA i/ili njihovim horizontalnim prijenosom u nove domaćine. Budući da je

povećano horizontalno širenje gena za makrolidnu otpornost već potvrđeno u nizvodnim riječnim sedimentima u ovom radu, procijenjeno je da je postojanost tih gena na nizvodnim lokacijama vjerojatno posljedica njihova horizontalnog prijenosa među bakterijama.

Slično kao u sedimentu rijeke Save, i u sedimentu potoka Kalinovica u koji se ispuštaju otpadne vode Industrije 2 došlo je do značajnih prostornih promjena u strukturi i sastavu bakterijskih zajednica. Rezultati NMDS analize ukazali su na različitu strukturu zajednica na sve 3 istraživane lokacije (uzvodna, ispust, nizvodna) budući da su se zajednice s tih lokacija grupirale odvojeno. Nasuprot tome, sezonsko grupiranje nije zamijećeno, što ukazuje da se struktura bakterijskih zajednica na pojedinoj lokaciji ne mijenja promjenom sezone (zima/ljeto). Interesantno, sastav bakterijske zajednice sedimenta na lokaciji ispusta bio je sličniji taksonomskom sastavu otpadne vode negoli sastavu uzvodnog ili nizvodnog sedimenta. Tako su koljena *Firmicutes* i *Epsilonbacteraeota* koja su dominirala u otpadnoj vodi Industrije 2, ali i u drugim otpadnim vodama farmaceutskih industrija (Kristiansson i sur., 2011.; Li i sur., 2010.), bila značajno povišena u relativnom udjelu u sedimentu na lokaciji ispusta u odnosu na uzvodnu lokaciju tijekom obje sezone, što upućuje na akumulaciju bakterija iz otpadne vode u sedimentu. Međutim, u sedimentu na nizvodnoj lokaciji udio navedenih koljena značajno se smanjio (< 1 %) ukazujući na neuspješan prijenos ili slabo preživljavanje bakterija porijeklom iz otpadne vode u tim sedimentima. Nasuprot tome, u nizvodnom sedimentu došlo je do značajnog povećanja udjela drugih koljena poput *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, *Gemmatimonadetes*, *Latescibacteria* i razreda *α-Proteobacteria* u usporedbi s uzvodnom lokacijom i lokacijom ispusta. Takav promijenjen taksonomski sastav sedimenta na nizvodnoj lokaciji može biti posljedica izloženosti bakterija tog sedimenta visokim razinama sulfonamida (ukupno do 1,2 mg kg^{-1} tijekom obje sezone), ali i drugim eventualno prisutnim onečišćivalima uključujući metale. Pored toga, okolišni faktori poput različitih hranjivih tvari i osobina staništa nizvodne lokacije mogu također pridonijeti takvom promijenjenom sastavu zajednice. Međutim, unatoč promjenjenom taksonomskom sastavu sedimenta s lokacije ispusta i nizvodne lokacije, nisu uočene značajne promjene u bioraznolikosti tih sedimenata u odnosu na sediment uzvodne lokacije, što ukazuje na prilagodbu širokog spektra okolišnih bakterija na onečišćenje iz farmaceutskih pogona. Kao što je ranije spomenuto, slična zapažanja zabilježena su u sedimentima rijeke Save u ovom radu te u drugim istraživanjima toga tipa u Aziji (Kristiansson i sur., 2011.; Li i sur., 2010.). Budući da literaturni podaci navode važnu ulogu sastava bakterijskih zajednica u oblikovanju profila otpornosti na antibiotike u okolišu onečišćenom antibioticima (Forsberg i sur., 2014.; Su i sur., 2015.), detaljnije smo proučili povezanost

varijacija u količini analiziranih gena otpornosti sa dinamikom bakterijske zajednice. Rezultati provedene mrežne analize (engl. *Network Analysis*) ukazali su na povezanost nekih klinički relevantnih gena otpornosti i bakterija obogaćenih u sedimentu na lokaciji ispusta. Tako je, primjerice, uočena pozitivna korelacija rodova *Azoarcus* i *Aeromonas* s genom za otpornost na β-laktame bla_{GES} te roda *Sulfuricurvum* s genom bla_{OXA-2}. Dosadašnja istraživanja ukazala su na plazmidni smještaj gena bla_{GES} u vrsti *Aeromonas* spp. izoliranoj iz rijeke (Girlich i sur., 2011.) i uređaja za obradu komunalnih otpadnih voda (Piotrowska i sur., 2017.), što upućuje na to da bi rod *Aeromonas* mogao biti važan spremnik gena za β-laktamaze proširenog spektra u okolišu (Harnisz i Korzeniewska, 2018.). Pored toga, uočeno je da su pojedini bakterijski rodovi pozitivno korelirali s više različitih gena za antibiotičku otpornost kao npr. rod *Arcobacter* s genima bla_{GES}, *tet*39 i *erm*B, rod *Acinetobacter* s genima bla_{GES}, bla_{OXA-2} i *tet*39 te rod *Shewanella* s genima bla_{GES}, bla_{OXA-1}, *tet*39 i *erm*B. Svi su ti rodovi, zajedno s rodom *Aeromonas*, okarakterizirani kao oportunistički ljudski patogeni (Ferreira i sur., 2015.; Janda, 2014.; Janda i Abbott, 2010.; Wong i sur., 2017.), što upućuje na to da industrijski ispusti povećavaju prevalenciju patogenih bakterija koje nose višestruke i klinički relevantne gene za antibiotičku otpornost u potočnom sedimentu. To nadalje ukazuje na rizik mogućeg prijenosa tih patogena otpornih na više skupina antibiotike na ljude. Međutim, od tri gena za otpornost koji su nađeni značajno povišeni na nizvodnoj lokaciji u odnosu na uzvodnu (*sul*2, *mph*G i *msr*E), pozitivna korelacija uočena je samo između gena *sul*2 i bakterija iz porodice *Sphingomonadaceae,* za koje se smatra da su sklone stjecanju gena *sul*, a time i otpornosti na sulfonamide (Narciso-da-Rocha i sur., 2014.; Vaz-Moreira i sur., 2011.). Međutim, važno je istaknuti da mnoge bakterije koje su nađene u značajno višem udjelu u sedimentu nizvodne lokacije u odnosu na uzvodnu lokaciju i lokaciju ispusta nije bilo moguće klasificirati do razine roda ili vrste.

Zaključno se može reći da se na temelju rezultata dobivenih ovim dijelom istraživanja hipoteza H4 „Industrijski ispusti mijenjaju strukturu i sastav izloženih prirodnih bakterijskih zajednica“ prihvaća.

ZAKLJUČCI

5. ZAKLJUČCI

1. Otpadne vode lokalnih farmaceutskih industrija uključenih u proizvodnju azitromicina i formulaciju veterinarskih lijekova predstavljaju značajne točkaste izvore antibiotika i bakterija otpornih na te antibiotike. Koncentracije pojedinačnih antibiotika u tim otpadnim vodama ocijenjene su kao selektivne za razvoj otpornosti na antibiotike. Pritom su znatno niže koncentracije antibiotika utvrđene u otpadnim vodama iz formulacije lijekova (μg L^{-1}) u usporedbi s koncentracijama u otpadnim vodama iz proizvodnje azitromicina (mg L^{-1}).
2. Onečišćenje rijeke Save makrolidima, metalima te determinantama makrolidne otpornosti ovisi dominantno o ispustima otpadnih voda iz proizvodnje azitromicina, dok drugi lokalni izvori vezani uz terapijsku primjenu makrolida manjim dijelom pridonose tom onečišćenju. Kemijsko i mikrobiološko opterećenje potoka Kalinovica ne ovisi dominantno o ispustima otpadnih voda iz formulacije veterinarskih lijekova, već se radi i o doprinosu sa uzvodnog područja zbog odlaganja otpada ili ispiranja s tla gnojenog stajskim gnojivom.
3. Industrijske otpadne vode i sedimenti njihovih recipijentnih voda su spremnici poznatih i potencijalno novih gena za otpornost na makrolide odnosno β-laktame, 2,4-diaminopirimidine, sulfonamide i tetracikline. Česta povezanost identificiranih gena s pokretnim genetičkim elementima poput plazmida i integrona skupine 1 te genima za otpornost na istu ili druge skupine antibiotika ukazuje na potencijal zajedničke mobilnosti višestruke otpornosti među bakterijama.
4. Relativna zastupljenost ciljanih gena za otpornost na antibiotike značajno je povišena u sedimentima izloženim industrijskim ispustima u usporedbi sa sedimentima referentne lokacije uzvodno od ispusta. Pritom je puno izraženiji utjecaj industrijskog ispusta utvrđen za riječne negoli potočne sedimente zbog nižih pozadinskih razina istraživanih gena u samoj rijeci.
5. Industrijski ispusti potiču širenje gena za otpornost na antibiotike putem plazmidnog prijenosa među bakterijama sedimenta. Promiskuitetni plazmidi, naročito plazmidi IncP-1 skupine inkompatibilnosti velikog potencijala širenja među širokim rasponom bakterija, mogu bitno pridonijeti prilagodbi lokalnih bakterija na okoliš prepun antibiotika. Ti plazmidi često nose višestruke gene za otpornost na više različitih skupina antibiotika u sklopu genskih kazeta integrona skupine 1.

6. Utvrđena visoka sličnost nukleotidnih sekvenci gena *erm*B između okolišnih bakterijskih izolata porijeklom iz sedimenta rijeke Save izrazito onečišćenog makrolidima i kliničkih izolata streptokoka ukazuje na mogući prijenos ovog gena između bakterija kliničkog i okolišnog podrijetla.
7. Utvrđena slična bioraznolikost sedimenata s lokacija industrijskih ispusta te uzvodno i nizvodno od tih ispusta ukazuje da širok spektar bakterija može preživjeti u okolišu pri razmjerno visokim koncentracijama antibiotika.
8. Značajno različit sastav bakterijskih zajednica, potvrđen u sedimentima na obje istraživane lokacije ispusta u usporedbi s referentnom lokacijom, upućuje na unos bakterija otpadnom vodom te selekciju bakterija iz sedimenta. Sezonske promjene taksonomskog sastava, potvrđene samo u riječnom sedimentu, uzrokovane su promjenama u sastavu otpadne vode i kemijskim značajkama sedimenta. Oporavak bakterijskih zajednica potvrđen je u riječnim sedimentima nizvodno od ispusta, za razliku od potočnog sedimenta kojeg i dalje karakterizira značajno promjenjen sastav na nizvodnoj lokaciji.

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PRILOZI

7. PRILOZI

7.1. Dodatne informacije uz rad:

Bielen A, Šimatović A, Kosić-Vukšić J, Senta I, Ahel M, Babić S, Jurina T, González Plaza JJ, **Milaković M**, Udiković-Kolić N: Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries. Water Research 126 (2017): 79-87. doi: 10.1016/j.watres.2017.09.019

Tablica 1. Fizikalno-kemijski pokazatelji i popis međunarodno standardiziranih (ISO) metoda korištenih pri analizi otpadnih voda iz farmaceutskih industrija. Oznaka tablice u radu **Table S1**.

Parameter	Unit	ISO standard	Methodology
pH		HRN EN ISO 10523:2012	Electrochemistry
Conductivity	µS/cm	HRN EN 27888:2008	Electrochemistry
Chemical oxygen demand (COD)	mg/L O_2	DIN 38409 T41:1980	Electrochemistry
Biochemical oxygen demand (BOD_5)	mg/L O_2	HRN EN 1899-1:2004	Electrochemistry
Total organic carbon (TOC)	mg/L	HRN EN 1484:2002	IR spectrometry
Orthophosphate, Total phosphorus	mg/L P	HRN EN ISO 6878:2008	Spectrophotometry
Nitrite, N	mg/L	HRN EN ISO 10304-1:2009; HRN EN ISO 10304-1:2009/Ispr.1:2012	Ion chromatography
Nitrate, N	mg/L		
Sulfate, SO_4^{2-}	mg/L		
Ammonium, N	mg/L	HRN EN ISO 14911:2001	Ion chromatography

Tablica 2. Fizikalno-kemijski pokazatelji otpadnih voda Industrije 1 i 2 koje se ispuštaju u površinske vode rijeke Save i potoka Kalinovica. Oznaka tablice u radu **Table S2**.

Parameters	Company 1 sampling campaigns				Company 2 sampling campaigns				Maximal permitted value[a]
	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	
Temperature °C	13.5	17.2	21.1	14.5	11	12.7	20.7	16.7	30
pH	8	7.3	7.8	8.3	7.8	7.9	7.6	7.8	6.5 – 9
Conductivity µS/cm	3 590	2 085	2 303	2 905	1 678	1 837	2 153	1 288	-
Orthophosphate mg P/L	6.32	2.43	4.39	<0.03	0.54	0.28	0.005	0.16	-
Sulfates mg SO_4^{2-}/L	176.4	233.3	123.3	219.6	68.0	24.5	26.0	36.3	250
Ammonium mg N/L	44.5	0.6	27.1	51.6	3.0	2.8	4.3	5.1	-

[a] maksimalno dozvoljene koncentracije prema Pravilniku o graničnim vrijednostima emisije otpadnih voda NN 80/13 (43/14, 27/15, 3/16)

Tablica 3. Fiziološki letalni učinci izmjereni 48 i 72 hpf (sati nakon oplodnje) nakon izlaganja embrija *D. rerio* nerazrjeđenim te 2x i 4x razrjeđenim uzorcima industrijskih otpadnih voda. Statistička analiza provedena je pomoću *t* testa uz razinu značajnosti $p \leq 0.05$. Kontrolni embriji su bili izloženi destiliranoj vodi. Oznaka tablice u radu **Table S3**.

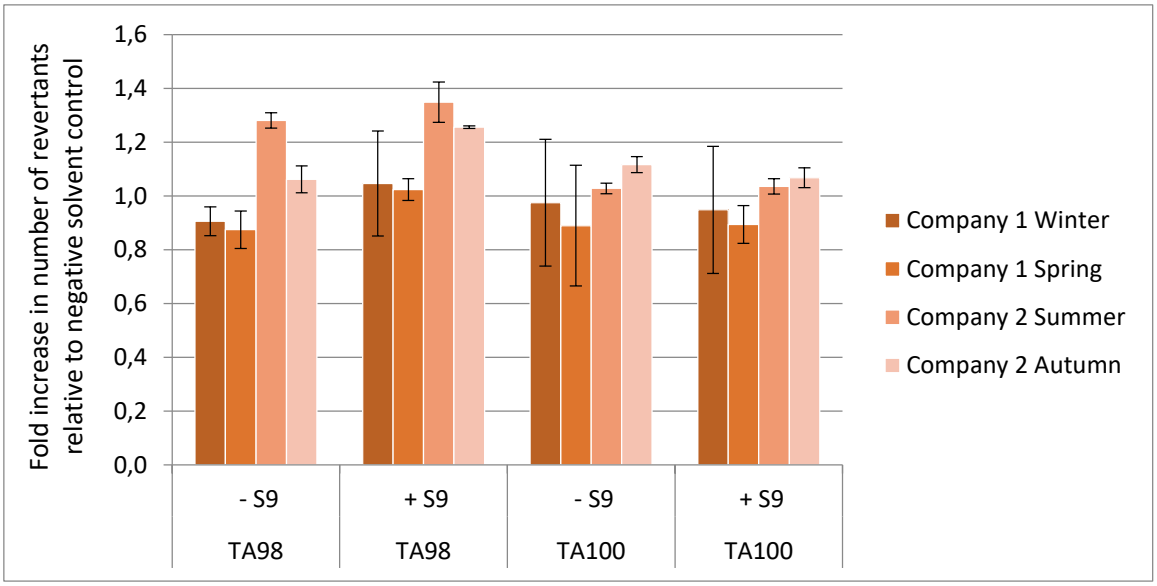
			Measured endpoint		
		Sample dilution	**Heart beat rate (beats / 15 sec)**	**Pigmentation formation (scored 1-3)**	**Hatching rate (%)**
Company 1 Winter	**48 hpf**	Control	35.00 ± 0.82	2.93 ± 0.27	-
		Diluted 2 x	23.60 ± 5.10*	1.71 ± 1.38*	-
		Diluted 4 x	26.25 ± 5.56*	1.82 ± 1.24*	-
	72 hpf	Control	36.00 ± 0.71	2.91 ± 0.29	100.00 ± 0.00
		Diluted 2 x	29.67 ± 3.51*	2.00 ± 1.41	58.33 ± 4.12*
		Diluted 4 x	32.75 ± 2.29*	2.00 ± 1.07	73.63 ± 3.87*
Company 1 Spring	**48 hpf**	Control	35.60 ± 0.89	2.94 ±0.25	-
		Diluted 2 x	37.50 ± 3.46	2.55 ± 0.93	-
		Diluted 4 x	32.41 ± 4.83	2.53 ± 0.85	-
	72 hpf	Control	36.14 ± 0.69	2.96 ± 0.12	100 ± 0.00
		Diluted 2 x	40.86 ± 2.48*	2.46 ± 0.14	33.00 ± 3.94*
		Diluted 4 x	43.17 ± 2.14*	2.54 ± 0.21	62.50 ± 2.80*
Company 2 Summer	**48 hpf**	Control	36.40 ± 0.55	2.92 ± 0.26	-
		Undiluted	44.20 ± 1.64*	2.60 ± 0.55	-
		Diluted 2 x	42.00 ± 3.40*	2.67 ± 0.50	-
		Diluted 4 x	38.80 ± 1.64	2.75 ± 0.46	-
	72 hpf	Control	36.33 ± 0.82	3.00 ± 0.00	100 ± 0.00
		Undiluted	45.25 ± 0.5*	2.60 ± 0.55	100 ± 0.00
		Diluted 2 x	46.00 ± 1.26*	2.67 ± 0.50	100 ± 0.00
		Diluted 4 x	44.83 ± 1.15*	2.75 ± 0.46	100 ± 0.00
Company 2 Autumn	**48 hpf**	Control	36.17 ± 0.75	3.00 ± 0.00	-
		Undiluted	40.20 ± 2.49*	2.67 ± 0.52	-
		Diluted 2 x	40.83 ± 1.83*	2.75 ± 0.46	-
		Diluted 4 x	40.80 ± 1.10*	2.80 ± 0.45	-
	72 hpf	Control	36.17 ± 0.75	3.00 ± 0.00	100 ± 0.00
		Undiluted	43.83 ± 1.17*	2.63 ± 0.51	40.00 ± 9.43*
		Diluted 2 x	43.80 ± 0.84*	3.00 ± 0.00	90.00 ± 4.71
		Diluted 4 x	44.83 ± 1.17*	3.00 ± 0.00	96.67 ± 4.71

* statistički značajna razlika u odnosu na kontrolu

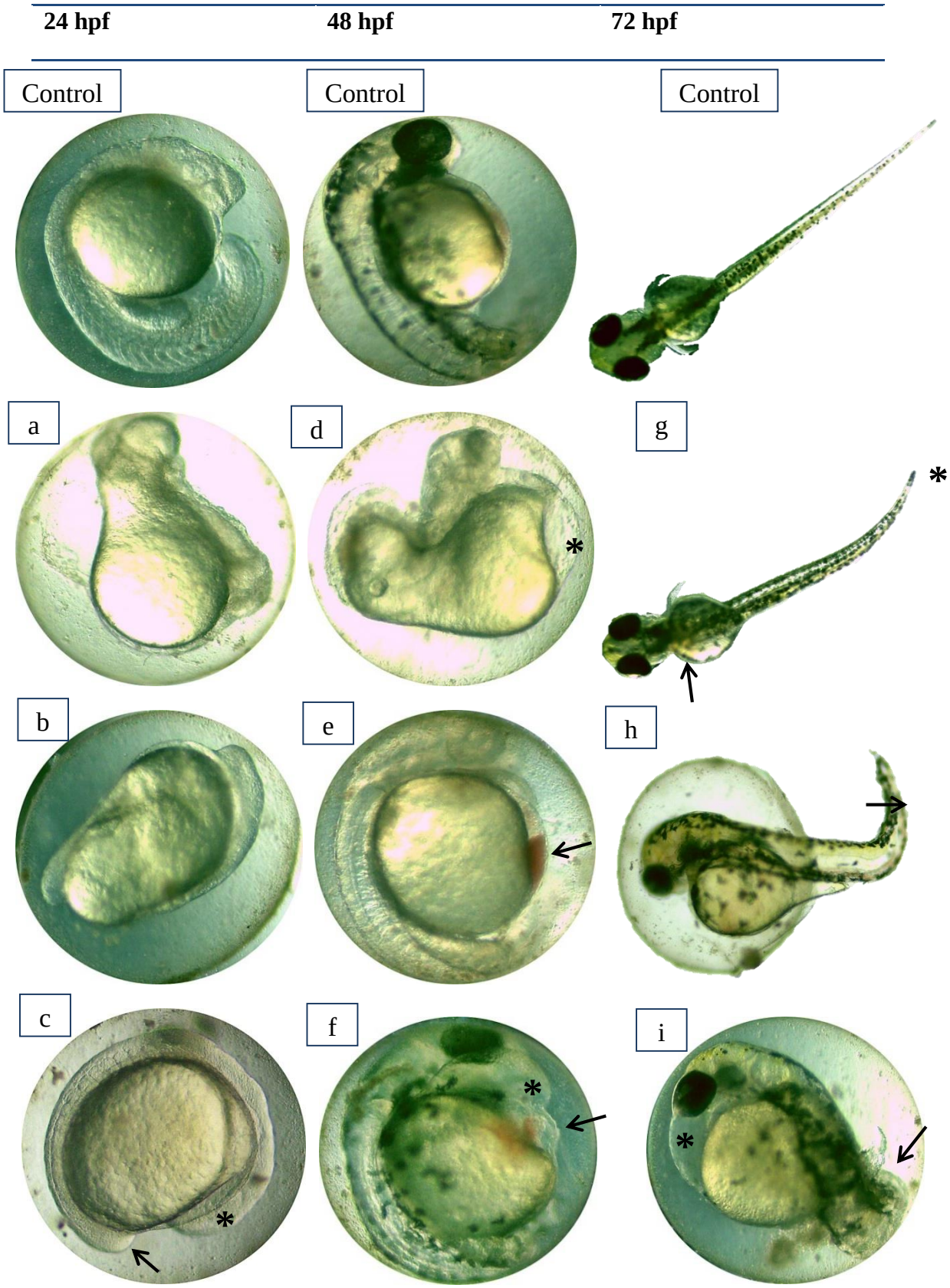
Tablica 4. Vrijednosti akutne toksičnosti antibiotika prisutnih u otpadnim vodama Industrije 1 2 za slatkovodne organizme. Oznaka tablice u radu **Table S4**.

Anti-microbial class	**Compound**	**Acronym**	**Test organism**	**EC50 (μg/L)**	**Reference**
Sulfonamides	Sulfadiazine	SDZ	*algae*	7 800	(Lützhøft et al. 1999)
			daphnid	112 000	(Sanderson and Thomsen 2009)
			fish	n.a.	
	Sulfamethazine	SMZ	*algae*	8 700	(Yang et al. 2008)
			daphnid	202 000	(De Liguoro et al. 2009)
			fish	> 100 000	(Sanderson and Thomsen 2009)
	Sulfamethoxazole	SMX	*algae*	30	(Sanderson and Thomsen 2009)
			daphnid	10 000	(Sanderson and Thomsen 2009)
			fish	n.e. 1 000 000	(Isidori et al. 2005)
Diamino-pyridines	Trimethoprim	TMP	*algae*	110 000	(Sanderson and Thomsen 2009)
			daphnid	> 100 000	(Sanderson and Thomsen 2009)
			fish	> 100 000	(Halling-Sørensen 2000)
Fluoroquionolones	Norfloxacin	NOR	*algae*	18 000	(Yang et al. 2008)
			daphnid	527 500	(Pan et al. 2017)
			fish	n.a.	
	Ciprofloxacin	CIP	*algae*	3 000	(Sanderson and Thomsen 2009)
			daphnid	60 000	(Sanderson and Thomsen 2009)
			fish	> 10 000	(Robinson et al. 2005)
	Enrofloxacin	ENR	*algae*	3 100	(Robinson et al. 2005)
			daphnid	> 10 000	(Robinson et al. 2005)
			fish	> 10 000	(Robinson et al. 2005)
Macrolides	Azithromycin	AZI	*algae*	19	(Harada et al. 2008)
			daphnid	120 000	(Sanderson and Thomsen 2009)
			fish	n.a.	
	Erythromycin	ERY	*algae*	20	(Sanderson and Thomsen 2009)
			daphnid	940	(Sanderson and Thomsen 2009)
			fish	80 000	(Sanderson and Thomsen 2009)
Tetra-cyclines	Oxytetracycline	OTC	*algae*	170	(Sanderson and Thomsen 2009)
			daphnid	18 700	(Sanderson and Thomsen 2009)
			fish	62 500	(Sanderson and Thomsen 2009)

n.e. – nema efekta; n.a. – nije dostupno



Slika 1. Potencijalni genotoksični utjecaji otpadnih voda Industrije 1 2. Rezultati su prikazani kao omjer broja *Salmonella typhimurium* TA98 i TA100 revertanata nakon izlaganja otpadnim vodama i broja spontatnih revertanata nakon izlaganja destiliranoj vodi (negativna kontrola). Testovi su provedeni u prisustvu ili odsustvu metaboličkog aktivatora (frakcija S9 jetre štakora). Barovi predstavljaju srednje vrijednosti tri pokusa ± SD. Oznaka slike u radu **Figure S1**.

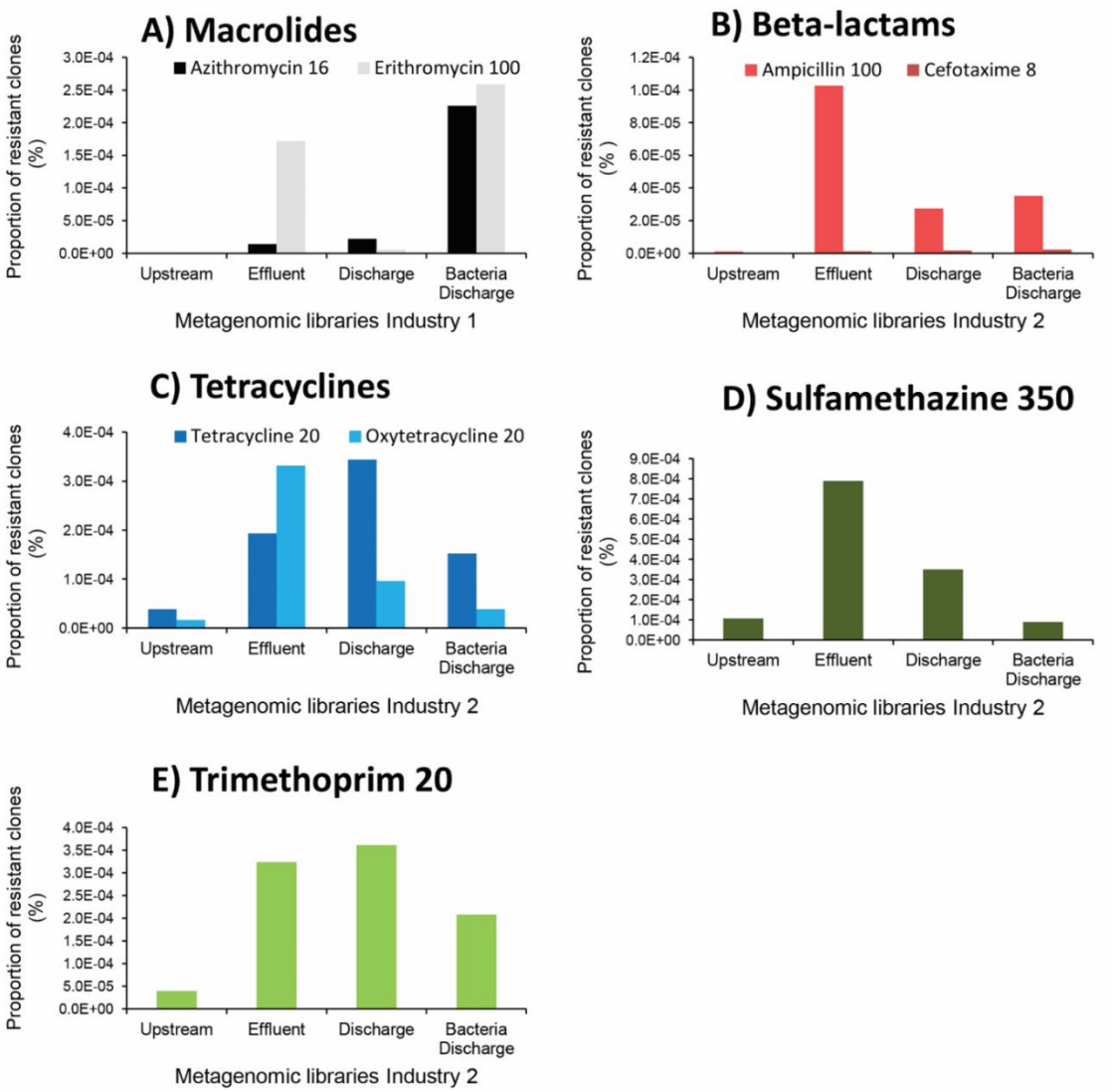


Slika 2. Deformacije embrija zebrica izloženih seriji razrjeđenja otpadnih voda iz farmaceutskih industrija nakon 24, 48 i 72 hpf (sati nakon oplodnje). Kontrolni embriji predstavljaju normalno razvijene embrije nakon 24, 48 i 72 hpf. a) deformacija cijelog embrija;

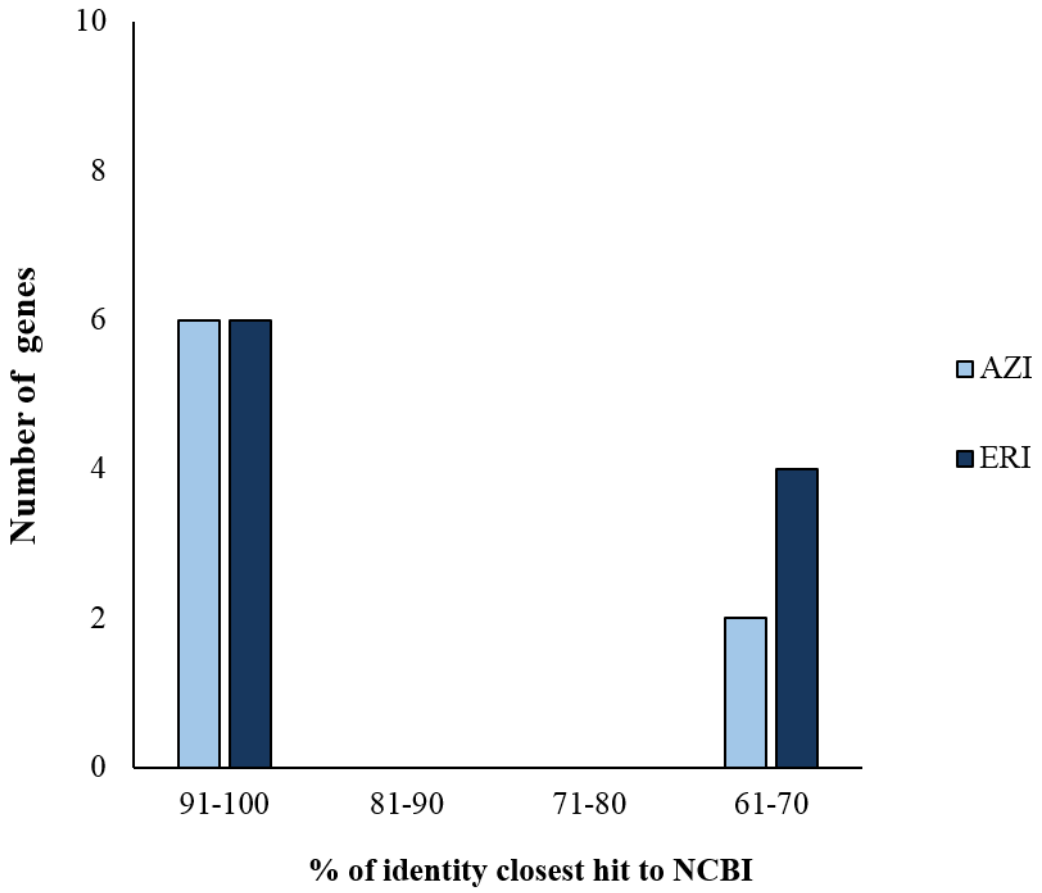
b) nerazvijena regija glave i repa, deformacija žumanjka i nerazvitak somita; c) nerazvijena regija glave (zvjezdica) i neodvajanje repa (strelica); d) perikardijalni edem (zvjezdica), nerazvijena i nepravilna regija glave i repa, deformitet žumanjka i nedostatak pigmentacije; e) zgrušavanje krvi (strelica) i nedostatak pigmentacije; f) perikardijalni edem (zvjezdica) i edem žumanjčane vreće (strelica); g) izlegla riba sa savijenom kralježnicom (zvjezdica) i neformiranom perajom (strelica); h) nepravilno oblikovana kralježnica (strelica); i) neizlegla riba sa perikardijalnim edemom (zvjezdica), deformacijom kralježnice (strelica) i kašnjenjem ili anomalijom u absorpciji žumanjčane vreće. Oznaka slike u radu **Figure S2**.

7.2. Dodatne informacije uz rad:

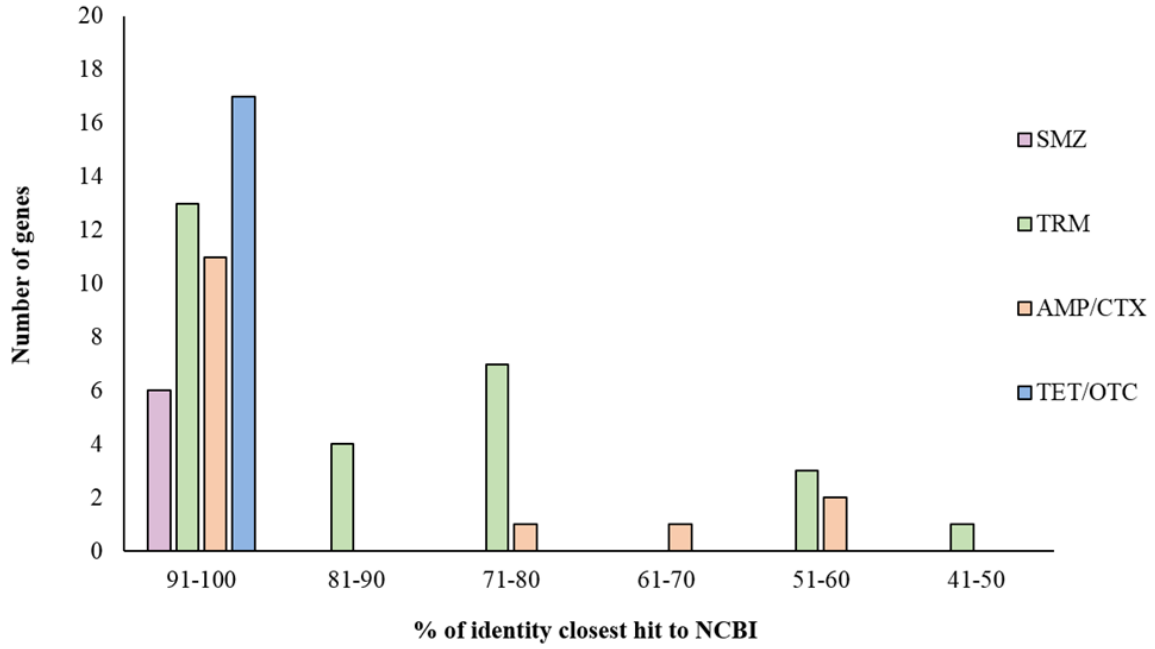
González Plaza JJ, Šimatović A, **Milaković M**, Bielen A, Wichmann F, Udiković Kolić N: Functional repertoire of antibiotic resistance genes in antibiotic manufacturing effluents and receiving freshwater sediments. Frontiers in Microbiology 8 (2018): 2675. doi: 10.3389/fmicb.2017.02675



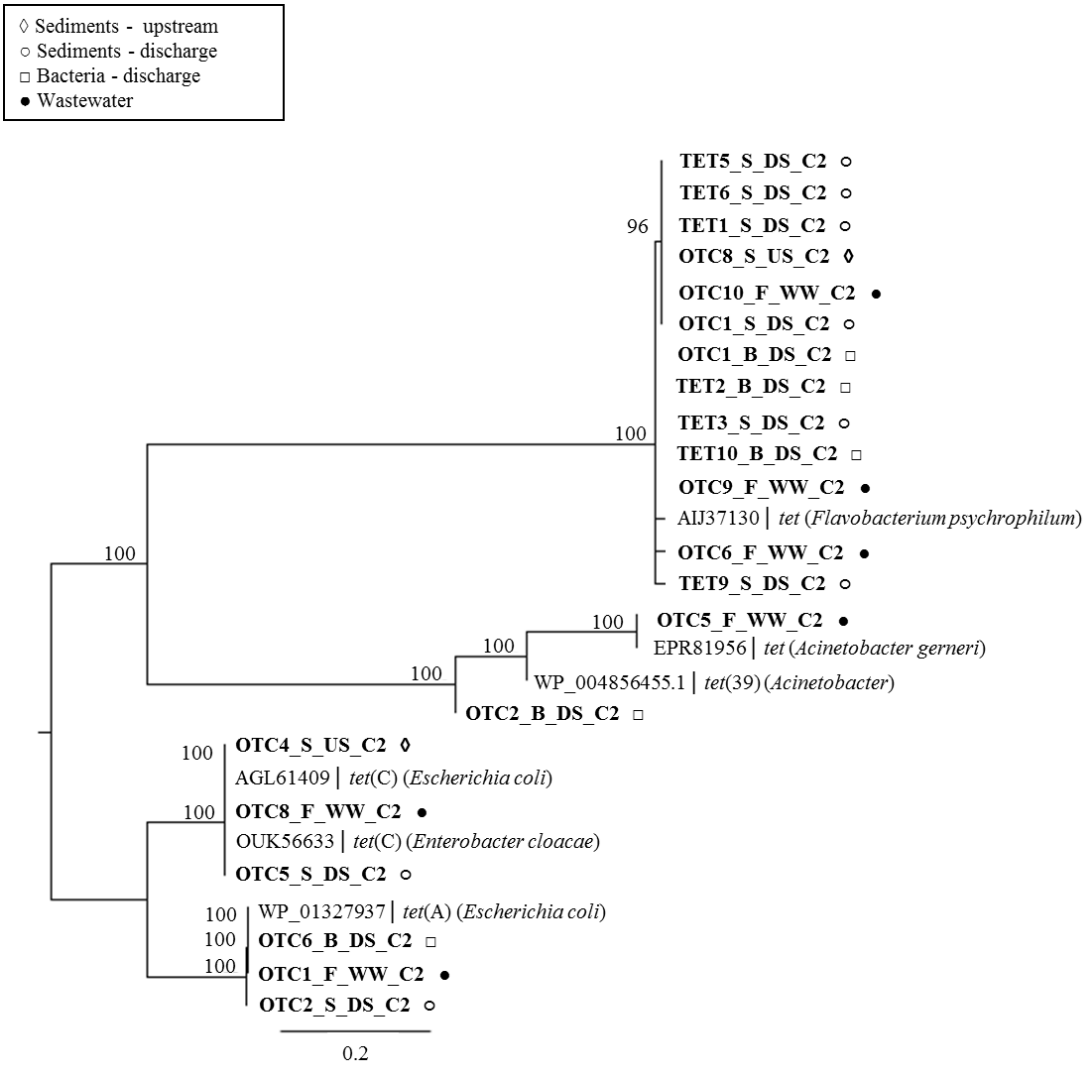
Slika 3. Udio metagenomskih klonova (%) otpornih na antibiotike dobivenih pomoću funkcionalne metagenomike iz otpadne vode Industrije 1 i sedimenata rijeke Save (**A**) te otpadne vode Industrije 2 i sedimenata potoka Kalinovica (**B, C, D, E** i **F**). Antibiotici i koncentracija antibiotika (mg L^{-1}) korišteni za karakterizaciju knjižnica navedeni su iznad svakog grafa. Udio klonova otpornih na antibiotike u svakoj knjižnici prikazan je kao omjer klonova otpornih na antibiotike i ukupnog broja klonova. Oznaka slike u radu **Supplementary Figure 1**.



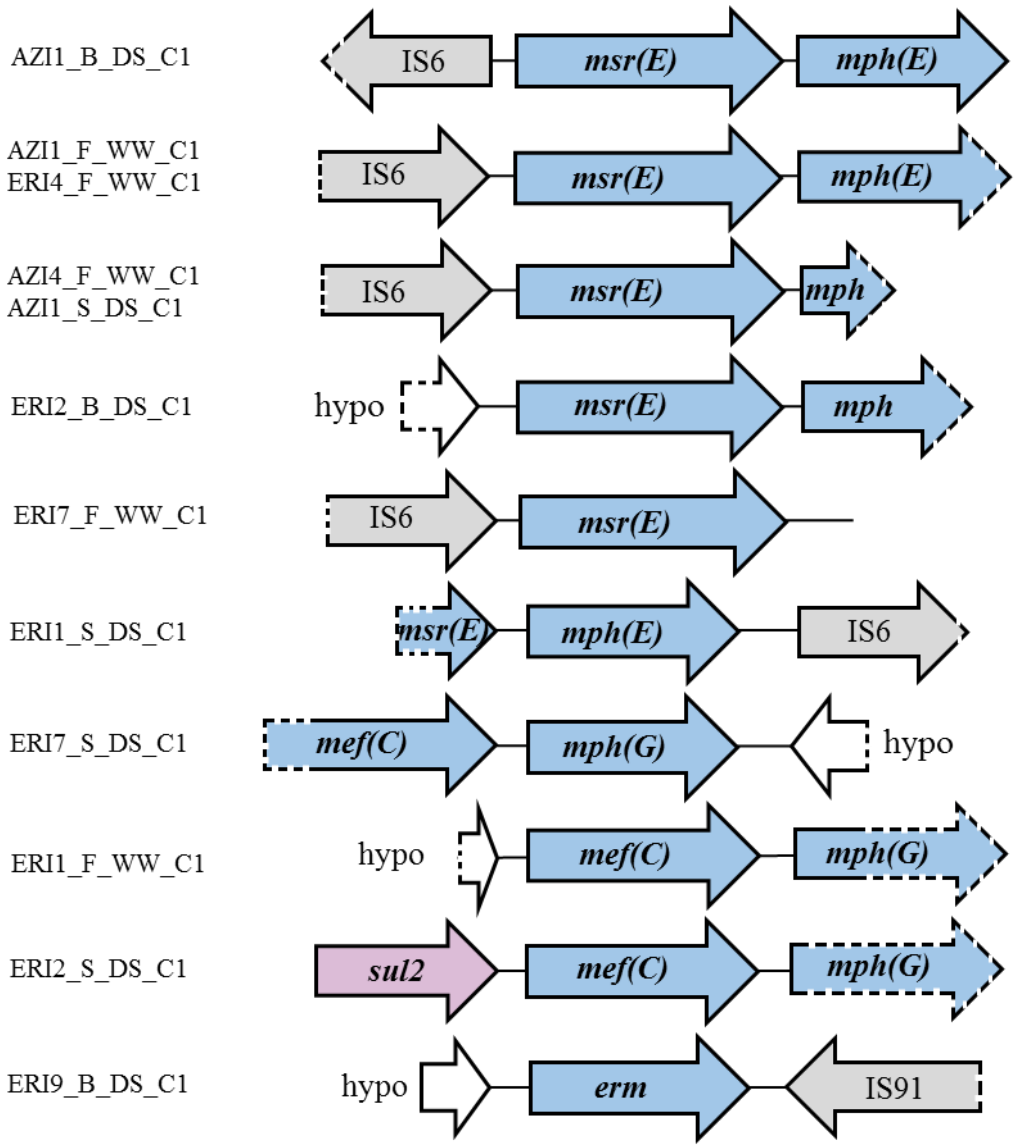
Slika 4. Raspodjela postotka sličnosti proteinskih sekvenci gena za otpornost na azitromicin (AZI) i eritromicin (ERI) u usporedbi sa sekvencama u NCBI bazi. Oznaka slike u radu **Supplementary Figure 2.**



Slika 5. Raspodjela sličnosti proteinske sekvence gena za otpornost na antibiotike iz skupine sulfonamida (sulfametazin, SMZ), 2,4-diaminopirimidina (trimetoprim, TMP), β-laktama (ampicilin, AMP; cefotaksim, CTX) i tetraciklina (tetraciklin, TET; oksitetraciklin, OTC) u odnosu na sekvence pohranjene u NCBI bazi. Oznaka slike u radu **Supplementary Figure 3**.



Slika 6. Filogenetsko stablo proteinskih sekvenci gena za otpornost na tetraciklin (TET) i oksitetraciklin (OTC). Najbolji BLAST rezultati i reprezentativne proteinske sekvence gena preuzete su iz NCBI baze podataka. Stablo je izgrađeno metodom maksimalne sličnosti (engl. *Maximum Likelihood*) u Geneious programu. Podrška za grananja unutar stabla određena je na temelju 1.000 ponavljanja, a prikazane su vrijednosti više od 80%. Oznaka slike u radu **Supplementary Figure 4**.



Slika 7. Okolne regije gena odgovornih za otpornost na makrolide iz metagenomskih knjižnica porijeklom iz otpadne vode Industrije 1 i recipijentnih sedimenata. Okviri čitanja gena (engl. *Open Reading Frame*, ORF) prikazani su strelicama. Plavom su bojom označeni geni za otpornost na makrolide, ljubičastom geni za otpornost na sulfonamide, sivom su bojom označeni pokretni genetički elementi, a bijelom hipotetski proteini. Isprekidani dijelovi strelica označavaju nepotpune sekvence. Oznaka slike u radu **Supplementary Figure 5.**

Tablica 5. Broj jedinstvenih gena za otpornost na antibiotike u metagenomskim knjižnicama porijeklom iz sedimenata sa uzvodne lokacije i lokacije ispusta te otpadne vode Industrije 1 (A) i Industrije 2 (B). Geni su klasificirani obzirom na mehanizam otpornosti za kojeg kodiraju. Oznaka tablice u radu **Supplementary Table 1**.

A)

Industry area 1		
Library	**Mechanism of macrolide resistance**	**Number of unique ARGS**
S_US_C1	Unknown- GTPase binding protein	**4**
F_WW_C1	Efflux pump	**1**
	Ribosomal protection	**3**
B_DS_C1	Efflux pump	**1**
	Macrolide inactivation	**1**
	Methylation of rRNA	**1**
	Ribosomal protection	**1**
S_DS_C1	Efflux pump	**1**
	Macrolide inactivation	**2**
	Ribosomal protection	**1**
	TOTAL:	**16**

B)

Industry area 2		
Library	**Mechanism of antibiotic resistance**	**Number of unique ARGs**
S_US_C2	Modification of target site - dihydropteroate synthase	**1**
	Modification of target site - dihydrofolate reductase	**4**
	Modification of target site - thymidylate synthase	**3**
	Efflux - tetracycline transporter	**2**
	Antibiotic inactivation - beta-lactamase	**2**
F_WW_C2	Modification of target site - dihydropteroate synthase	**1**
	Modification of target site - dihydrofolate reductase	**5**
	Modification of target site - thymidylate synthase	**1**
	Efflux - tetracycline transporter	**6**
	Antibiotic inactivation - beta-lactamase	**3**
B_DS_C2	Modification of target site - dihydropteroate synthase	**2**
	Modification of target site - dihydrofolate reductase	**3**
	Efflux - tetracycline transporter	**4**
	Antibiotic inactivation - beta-lactamase	**1**
S_DS_C2	Modification of target site - dihydropteroate synthase	**2**
	Modification of target site - dihydrofolate reductase	**11**
	Modification of target site - thymidylate synthase	**1**
	Efflux - tetracycline transporter	**5**
	Antibiotic inactivation - beta-lactamase	**9**
	TOTAL:	**66**

Tablica 6. Geni za otpornost na antibiotike iz metagenomskih knjižnica porijeklom iz otpadne vode Industrije 2 te sedimenata s uzvodne lokacije i lokacije ispusta potoka Kalinovica. Prikazani su klonovi koji ispoljavaju otpornost na sulfametazin (SMZ), trimetoprim (TMP), oksitetraciklin (OTC), tetraciklin (TET), ampicilin (AMP) i cefotaksim (CTX). MIC: minimalna inhibitorna koncentracija (engl. *Minimum Inhibitory Concentration*). Jedinstveni geni (nukleotidna sekvenca) iz iste knjižnice su označeni sa*. Oznaka tablice u radu **Supplementary Table 2**.

Antibiotic used for selection	**Clone designation / origin**	**MIC (mg L^{-1})**	**Gene length (bp)**	**Gene annotation (Closest BLASTX hit in NCBI)**	**% amino acid identity.**	**GenBank Accession #**
Sulfamethazine	SMZ2_S_US_C2/ Upstream sediment	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1) *	99	MG585961
	SMZ7_S_US_C2/ Upstream sediment	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1)	99	MG585962
	SMZ10_S_US_C2/ Upstream sediment	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1)	99	MG585963
	SMZ5_F_WW_C2/ Effluent	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1) *	99	MG585964
	SMZ6_F_WW_C2/ Effluent	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1)	99	MG585965
	SMZ10_F_WW_C2/ Effluent	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1)	99	MG585966
	SMZ1_B_DS_C2/ Sediment bacteria	>1 024 (SMZ)	852	Dihydropteroate synthase Sul2 (*Escherichia coli* HVH 213 EQU75297.1) *	99	MG585967
	SMZ5_B_DS_C2/ Sediment bacteria	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1) *	99	MG585968
	SMZ9_B_DS_C2/ Sediment bacteria	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1)	99	MG585969
	SMZ6_S_DS_C2/ Discharge sediment	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Acinetobacter baumannii* ADX02776.1) *	99	MG585970

	SMZ8_S_DS_C2/ Discharge sediment	>1 024 (SMZ)	927	Dihydropteroate synthase Sul1 (*Enterobacter cloacae* WP_012695459.1) *	99	MG585971
Trimethoprim	TRM2_S_US_C2/ Upstream sediment	512 (TRM)	795	Thymidylate synthase (*Bacteroidetes bacterium* GWB2_41_8 OFX59224) *	90	MG585972
	TRM5_S_US_C2/ Upstream sediment	>512 (TRM)	795	Thymidylate synthase (*Neisseria* sp. HMSC15C08 WP_070491626.1) *	80	MG585973
	TRM6_S_US_C2/ Upstream sediment	>512 (TRM)	504	Dihydrofolate reductase (*Nitrosomonas nitrosa* SFM09632.1) *	53	MG585975
	TRM7_S_US_C2/ Upstream sediment	>512 (TRM)	483	Dihydrofolate reductase (*Methylotenera versatilis* WP_047542178.1)*	74	MG585976
	TRM8_S_US_C2/ Upstream sediment	>512 (TRM)	528	Dihydrofolate reductase (*Lactonifactor longoviformis* WP_072849072.1) *	49	MG585977
	TRM10_S_US_C2/ Upstream sediment	>512 (TRM)	498	Dihydrofolate reductase (*Lentimicrobium saccharophilum* WP_062039092.1) *	57	MG585978
			795	Thymidylate synthase (*Bacteroidetes bacterium* GWA2_40_15 OFX42999.1) *	80	MG585974
	TRM2_F_WW_C2/ Effluent	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1) *	100	MG585979
	TRM6_F_WW_C2/ Effluent	>512 (TRM)	294	Dihydrofolate reductase DHFR2 (*Pseudomonas aeruginosa* CAQ52800.1) *	99	MG585986
	TRM8_F_WW_C2/ Effluent	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Salmonella enterica* NP_569370.1) *	100	MG585980
	TRM10_F_WW_C2/ Effluent	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Salmonella enterica* NP_569370.1) *	99	MG585981
	TRM13_F_WW_C2/ Effluent	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1)	100	MG585982

	TRM16_F_WW_C2/ Effluent	>512 (TRM)	486	Dihydrofolate reductase (*Flavobacterium sasangense* WP_026725269.1) *	93	MG585985
	TRM17_F_WW_C2/ Effluent	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Salmonella enterica* NP_569370.1)	100	MG585983
	TRM19_F_WW_C2/ Effluent	>512 (TRM)	843	Thymidylate synthase (*Acinetobacter lwoffii* WP_004729505.1) *	100	MG585984
	TRM1_B_DS_C2/ Sediment bacteria	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1) *	100	MG585987
	TRM2_B_DS_C2/ Sediment bacteria	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1)	100	MG585988
	TRM4_B_DS_C2/ Sediment bacteria	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1)	100	MG585989
	TRM5_B_DS_C2/ Sediment bacteria	>512 (TRM)	486	Dihydrofolate reductase (*Neisseria wadsworthii* WP_040666967.1) *	78	MG585992
	TRM6_B_DS_C2/ Sediment bacteria	>512 (TRM)	459	Dihydrofolate reductase DfrA12 (*Escherichia coli* WP_071846383.1) *	100	MG585991
	TRM7_B_DS_C2/ Sediment bacteria	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1)	100	MG585990
	TRM2_S_DS_C2/ Discharge sediment	512 (TRM)	483	Dihydrofolate reductase (*Flavobacterium sinopsychrotolerans* WP_091173155.1) *	84	MG585993
	TRM3_S_DS_C2/ Discharge sediment	>512 (TRM)	486	Dihydrofolate reductase (*Flavobacterium sasangense* WP_026725269.1) *	94	MG585994
	TRM4_S_DS_C2/ Discharge sediment	>512 (TRM)	510	Dihydrofolate reductase (*Ruminococcaceae bacterium* YRB3002 SCW70680.1) *	59	MG585995
	TRM5_S_DS_C2/ Discharge sediment	>512 (TRM)	795	Thymidylate synthase (*Bdellovibrio exovorus* WP_015470741.1) *	87	MG586006

			507	Dihydrofolate reductase (uncultured bacteria AIA18652.1) *	72	MG585996
	TRM6_S_DS_C2/ Discharge sediment	>512 (TRM)	702	Dihydrofolate reductase DfrA17 (*Klebsiella pneumoniae* WP_013023850.1) *	93	MG586003
	TRM7_S_DS_C2/ Discharge sediment	>512 (TRM)	477	Dihydrofolate reductase DfrA1 (*Citrobacter freundii* WP_053764311.1) *	99	MG586002
	TRM8_S_DS_C2/ Discharge sediment	512 (TRM)	486	Dihydrofolate reductase (*Flavobacterium sasangense* WP_026725269.1)	94	MG585997
	TRM9_S_DS_C2/ Discharge sediment	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Escherichia coli* YP_006953259.1) *	100	MG586004
	TRM10_S_DS_C2/ Discharge sediment	>512 (TRM)	498	Dihydrofolate reductase (*Paludibacter propionicigenes* WP_013444380) *	75	MG585998
	TRM11_S_DS_C2/ Discharge sediment	512 (TRM)	483	Dihydrofolate reductase (*Flavobacterium sinopsychrotolerans* WP_091173155.1)	84	MG585999
	TRM12_S_DS_C2/ Discharge sediment	>512 (TRM)	483	Dihydrofolate reductase (*Flavobacterium sinopsychrotolerans* WP_091173155.1) *	84	MG586000
	TRM14_S_DS_C2/ Discharge sediment	>512 (TRM)	483	Dihydrofolate reductase DfrA14 (*Salmonella enterica* NP_569370.1) *	100	MG586005
	TRM19_S_DS_C2/ Discharge sediment	>512 (TRM)	495	Dihydrofolate reductase (*Flavobacterium antarcticum* WP_022826597.1) *	79	MG586001
Oxytetracycline	OTC4_S_US_C2/ Upstream sediment	128 (OTC) 64 (TET)	1 191	Tetracycline MFS efflux pump (*Escherichia coli* AGL61409) *	100	MG586007
	OTC8_S_US_C2/ Upstream sediment	128 (OTC) 128 (TET)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	97	MG586008
	OTC1_F_WW_C2/ Effluent	512 (OTC) 256 (TET)	1 275	Tetracycline MFS efflux pump (*Escherichia coli* WP_01327937.1) *	100	MG586009

	OTC5_F_WW_C2/ Effluent	128 (OTC) 128 (TET)	1 122	Tetracycline MFS efflux pump (*Acinetobacter gerneri* EPR81956.1) *	100	MG586011
	OTC6_F_WW_C2/ Effluent	128 (OTC) 128 (TET)	1 317	Tetracycline efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	94	MG586012
	OTC8_F_WW_C2/ Effluent	128 (OTC) 64 (TET)	1 191	Tetracycline MFS efflux pump (*Enterobacter cloacae* OUK56633.1) *	100	MG586010
	OTC9_F_WW_C2/ Effluent	128 (OTC) 128 (TET)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	99	MG586013
	OTC10_F_WW_C2/ Effluent	64 (OTC) 64 (TET)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	97	MG586014
	OTC1_B_DS_C2/ Sediment bacteria	512 (OTC) 128 (TET)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	99	MG586029
	OTC2_B_DS_C2/ Sediment bacteria	128 (OTC) 128 (TET)	1 188	Tetracycline MFS efflux pump (*Acinetobacter* WP_004856455) *	99	MG586033
	OTC6_B_DS_C2/ Sediment bacteria	128 (OTC) 64 (TET)	1 275	Tetracycline MFS efflux pump (*Escherichia coli* WP_01327937.1) *	100	MG586032
	OTC1_S_DS_C2/ Discharge sediment	128 (OTC) 128 (TET)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	97	MG586015
	OTC2_S_DS_C2/ Discharge sediment	128 (OTC) 128 (TET)	1 275	Tetracycline MFS efflux pump (*Escherichia coli* WP_01327937.1) *	100	MG586022
	OTC5_S_DS_C2/ Discharge sediment	64 (OTC) 64 (TET)	1 191	Tetracycline MFS efflux pump (*Escherichia coli* AGL61409) *	100	MG586021
Tetracycline	TET2_B_DS_C2/ Sediment bacteria	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1)	99	MG586030
	TET10_B_DS_C2/ Sediment bacteria	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	98	MG586031

	TET1_S_DS_C2/ Discharge sediment	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1)	97	MG586016
	TET3_S_DS_C2/ Discharge sediment	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	99	MG586017
	TET5_S_DS_C2/ Discharge sediment	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1)	97	MG586018
	TET6_S_DS_C2/ Discharge sediment	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1)	99	MG586019
	TET9_S_DS_C2/ Discharge sediment	64 (TET) 64 (OTC)	1 320	Tetracycline MFS efflux pump (*Flavobacterium psychrophilum* AIJ37130.1) *	98	MG586020
Ampicillin	AMP1_S_US_C2/ Upstream sediment	>1 024 (AMP) <8 (CTX)	777	Class D beta-lactamase OXA-198 (*Pseudomonas aeruginosa* WP_094865115.1) *	70	MG586024
	AMP6_S_US_C2/ Upstream sediment	>1 024 (AMP) <8 (CTX)	828	Class D beta-lactamase OXA-10 (*Klebsiella pneumoniae* WP_020442392.1) *	99	MG586023
	AMP6_F_WW_C2/ Effluent	>1 024 (AMP) <0.25 (CTX)	849	Class D beta-lactamase (uncultured bacterium AMP47957.1) *	99	MG586025
	AMP7_F_WW_C2/ Effluent	>1 024 (AMP) <0.25 (CTX)	828	Class D beta-lactamase OXA-10 (*Klebsiella pneumoniae* WP_020442392.1) *	99	MG586028
	AMP11_F_WW_C2/ Effluent	>1 024 (AMP) <0.25 (CTX)	741	Subclass B1 metallo-beta-lactamase (*Flavobacterium plurextorum* WP_089058075) *	98	MG586026
	AMP18_F_WW_C2/ Effluent	>1 024 (AMP) <0.25 (CTX)	849	Class D beta-lactamase (uncultured bacterium AMP47957.1)	99	MG586027
	AMP2_S_DS_C2/ Discharge sediment	>1 024 (AMP) >16 (CTX)	864	Class A extended-spectrum beta-lactamase GES-1 (*Klebsiella pneumoniae* AAO32356.1) *	100	MG586040
	AMP3_S_DS_C2/ Discharge sediment	>1 024 (AMP) 8 (CTX)	1 152	Class C beta-lactamase CMY-10 (*Acinetobacter baumannii* EXB07206.1) *	99	MG586041

	AMP4_S_DS_C2/ Discharge sediment	>1 024 (AMP) >16 (CTX)	906	Class A beta-lactamase (*Pedobacter* sp. PACM 27299 ALL06350.1)*	59	MG586035
	AMP6_S_DS_C2/ Discharge sediment	>1 024 (AMP) <0.25 (CTX)	786	Class D beta-lactamase (*Desulforegula conservatrix* WP_027358149.1) *	55	MG586036
	AMP7_S_DS_C2/ Discharge sediment	>1 024 (AMP) <0.25 (CTX)	903	Class D beta-lactamase (uncultured bacterium AMP47162.1) *	100	MG586037
	AMP8_S_DS_C2/ Discharge sediment	>1 024 (AMP) 8 (CTX)	855	BJP beta-lactamase (uncultured bacterium AIA18864) *	74	MG586038
	AMP9_S_DS_C2/ Discharge sediment	512 (AMP) <0.25 (CTX)	876	Beta-lactamase OXA-1 precursor (*Acinetobacter baumannii* SCY69726.1) *	99	MG586043
	AMP10_S_DS_C2/ Discharge sediment	>1 024 (AMP) 32 (CTX)	900	Class A extended-spectrum beta-lactamase VEB-9 (*Pseudomonas aeruginosa* OOK92124.1) *	100	MG586042
	AMP12_S_DS_C2/ Discharge sediment	>1 024 (AMP) 8 (CTX)	741	Subclass B1 metallo-beta-lactamase (*Flavobacterium oncorhynchi* WP_089052251.1) *	97	MG586039
	AMP18_S_DS_C2/ Discharge sediment	1 024 (AMP) >0.25 (CTX)	876	Class D beta-lactamase OXA-1 (*Acinetobacter baumannii* SCY69726.1)	99	MG586044
Cefotaxime	CTX2_B_DS_C2/ Sediment bacteria	32 (CTX)	1 152	CMY-1/MOX family class C beta-lactamase MOX-9 (*Citrobacter freundii* AIG22447.1) *	98	MG586034

7.3. Dodatne informacije uz rad:

Milaković M, Vestergaard G, González Plaza JJ, Petrić I, Šimatović A, Senta I, Kublik S, Schloter M, Udiković Kolić N: Pollution from azithromycin-manufacturing promotes macrolide-resistance gene propagation and induces spatial and seasonal bacterial community shifts in receiving river sediments. Environment International 123 (2019): 501-511. doi: 10.1016/j.envint.2018.12.050

Tablica 7. Fizikalno-kemijski pokazatelji i međunarodno standardizirane (ISO) metode korištene za analizu riječnih sedimenata. Oznaka tablice u radu **Table S1**.

Parameter	ISO standard	Methodology
pH (H_2O)	HRN ISO 10390	Electrochemistry
Conductivity	HRN EN 27888:2008	Electrochemistry
Total organic carbon (TOC)	HRN EN 1484:2002	IR spectrometry
Total carbon (TC)	HRN EN 1484:2002	IR spectrometry
Total nitrogen (TN)	HRN ISO 1871:1999	
Total phosphorus (TP)	HRN EN ISO 6878:2008	Spectrophotometry
Nitrite (NO_2^-)	HRN EN ISO 10304-1:2009	Ion chromatography
Nitrate (NO_3^-)	HRN EN ISO 10304-1:2009	Ion chromatography
Ammonium (NH_4^+)	HRN EN ISO 14911:2001	Ion chromatography

Tablica 8. Parovi početnica korišteni za kvantifikaciju 16S rRNA gena, gena za otpornost na makrolidne antibiotike i integrona skupine 1. Točnost (R^2) i učinkovitost (%) svake amplifikacijske reakcije prikazani su za zimsku (Assay 1) i ljetnu (Assay 2) sezonu. Oznaka tablice u radu **Table S2**.

Target gene	Primer name	Primer sequence 5' → 3'	Amplicon size (bp)	Tm °C*	Accuracy (R^2)		Efficiency [%]		Reference
					Assay1	Assay2	Assay1	Assay2	
*mph*G	PrFW024	GGTATAAGTGAGCAATTGGAAAC	128	60	0.9946	0.9916	108.99	90.55	this study**
	PrFW025	GCTCCATCCTTTGAAGCTAG							
*mph*E	PrFW028	CTGTTTTCGGTGAAGAAAGTC	124	60	0.9931	0.9916	94.33	102.29	this study**
	PrFW029	CCATAAGCTAGAGGTGCGG							
*msr*E	PrFW030	CAATGTTATCTCGCCTTGGTG	127	60	0.9956	0.9945	85.47	98.61	this study**
	PrFW031	GTTGGTTCATCCGCTAGAATG							
*mef*C	PrFW036	GCTTACAAGTTATGCTGTTCAG	195	60	0.9970	0.9916	81.24	90.55	this study**
	PrFW037	CAGAGAGCTATAAAAGCATCC							
*erm*B	erm(B)-91f	GATACCGTTTACGAAATTGG	364	58	0.9836	0.9916	94.71	105.15	Chen et al., 2007
	erm(B)-454r	GAATCGAGACTTGAGTGTGC							
16S rRNA	534R	CCTACGGGAGGCAGCAG	174	60	0.9997	0.9934	102.11	83.65	Lopez et al., 2004
	341F	ATTACCGCGGCTGCTGGCA							
*int*I1	intI1_LC1	GCCTTGATGTTACCCGAGAG	196	60	0.9956	0.9970	83.02	86.56	Barraud et al., 2010
	intI1_LC5	GATCGGTCGAATGCGTGT							

***** temperatura sparivanja parova početnica

****** parovi početnica su konstruirani koristeći sekvence prethodno identificiranih gena (González-Plaza i sur., 2018) pomoću Geneiosus programa (v6.0.5)

Tablica 9. Fizikalno-kemijski pokazatelji sedimenata rijeke Save tijekom zimske i ljetne sezone. Oznaka tablice u radu **Table S3**.

Parameter	Sampling campaign	Sampling site					
		UP7500	DW0	DW300	DW700	DW4500	DW11000
pH (H_2O)	Winter	8.63	8.53	8.80	8.41	9.12	8.37
	Summer	8.76	7.67	8.78	8.35	8.58	8.28
T (°C)	Winter	9.5	9.5	10.1	8.5	9.8	9.7
	Summer	24.8	24.1	24.0	23.5	25.0	24.7
Conductivity (KCl) (mS)	Winter	48.3	115.2	223.0	52.7	115.0	56.9
	Summer	59.5	951.0	56.5	122.6	65.5	162.8
Sand (%)	Winter	85.2	56.4	70.7	71.4	82.1	79.4
	Summer	78.5	69.9	63.4	47.4	64.9	81.8
Silt (%)	Winter	12.8	37.9	25.5	25.2	13.3	17.6
	Summer	18.1	27.9	32.2	47.3	30.3	15.7
Clay (%)	Winter	2.0	5.7	3.8	3.4	2.6	3.0
	Summer	3.4	2.2	4.4	5.3	4.8	2.5
Total organic carbon (%)	Winter	0.97	1.35	0.93	2.10	3.70	0.78
	Summer	0.90	2.83	0.54	1.65	1.49	1.12
Total carbon (%)	Winter	7.50	9.06	8.20	8.11	6.98	6.42
	Summer	5.58	8.37	6.53	6.83	6.12	3.68
Total nitrogen (%)	Winter	0.04	0.12	0.05	0.10	0.04	0.04
	Summer	0.05	0.26	0.02	0.22	0.07	0.06
Total phosphorus (%)	Winter	0.38	0.39	0.40	0.30	0.35	0.49
	Summer	0.49	0.47	0.48	0.33	0.31	0.35
NO_2^- (mg/kg)	Winter	<0.03	10	<0.03	<0.03	<10	<0.03
	Summer	0.60	7.4	0.60	1.2	<0.30	0.80
NO_3^- (mg/kg)	Winter	2.26	<10	3.51	4.03	<10	2.41
	Summer	44.0	23.0	56.0	<10	24.0	31.0
NH_4^+ (mg/kg)	Winter	<0.05	25	0.05	0.23	<10	0.26
	Summer	0.50	296	1.60	1.90	1.50	11.0

Lokacije uzorkovanja: UP7500, 7500 m uzvodno od ispusta (referentna lokacija); DW0, ispust; DW300, 300 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta; DW4500, 4500 m nizvodno od ispusta; DW11000, 11 000 m nizvodno od ispusta.

Tablica 10. Relativna zastupljenost rodova (izražena u postotku) koji su se značajno povećali na nizvodnim lokacijama (DW) u odnosu na referentnu lokaciju (UP7500) tijekom zimske sezone. Značajna razlika u relativnoj zastupljenosti rodova između nizvodnih lokacija (DW) i referentne lokacije (UP7500) je označena podebljano ($p < 0.05$). Oznaka tablice u radu **Table S4**.

Phylum	Class	Order	Family	Genus	Sampling site						
					UP 7500	**WW**	**DW0**	**DW 300**	**DW 700**	**DW 4500**	**DW 11000**
Bacteroidetes	Bacteroidetes vadinHA17	Uncultured	Uncultured	Uncultured	0.00± 0.00	0.00± 0.00	**0.68± 0.23**	0.00± 0.00	0.01± 0.02	0.00± 0.00	0.01± 0.01
	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.00± 0.00	7.16± 2.21	**1.08± 0.60**	0.00± 0.00	0.01± 0.03	0.00± 0.00	0.00± 0.00
			Tannerellaceae	*Macellibacteroidetes*	0.00± 0.00	1.08± 0.14	**2.66± 0.99**	0.00± 0.00	0.06± 0.02	0.02± 0.01	0.01± 0.00
			Paludibacteraceae	*Paludibacter*	0.00± 0.00	0.19± 0.10	**1.35± 0.84**	0.00± 0.00	0.02± 0.03	0.00± 0.00	0.00± 0.00
			Rikenellaceae	*vadinBC27* wastewater-sludge group	0.00± 0.00	0.39± 0.04	**1.20± 0.30**	0.00± 0.00	0.02± 0.03	0.00± 0.00	0.00± 0.00
				Anaerocella	0.00± 0.00	0.00± 0.00	**0.47± 0.24**	0.01± 0.01	0.01± 0.01	0.01± 0.01	0.00± 0.00
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.79± 0.80	0.14± 0.06	1.29± 0.84	2.84± 2.92	0.95± 0.49	0.80± 0.16	**12.55± 9.75**
	Sphingobacteriia	Sphingobacteriales	WCHB1-69	Uncultured *Sphingobacteirales* bacterium	0.00± 0.00	0.00± 0.00	**0.56± 0.30**	0.03± 0.04	0.02± 0.01	0.03± 0.02	0.05± 0.04
				Uncultured bacterium	0.00± 0.00	0.00± 0.00	**0.62± 0.11**	0.01± 0.00	0.01± 0.02	0.00± 0.00	0.00± 0.00
Cyanobacteria	Oxyphotobacteria	Nostocales	Xenococcaceae	*Pleurocapsa*	0.01± 0.03	0.00± 0.00	0.02± 0.03	0.02± 0.01	0.06± 0.08	**1.20± 2.10**	0.10± 0.12
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Trichococcus*	0.06± 0.03	1.09± 0.09	**4.23± 1.20**	0.19± 0.09	0.16± 0.08	0.37± 0.02	0.32± 0.28
	Clostridia	Clostridiales	Christensenellaceae	*Christensenellacae R-7* Group	0.00± 0.00	0.92± 0.30	**0.54± 0.11**	0.00± 0.00	0.01± 0.02	0.00± 0.00	0.01± 0.01
			Family XII	*Guggenheimella*	0.00± 0.0	0.00± 0.00	**1.40± 0.54**	0.00± 0.00	0.02± 0.02	0.00± 0.00	0.00± 0.00
				Fusibacter	0.01± 0.00	0.09± 0.01	**1.46± 0.88**	0.02± 0.02	0.02± 0.02	0.02± 0.01	0.02± 0.01
			Family XIII	*Anaerovorax*	0.00± 0.00	0.04± 0.01	**0.48± 0.10**	0.00± 0.00	0.01± 0.01	0.00± 0.00	0.00± 0.00
	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaeae	*Erysipelothrix*	0.01± 0.01	0.10± 0.01	**0.94± 0.48**	0.01± 0.01	0.02± 0.02	0.02± 0.01	0.06± 0.02

Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	0.16± 0.18	0.06± 0.02	0.49± 0.26	0.33± 0.26	0.52± 0.27	0.44± 0.20	**0.88± 0.47**
		Rhodobacterales	Rhodobacteraceae	*Gemmobacter*	0.01± 0.01	0.57± 0.07	**0.78± 0.32**	0.03± 0.04	0.05± 0.02	0.06± 0.02	0.04± 0.02
				Pseudorhodobacter	0.04± 0.02	0.03± 0.01	0.07± 0.07	0.23± 0.29	0.12± 0.07	**0.59± 0.08**	0.28± 0.18
				Paracoccus	0.03± 0.02	0.13± 0.01	**0.38± 0.14**	0.07± 0.06	0.22± 0.14	0.07± 0.08	0.09± 0.07
				Tabrizicola	0.12± 0.06	0.04± 0.01	0.12± 0.10	0.16± 0.11	0.25± 0.17	0.52± 0.10	**0.63± 0.42**
				Other	0.10± 0.07	0.68± 0.19	0.34± 0.09	0.34± 0.12	0.22± 0.12	**0.75± 0.22**	0.64± 0.21
	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	*Desulfobacter*	0.00± 0.00	0.00± 0.00	**1.21± 0.66**	0.00± 0.00	0.01± 0.00	0.00± 0.00	0.00± 0.00
				Desulfobulbus	0.00± 0.00	0.02± 0.00	**0.85± 0.21**	0.02± 0.02	0.03± 0.03	0.05± 0.04	0.07 ±0.05
		Desulfuromonadales	Geobacteraceae	*Geobacter*	0.11± 0.04	0.02± 0.01	**0.63± 0.46**	0.04± 0.01	0.08± 0.01	0.20± 0.11	0.18± 0.06
	Gammaproteobacteria	Betaproteobacteriales	Comamonadaceae	*Comamonas*	0.75± 0.17	6.06± 1.14	**5.28± 1.54**	0.77± 0.25	0.84± 0.19	1.06± 0.16	0.89± 0.11
				Rhizobacter	0.14± 0.09	0.00± 0.00	**0.58± 0.41**	0.38± 0.25	0.25± 0.13	0.21± 0.09	0.15± 0.08
				Variovorax	0.17± 0.11	2.19± 0.35	**2.61± 1.08**	0.57± 0.41	0.38± 0.16	0.24± 0.12	0.21± 0.14
			Hydrogenophilaceae	*Thiobacillus*	0.03± 0.02	0.00± 0.00	**1.36± 0.77**	0.04± 0.04	0.09± 0.05	0.11± 0.04	0.12± 0.08
				Uncultured	0.00± 0.00	0.02± 0.02	**0.57± 0.28**	0.00± 0.00	0.01± 0.01	0.02± 0.01	0.00± 0.00
			Methylophilaceae	*Methylotenera*	0.12± 0.02	0.00± 0.00	**0.52± 0.86**	0.10± 0.04	0.04± 0.04	0.13± 0.04	0.03± 0.01
			Rhodocyclaceae	*Thauera*	0.00± 0.01	1.03± 0.09	**5.97± 1.66**	0.13± 0.14	0.15± 0.17	0.15± 0.13	0.09± 0.03
				Dechloromonas	0.37± 0.06	0.04± 0.01	**1.07± 0.64**	0.43± 0.37	0.30± 0.18	0.38± 0.14	0.72± 0.30
				Propionivibrio	0.05± 0.02	0.16± 0.02	**0.59± 0.06**	0.02± 0.01	0.04± 0.02	0.15± 0.08	0.03± 0.02
		Methylococcales	Methylomonaceae	*Methylomonas*	0.00± 0.00	0.00± 0.00	**1.51± 1.05**	0.00± 0.00	0.05± 0.08	0.00± 0.00	0.00± 0.00
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.01± 0.00	0.25± 0.02	**0.97± 0.43**	0.02± 0.01	0.03± 0.04	0.06± 0.04	0.02± 0.01
			Pseudomonadaceae	*Pseudomonas*	0.63± 0.25	1.69± 0.12	**3.61± 0.78**	1.22± 1.40	0.77± 1.18	0.54± 0.17	0.22± 0.17
		Xanthomonadales	Xanthomonadaceae	*Arenimonas*	0.81± 0.19	0.02± 0.00	0.96± 0.48	0.89± 0.28	1.15± 0.75	1.32± 0.24	**1.56± 1.22**

		Other	Other	Other	0.28± 0.01	0.06± 0.04	0.34± 0.02	0.36± 0.20	0.37± 0.09	0.49± 0.09	**0.61± 0.23**
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Thiovulaceae	*Sulfuricurvum*	0.00± 0.00	0.01± 0.01	**2.37± 3.46**	0.00± 0.00	0.07± 0.14	0.00± 0.00	0.00± 0.00
			Arcobacteraceae	*Arcobacter*	0.01± 0.00	16.43±2 .86	**8.17± 3.62**	0.02± 0.01	0.11± 0.17	0.14± 0.08	0.02± 0.01
Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaecae	Uncultured	0.00± 0.00	0.14± 0.04	**0.92± 0.31**	0.00± 0.00	0.01± 0.01	0.11± 0.10	0.00± 0.00
				Sphaerochaeta	0.00± 0.00	0.32± 0.06	**0.46± 0.22**	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
				Treponema	0.00± 0.00	0.01± 0.01	**0.45± 0.27**	0.00± 0.00	0.00± 0.00	0.01± 0.01	0.00± 0.00
Synergistetes	Synergistia	Synergistales	Synergistaceae	Uncultured	0.00± 0.00	1.13± 0.23	**1.59± 0.04**	0.00± 0.00	0.03± 0.01	0.00± 0.00	0.01± 0.00

Lokacije uzorkovanja: UP7500, 7500 m uzvodno od ispusta (referentna lokacija); DW0, ispust; DW300, 300 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta; DW4500, 4500 m nizvodno od ispusta; DW11000, 11 000 m nizvodno od ispusta.

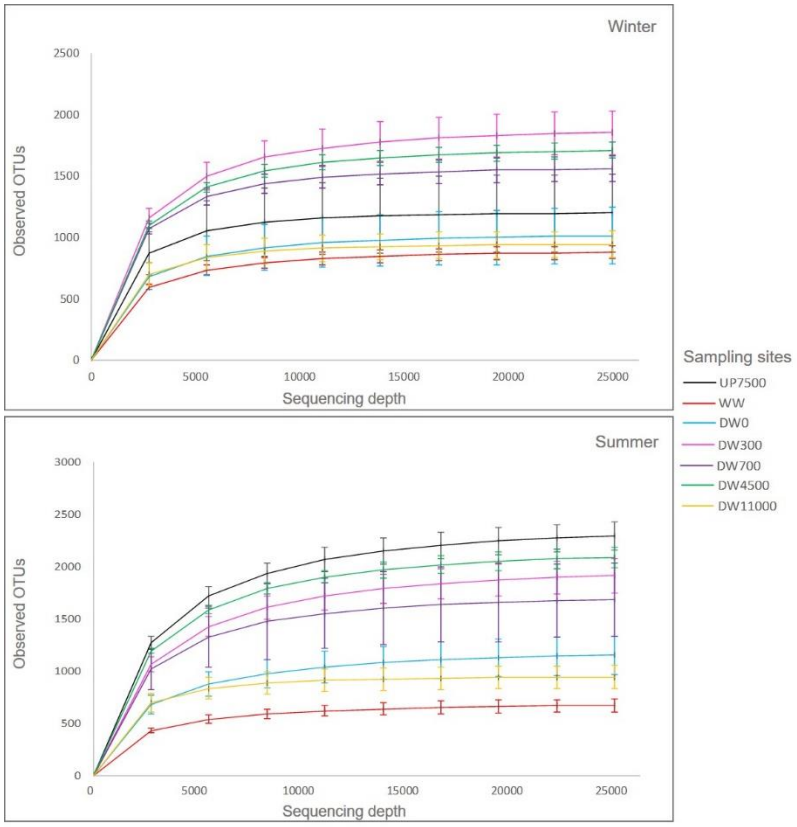
Tablica 11. Relativna zastupljenost rodova (izražena u postotku) koji su se značajno povećali na nizvodnim lokacijama (DW) u odnosu na referentnu (UP7500) tijekom ljetne sezone. Značajna razlika u relativnoj zastupljenosti rodova između nizvodnih lokacija (DW) i referentne lokacije (UP7500) je označena podebljano ($p < 0.05$). Oznaka tablice u radu **Table S5**.

Phylum	**Class**	**Order**	**Family**	**Genus**	**Sampling site**						
					UP 7500	**WW**	**DW0**	**DW 300**	**DW 700**	**DW 4500**	**DW 11000**
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.00± 0.00	4.59± 0.06	**0.75± 0.08**	0.14± 0.05	0.00± 0.00	0.00± 0.00	0.00± 0.00
			Paludibacteraceae	*Paludibacter*	0.00± 0.00	0.19± 0.02	**0.86± 0.11**	0.08± 0.05	0.02± 0.01	0.00± 0.00	0.00± 0.00
			Rikenellaceae	*vadinBC27* wastewater-sludge group	0.00± 0.00	0.50± 0.05	**3.27± 0.52**	0.07± 0.03	0.02± 0.02	0.00± 0.00	0.00± 0.00
		Flavobacteriales	Weeksellaceae	*Cloacibacterium*	0.00± 0.00	0.00± 0.00	**0.68± 0.05**	0.15± 0.01	0.01± 0.01	0.00± 0.00	0.01± 0.01
	Sphinobacteriia	Sphingobacteriales	ST-12K33	Uncultured	0.00± 0.00	0.00± 0.00	**2.34± 0.14**	0.05± 0.02	0.04± 0.02	0.00± 0.00	0.00± 0.00
			WCHB1-69	Uncultured	0.00± 0.00	0.00± 0.00	**0.78± 0.13**	0.02± 0.02	0.01± 0.02	0.00± 0.00	0.01± 0.01
Cloacimonetes	W27	Uncultured	Uncultured	Uncultured	0.00± 0.00	4.54± 0.00	**2.88± 0.56**	0.05± 0.02	0.02± 0.02	0.00± 0.00	0.00± 0.00
Cyanobacteria	Oxyphotobacteria	Subsection I	Family I	*Cyanobacterium*	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	**0.60± 0.49**
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Trichococcus*	0.06± 0.06	0.07± 0.01	**2.06± 0.73**	0.39± 0.19	0.03± 0.01	0.04± 0.01	0.07± 0.00
			Enterococcaceae	*Enterococcus*	0.00± 0.00	0.03± 0.00	**1.85± 0.70**	0.17± 0.05	0.01± 0.01	0.00± 0.00	0.00± 0.00
			Family XIII	*Anaerovorax*	0.00± 0.00	0.05± 0.00	**1.06± 0.05**	0.02± 0.01	0.02± 0.02	0.00± 0.00	0.01± 0.01
	Negativicutes	Selenomonadales	Veillonellaceae	Uncultured	0.00± 0.00	0.08± 0.00	**0.59± 0.19**	0.01± 0.01	0.01± 0.01	0.00± 0.00	0.00± 0.00
			Uncultured	Bacterium enrichment	0.00± 0.00	0.07± 0.00	**1.25± 0.18**	0.00± 0.00	0.01± 0.00	0.00± 0.00	0.00± 0.00

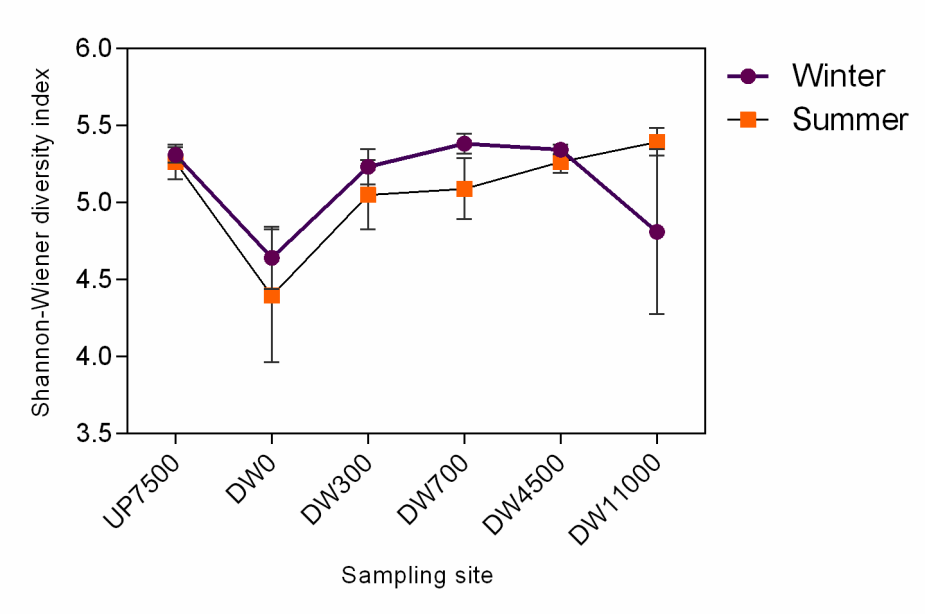
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Falsirhodobacter*	0.00± 0.00	0.11± 0.01	**1.32± 1.45**	0.01± 0.01	0.01± 0.01	0.00± 0.00	0.00± 0.00
				Gemmobacter	0.02± 0.01	0.49± 0.08	**0.42± 0.01**	0.14± 0.03	0.02± 0.01	0.02± 0.02	0.01± 0.00
	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	*Desulfatiferula*	0.00± 0.00	0.00± 0.00	**0.70± 0.05**	0.00± 0.00	0.01± 0.01	0.00± 0.00	0.00± 0.00
				Desulfobacter	0.00± 0.00	0.01± 0.00	**2.09± 0.34**	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
				Uncultured	0.00± 0.00	0.00± 0.00	**0.61± 0.01**	0.01± 0.01	0.03± 0.02	0.02± 0.01	0.20± 0.09
			Desulfobulbaceae	*Desulfobulbus*	0.01± 0.09	0.01± 0.01	**1.21± 0.18**	0.14± 0.06	**0.77± 0.25**	0.23± 0.13	**0.59± 0.60**
				Uncultured	0.06± 0.02	0.00± 0.00	0.17± 0.01	0.15± 0.12	0.22± 0.07	0.17± 0.11	**0.58± 0.41**
			Nitrospinaceae	Uncultured	0.06± 0.03	0.00± 0.00	0.00± 0.00	0.09± 0.07	0.05± 0.04	0.05± 0.03	**0.54± 0.28**
		Desulfuromonadales	Geobacteraceae	*Geobacter*	0.19± 0.14	0.04± 0.01	0.23± 0.07	0.14± 0.11	0.27± 0.06	0.26± 0.08	**0.55± 0.24**
		Myxococcales	Cystobacteraceae	*Anaeromyxobacter*	0.26± 0.11	0.00± 0.00	0.01± 0.00	0.36± 0.21	**0.41± 0.33**	**0.62± 0.27**	**1.48± 0.71**
				Cystobacter	0.10± 0.08	0.00± 0.00	0.01± 0.02	0.25± 0.27	**0.73± 0.38**	0.14± 0.03	0.14± 0.09
	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Sphaerotilus*	0.01± 0.00	0.04± 0.01	0.02± 0.01	**8.92± 6.39**	0.22± 0.25	0.04± 0.00	0.01± 0.00
			Hydrogenophilaceae	*Thiobacillus*	0.04± 0.01	0.00± 0.00	0.11± 0.01	0.13± 0.10	0.06± 0.06	0.08± 0.05	**0.74± 0.60**
			Rhodocyclaceae	*Dechloromonas*	0.75± 0.02	0.32± 0.04	0.96± 0.19	**2.01± 0.39**	1.82± 0.21	0.94± 0.27	1.65± 0.80
				Thauera	0.01± 0.01	0.78± 0.03	**13.26 ±2.54**	0.87± 0.16	0.46± 0.22	0.05± 0.01	0.09± 0.06
				Propionivibrio	0.63± 0.38	0.40± 0.04	**1.01± 0.18**	0.27± 0.02	0.73± 0.15	0.36± 0.20	0.20± 0.21
		Xanthomonadales	Xanthomonadales Incertae Sedis	*Candidatus* Competibacer	0.01± 0.01	0.00± 0.00	0.02± 0.01	0.03± 0.01	0.03± 0.03	0.03± 0.01	**0.51± 0.48**
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	0.35± 0.17	1.86± 0.49	**1.35± 0.16**	0.60± 0.03	0.38± 0.11	0.38± 0.04	0.15± 0.04
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.00± 0.00	44.39 ±1.43	**6.30± 0.71**	**1.46± 0.65**	0.09± 0.05	0.01± 0.00	0.03± 0.01
Spirochaetae	Spirochaetes	Spirochaetales	PL-11B10	Uncultured	0.00± 0.00	0.00± 0.00	**1.48± 0.32**	0.01± 0.00	0.03± 0.01	0.00± 0.00	0.00± 0.00
			Spirochaetae	*Spirochaeta 2*	0.09± 0.03	0.00± 0.00	**0.59± 0.04**	0.18± 0.01	0.19± 0.14	0.13± 0.09	**0.83± 0.18**
				Uncultured	0.04± 0.02	0.04± 0.16	**0.91± 0.10**	0.07± 0.00	0.26± 0.23	0.05± 0.04	**0.95± 0.07**

				Treponema	0.01± 0.00	0.01± 0.01	**0.33± 0.03**	0.01± 0.01	0.03± 0.04	0.02± 0.01	0.11± 0.08
Synergistetes	Synergistia	Synergistales	Synergistaceae	Uncultured	0.00± 0.00	0.32± 0.10	**2.80± 0.58**	0.03± 0.01	0.01± 0.00	0.00± 0.00	0.01± 0.01
				Thermovirga	0.00± 0.00	0.73± 0.07	**0.31± 0.07**	0.04± 0.01	0.00± 0.00	0.00± 0.00	0.00± 0.00

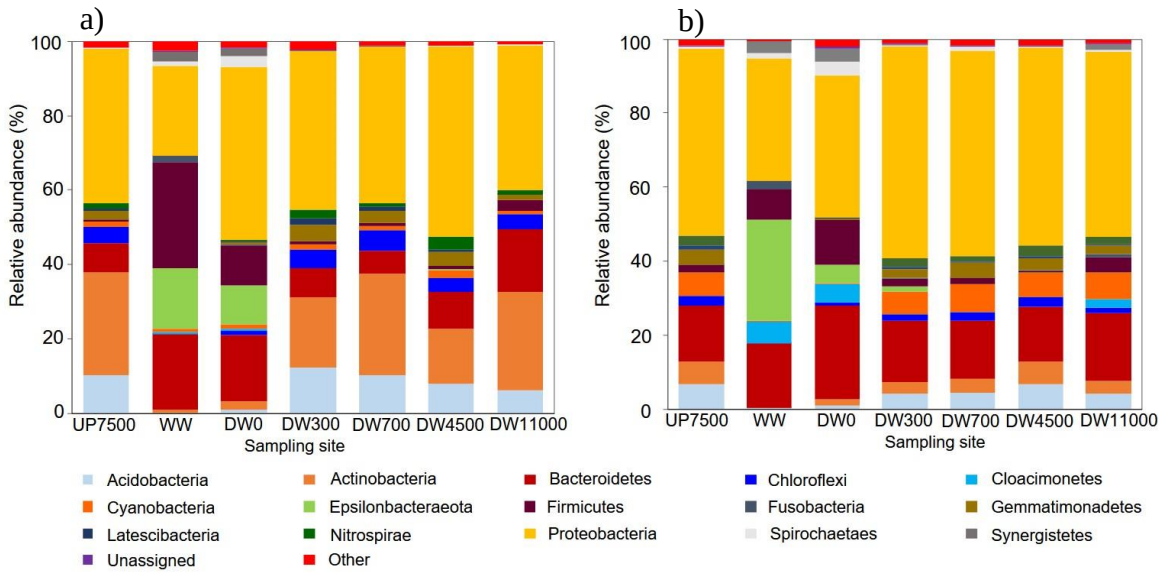
Lokacije uzorkovanja: UP7500, 7500 m uzvodno od ispusta (referentna lokacija); DW0, ispust; DW300, 300 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta; DW4500, 4500 m nizvodno od ispusta; DW11000, 11 000m nizvodno od ispusta.



Slika 8. Rarefakcijske krivulje koje prikazuju alfa raznolikost otpadne vode iz farmaceutske industrije koja proizvodi makrolidni antibiotik azitromicin (WW) i sedimenata rijeke Save dobivenih pomoću metrike „Observed ASVs (amplicon sequencing variants)“. Lokacije uzorkovanja: UP7500, 7500 m uzvodno od ispusta (referentna lokacija); DW0, ispust; DW300, 300 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta; DW4500, 4500 m nizvodno od ispusta; DW11000, 11 000 m nizvodno od ispusta. Oznaka slike u radu **Figure S1**.



Slika 9. Shannon-Wiener indeks raznolikosti sedimenata rijeke Save na različitim lokacijama uzorkovanja tijekom zimske i ljetne sezone. Lokacije uzorkovanja: UP7500, 7500 m uzvodno od ispusta (referentna lokacija); DW0, ispust; DW300, 300 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta; DW4500, 4500 m nizvodno od ispusta; DW11000, 11 000 m nizvodno od ispusta. Oznaka slike u radu **Figure S2**.



Slika 10. Taksonomska strukture bakterijske zajednice na razini koljena otpadne vode iz proizvodnje makrolidnog antibiotika azitromicina (WW) i sedimenata rijeke Save na različitim lokacijama uzorkovanja tijekom a) zime i b) ljeta. Bakterijska koljena sa relativnom zastupljenošću manjom od 1 % u svim uzorcima su grupirana u „Other“. Lokacije uzorkovanja: UP7500, 7500 m uzvodno od ispusta (referentna lokacija); DW0, ispust; DW300, 300 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta; DW4500, 4500 m nizvodno od ispusta; DW11000, 11 000 m nizvodno od ispusta. Oznaka slike u radu **Figure S3**.

7.4. Dodatne informacije uz rad:

González Plaza JJ, Blau K, **Milaković M**, Jurina T, Smalla K, Udiković Kolić N: Antibiotic-manufacturing sites are hot-spots for the release and spread of antibiotic resistance genes and mobile genetic elements in receiving aquatic environments. Environment International 130 (2019): 104735. doi: 10.1016/j.envint.2019.04.007

Tablica 12. Plazmidi dobiveni tijekom biparentalne konjugacije bakterija te integroni razreda 1 i geni za otpornost na antibiotike detektirani metodama PCRa, Southern blot hibridizacijom i lančanom reakcijom polimerazom u stvarno vremenu (engl. *Real Time PCR*, RT-PCR) u plazmidnoj DNA *E. coli* CV06 *gfp*+ transkonjuganata. Prisutnost gena je označena znakom +, dok je odsutnost gena označena znakom -. Oznaka tablice u radu **Table S1**.

Site	Ind. facility	Antibiotic for capture	# tc	*intI1*	*qacE/ qacEΔ1*	*mer RTΔP*	*sul1*	*sul2*	*tet*(A)	IncP-1 (*korB*)	IncP-1ε (*trfA*)	IncP-1β	IncN
UP	1	ERY	1	+	+	+	+	-	-	+	-	-	-
DW0	1	ERY	1	+	+	-	+	-	-	-	-	-	-
DW0	1	ERY	1	+	+	-	+	-	-	+	-	-	-
DW0	1	ERY	4	+	+	-	+	-	+	+	+	-	-
DW0	1	TET	2	+	+	-	+	-	+	+	+	-	-
DW0	1	TET	1	-	-	-	-	-	-	-	-	-	-
DW0	1	TET	12	+	+	-	+	-	+	+	+	-	-
DW0	1	TET	1	+	+	-	+	+	+	-	-	-	+
DW700	1	ERY	9	-	-	-	-	+	-	-	-	-	-
DW700	1	ERY	1	+	+	+	+	-	-	+	+	-	-
DW700	1	ERY	2	-	-	-	-	-	-	+	-	+	-
DW700	1	TET	33	-	-	-	-	-	-	-	-	-	-
DW700	1	TET	4	+	+	-	+	-	+	+	+	-	-
UP	2	TET	4	-	-	-	-	-	+	-	-	-	-
UP	2	TET	16	+	+	-	+	-	+	+	+	-	-
UP	2	TET	2	+	+	-	+	-	+	-	-	-	-
DW0	2	TET	1	+	+	-	+	-	+	-	-	-	-
DW0	2	TET	22	+	+	-	+	-	+	+	+	-	-
DW0	2	TET	1	-	-	-	-	-	+	-	-	-	-
DW3000	2	TET	11	-	-	-	-	+	-	-	-	-	-
DW3000	2	TET	1	-	-	+	-	+	-	-	-	-	-
WW2	2	TET	23	+	+	-	+	-	+	+	+	-	-

Industrija 1, proizvodnja azitromicina; Industrija 2, formulacija antibiotika. Lokacije: UP, uzvodno (referentna lokacija); DW0, ispust; WW2, otpadna voda iz formulacije antibiotika; DW3000, 3000 m nizvodno od ispusta; DW700, 700 m nizvodno od ispusta. TET: tetraciklin; ERY: eritromicin.

7.5. Dodatne informacije uz rad:

Milaković M, Vestergaard G, González-Plaza JJ, Petrić I, Kosić-Vukšić J, Senta I, Kublik S, Schloter M, Udiković-Kolić N: Effects of industrial effluents containing moderate levels of antibiotic mixtures on the abundance of antibiotic resistance genes and bacterial community composition in exposed creek sediments. Science of the Total Environment

Tablica 13. Parovi početnica i uvjeti reakcija za kvantifikaciju 16S rRNA bakterijskog gena i gena za antibiotičku otpornost. Oznaka tablice u radu **Table S1**.

Target gene	Resistance phenotype	Primer sequence 5' → 3'	Amplicon size (bp)	Tm* (°C)	Amplification accuracy/efficiency	Reference
***tet*C**	Tetracyclines	GGCATTCTGCATTCACTCGC GAAGCAAGCAGGACCATGATC	170	60	0.996 / 107.2%	This study**
***tet*39**	Tetracyclines	GCGGGAATTACAGGTGCCAAC GCAGCAAAGAACGGTGCATG	182	60	0.991 / 98.2%	This study**
bla_{GES}	ß -lactams	GCGGGTTTTCCTAAAGATTGG AATAACTTGACCGACAGAGGC	196	65	0.994 / 90.1%	Gatica et al., 2016
bla_{VEB}	ß -lactams	CGACTTCCATTTCCCGATGC CCAATATTGTCGCTCTCTGATAC	183	65	0.996 / 82.2%	Naas et al., 2000 (F)/This study** (R)
bla_{OXA-1}	ß -lactams	TATCTACAGCAGCGCCAGTG CGCATCAAATGCCATAAGTG	199	60	0.999 / 95.9%	Yang et al., 2012
bla_{OXA-2}	ß -lactams	TCTTCGCGATACTTTTCTCCA ATCGCACAGGATCAAAAACC	177	60	0.999 / 99.7%	Yang et al., 2012
***dfr*A14**	Trimethoprim	GTCGTTACCCGCTCAGGTTG GTCGATCGTCGATAAGTGGAG	177	67	0.992 / 104.1%	This study**
***fol*A**	Trimethoprim	CGAAGCTCCAGAAGGTTGTTTG CCGCTTCAATCGTAGTATGCAC	167	67	0.990 / 100.0%	This study**
***sul*1**	Sulfonamides	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT	965	65	0.999 / 101.6%	Heuer and Smalla, 2007
***sul*2**	Sulfonamides	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT	865	65	0.998 / 96.7%	Heuer and Smalla, 2007
***mph*G**	Macrolides	GGTATAAGTGAGCAATTGGAAAC GCTCCATCCTTTGAAGCTAG	128	60	0.998 / 105.4%	Milaković et al., 2019
***mph*E**	Macrolides	CTGTTTTCGGTGAAGAAAGTC CCATAAGCTAGAGGTGCGG	124	60	0.999 / 97.2%	Milaković et al., 2019
***msr*E**	Macrolides	CAATGTTATCTCGCCTTGGTG GTTGGTTCATCCGCTAGAATG	127	60	0.998 / 99.9%	Milaković et al., 2019
***mef*C**	Macrolides	GCTTACAAGTTATGCTGTTCAG CAGAGAGCTATAAAAGCATCC	195	60	0.996 / 104.4%	Milaković et al., 2019
***erm*B**	Macrolides	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	364	58	0.990 / 105.1%	Chen et al., 2007
16S rRNA		CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGCA	196	60	0.993 / 83.6%	López-Gutiérrez et al., 2004

'Resistance phenotype' označava za koju skupinu antibiotika kodira navedeni gen.
* temperatura sparivanja početnica
** parovi početnica su konstruirani na sekvencama prethodno identificiranih gena (González-Plaza i sur., 2018.) pomoću programa MEGA7 (verzija 7.0)

Tablica 14. Fizikalno-kemijski pokazatelji sedimenata na različitim lokacijama potoka Kalinovica tijekom zimske i ljetne sezone. Oznaka tablice u radu **Table S2**.

Parameter	Season	Sampling sites		
		UP	DW0	DW3000
pH (H_2O)	Winter	7.02	7.93	7.21
	Summer	7.62	7.98	6.81
T (°C)	Winter	10.4	6.9	3.0
	Summer	22.2	20.7	23.0
Conductivity (25°C) (µS/cm)	Winter	198.0	144.1	175.2
	Summer	283.0	309.0	386.0
Sand (%)	Winter	26.6	27.1	26.6
	Summer	30.2	29.7	43.0
Silt (%)	Winter	67.0	63.5	66.9
	Summer	64.9	64.6	53.4
Clay (%)	Winter	6.4	9.4	6.5
	Summer	4.9	5.7	3.6
Total organic carbon, TOC (%)	Winter	3.37	1.79	3.41
	Summer	3.93	2.13	6.23
Total carbon, TC (%)	Winter	4.13	2.14	5.05
	Summer	4.74	3.34	6.32
Total nitrogen, TN (%)	Winter	0.29	0.17	0.30
	Summer	0.39	0.25	0.48
Total phosphorus, TP (%)	Winter	0.09	0.03	0.06
	Summer	0.11	0.09	0.10
NO_2^- (mg/kg)	Winter	<0.3	<0.3	<0.3
	Summer	<0.3	<0.3	<0.3
NO_3^- (mg/kg)	Winter	22.8	21.0	11.0
	Summer	19.0	24.0	19.0
NH_4^+ (mg/kg)	Winter	9.77	16	<0.4
	Summer	55.0	23.0	21.0

Lokacije uzorkovanja: UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta.

Tablica 15. Koncentracija teških metala u sedimentima potoka Kalinovica sa tri različite lokacije tijekom zimske i ljetne sezone. Oznaka tablice u radu **Table S3**.

Metal	MCC*	Season	Sampling sites (mg/kg of dry sediment)		
			UP	DW0	DW3000
Cadmium (Cd)	1.0	Winter	0.37	0.10	0.31
		Summer	0.42	0.24	0.63
Chromium (Cr)	-	Winter	111	139	74
		Summer	102	98	83
Copper (Cu)	11.5	Winter	**125**	**43**	**82**
		Summer	**171**	**45**	**134**
Lead (Pb)	-	Winter	46	63	39
		Summer	53	52	43
Nickel (Ni)	-	Winter	46	69	53
		Summer	178	35	35
Zinc (Zn)	42.5	Winter	**445**	**186**	**276**
		Summer	**505**	**207**	**396**

UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta. Podebljane vrijednosti označavaju koncentracije teških metala iznad minimalne ko-selektivne koncentracije (engl. *Minimum co-selective concentration, MCC*), iznad koje se očekuje ko-selekcija za otpornost na antibiotike (Seiler i Berendonk, 2012).

Tablica 16. Prosječna relativna zastupljenost [$\log_{10}$ (kopija gena/*rrn* kopija)] kvantificiranih gena za otpornost na antibiotike u otpadnoj vodi iz formulacije antibiotika (WW) i sedimenata potoka Kalinovica tijekom zimske i ljetne sezone. Oznaka tablice u radu **Table S4.**

Target gene	Sampling campaign	Sampling sites			
		UP	**WW**	**DW0**	**DW3000**
***tet*C**	Winter	-2.59±0.33	-2.27±0.47	-2.78±0.65	-2.97±0.52
	Summer	-1.77±0.36	-0.95±0.02	-0.99±0.16	-1.51±0.31
***tet*39**	Winter	-3.99±0.09	-2.19±0.34	-3.47±0.71	<LOQ
	Summer	-0.98±0.01	-1.77±0.32	0.28±0.03	-2.56±0.32
***bla*$_{GES}$**	Winter	<LOQ	-4.67±0.64	-3.64±0.93	<LOQ
	Summer	<LOQ	-2.63±0.08	-2.24±0.23	<LOQ
***bla*$_{VEB}$**	Winter	-2.96±0.20	-2.85±0.40	-2.50±0.54	-2.85±0.48
	Summer	-1.36±0.07	-2.02±0.06	-1.49±0.06	-1.45±0.15
***bla*$_{OXA-1}$**	Winter	-3.86±0.32	-3.26±0.52	-2.55±0.80	-3.44±0.67
	Summer	-2.68±0.14	-1.89±0.12	-1.38±0.19	-2.24±0.18
***bla*$_{OXA-2}$**	Winter	-2.97±0.28	-2.82±0.36	-2.64±0.36	-2.61±0.67
	Summer	-2.33±0.20	-1.50±0.03	-1.46±0.46	-2.06±0.19
***fol*A**	Winter	-5.52±0.14	-5.05±0.12	-4.58±0.64	-5.61±0.16
	Summer	-3.58±0.13	-3.77±0.08	-3.88±0.13	-3.36±0.54
***dfr*A14**	Winter	-2.62±0.33	-2.20±0.47	-2.32±0.57	-3.00±0.51
	Summer	-1.80±0.36	-0.98±0.02	-1.0±0.16	-1.82±0.31
***sul*1**	Winter	-2.00±0.11	-1.39±0.54	-1.64±0.13	-1.68±0.63
	Summer	-1.12±0.24	-0.48±0.08	-0.38±0.09	-0.80±0.39
***sul*2**	Winter	-3.78±0.15	-1.64±0.64	-2.75±0.19	-2.31±0.54
	Summer	-2.42±0.25	-0.90±0.18	-1.62±0.11	-1.58±0.40
***mph*G**	Winter	-3.46±1.12	-2.34±0.96	-2.71±1.03	-3.14±0.65
	Summer	<LOQ	0.04±0.06	-1.04±0.24	-3.09±0.46
***mph*E**	Winter	<LOQ	-4.04±0.55	-4.10±0.93	<LOQ
	Summer	-2.32±0.28	-3.10±0.07	-2.70±0.09	-2.45±0.14
***msr*E**	Winter	-3.93±1.22	-2.49±1.17	-2.95±1.04	-3.50±0.67
	Summer	<LOQ	-0.10±0.07	-1.07±0.19	-2.78±0.44
***mef*C**	Winter	<LOQ	-3.35±0.59	-3.91±0.66	<LOQ
	Summer	-2.54±0.27	-2.86±0.05	-3.24±0.92	-2.63±0.17
***erm*B**	Winter	<LOQ	-4.00±0.60	-3.58±0.90	<LOQ
	Summer	<LOQ	-2.29±0.13	-2.16±0.22	<LOQ

< LOQ, ispod granice kvantifikacije. Lokacije uzorkovanja: UP, uzvodno; DW0, ispust, DW3000, 3000 m nizvodno od ispusta; WW, otpadna voda.

Tablica 17. Relativna zastupljenost rodova (izražena u postotku) karakteristična za uzvodni sediment (UP) u odnosu na otpadnu vodu (WW) i nizvodni sediment (DW) tijekom zimske sezone (DESeq 2, $p < 0,05$). Oznaka tablice u radu **Table S5**.

Phylum	Class	Order	Family	Genus	Sampling sites			
					UP	WW	DW0	DW3000
Acidobacteria	Subgroup 18	Uncultured Acidobacteria bacterium			0.37±0.11	0.00±0.00	0.00±0.00	0.20±0.16
	Holophagae	Subgroup 7	Uncultured bacterium SJA-36		1.17±0.22	0.00±0.00	0.93±0.37	0.82±0.13
Actinobacteria	Actinobacteria	Unassigned	Unassigned	Unassigned	0.34±0.06	0.00±0.00	0.04±0.02	0.14±0.22
		PeM15	metagenome		0.19±0.05	0.00±0.00	0.00±0.00	0.02±0.04
	Coriiobacteria	OPB41	Uncultured bacterium		0.25±0.08	0.00±0.00	0.03±0.03	0.03±0.02
			Unassigned		0.18±0.03	0.00±0.00	0.01±0.01	0.00±0.01
	Acidimicrobiia	Microtrichales	Ilumatobacteraceae	CL500-29 marine group	0.13±0.02	0.00±0.00	0.01±0.02	0.08±0.07
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidetes BD2-2	Uncultured bacterium	0.51±0.20	0.00±0.00	0.00±0.00	0.01±0.02
			Unassigned	Unassigned	1.00±0.17	0.22±0.06	0.37±0.03	0.38±0.13
			Prolixibacteraceae	BSV13	2.70±0.50	0.00±0.00	0.73±0.37	0.13±0.08
				Prolixibacter	1.12±0.19	0.00±0.00	0.00±0.00	0.13±0.04
				WCHB1-32	3.67±0.57	0.93±0.10	1.38±0.78	0.16±0.14
				Uncultured	2.70±0.38	0.00±0.00	0.03±0.01	0.22±0.09
				Unassigned	1.16±0.20	0.10±0.02	0.08±0.10	0.34±0.07
		Cytophagales	Cyclobacteraceae	*Ekhidna*	0.39±0.11	0.02±0.01	0.33±0.40	0.17±0.09
				Unassigned	1.67±0.28	0.02±0.00	0.23±0.24	0.06±0.07
	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	*Ignavibacterium*	1.03±0.15	0.00±0.00	0.02±0.03	0.29±0.19
Chloroflexi	Anaerolineae	SJA-15	Ambigous taxa		0.41±0.18	0.00±0.00	0.00±0.00	0.04±0.01
Cyanobacteria	Oxyphotobacteria	Chloroplast	Coelastrella sp. M60		0.28±0.12	0.00±0.00	0.00±0.00	0.00±0.00
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Thiovulaceae	*Sulfuricurvum*	1.50±0.92	0.01±0.01	0.75±0.35	0.53±0.54
Nitrospinae	P9X2b3D02	Uncultured bacterium			0.49±0.17	0.00±0.00	0.00±0.00	0.04±0.05
Proteobacteria	α-Proteobacteria	Acetobacterales	Acetobacteraceae	*Rhodovastum*	0.12±0.04	0.00±0.00	0.02±0.03	0.00±0.00
				Roseomonas	0.58±0.07	0.00±0.00	0.15±0.10	0.17±0.02
		Rhodobacterales	Rhodobacteraceae	*Planktomarina*	0.18±0.06	0.01±0.01	0.10±0.04	0.10±0.13
				Unassigned	0.57±0.21	0.30±0.01	0.18±0.12	0.06±0.07
	δ-Proteobacteria	Deltaproteobacteria Inc. Sedis	Syntrophorhabdaceae	*Syntrophorhabdus*	0.47±0.16	0.00±0.00	0.01±0.01	0.35±0.25
		Desulfobacterales	Desulfobacteraceae	[*Desulfobacterium*] catecholicum group	0.27±0.05	0.00±0.00	0.02±0.01	0.05±0.04
		Desulfuromonadales	Geobacteraceae	*Geobacter*	8.63±0.88	0.00±0.00	2.62±0.84	2.56±2.64
				Geothermobacter	0.90±0.19	0.00±0.00	0.02±0.01	0.06±0.05
			Sva1033	Uncultured bacterium	0.22±0.10	0.00±0.00	0.00±0.00	0.07±0.05
		Myxococcales	Archangiaceae	*Anaeromyxobacter*	1.14±0.26	0.00±0.00	0.15±0.22	0.50±0.26
			MidBa8	Uncultured deltaproteobacterium	0.28±0.09	0.00±0.00	0.01±0.01	0.01±0.01
	γ-Proteobacteria	Betaproteobacteriales	Burkholderiaceae	Unassigned	7.85±1.39	4.08±0.29	2.56±1.30	1.02±0.52
			Rhodocyclaceae	*Sulfuritalea*	0.31±0.12	0.00±0.00	0.02±0.04	0.25±0.18

		SAR86 clade	Uncultured marine eukaryote		0.24±0.07	0.00±0.00	0.04±0.04	0.10±0.06
		Steroidobacterales	Steroidobacteraceae	Uncultured	1.74±0.21	0.00±0.00	0.39±0.26	1.40±0.62
		Unassigned	Unassigned	Unassigned	2.11±0.29	0.05±0.01	0.57±0.76	1.33±0.28
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Sphaerochaeta*	2.12±0.80	0.00±0.00	0.01±0.01	0.35±0.23

Lokacije uzorkovanja: UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta; WW, otpadna voda.

Tablica 18. Relativna zastupljenost rodova (izražena u postotku) karakteristična za uzvodni sediment (UP) u odnosu na otpadnu vodu (WW) i nizvodni sediment (DW) tijekom ljetne sezone (DESeq 2, $p < 0,05$). Oznaka tablice u radu **Table S6**.

Phylum	Class	Order	Family	Genus	Sampling sites			
					UP	WW	DW0	DW3000
Acidobacteria	Subgroup 6	Uncultured bacterium	Unassigned	Unassigned	1.84±0.25	0.00±0.00	0.10±0.03	1.42±0.19
	Subgroup 18	Uncultured Acidobacteria bacterium			0.47±0.15	0.00±0.00	0.02±0.03	0.10±0.03
Actinobacteria	Actinobacteria	Catenulisporales	Actinospicae	*Actinospica*	0.13±0.04	0.00±0.00	0.02±0.02	0.05±0.04
		PeM15	metagenome		0.15±0.07	0.00±0.00	0.00±0.00	0.04±0.02
		Unassigned	Unassigned	Unassigned	0.35±0.09	0.01±0.01	0.09±0.01	0.06±0.05
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidetes BD2-2	Uncultured bacterium	0.38±0.05	0.00±0.00	0.00±0.00	0.00±0.00
			Prolixibacteraceae	BSV13	4.22±0.76	0.02±0.01	1.18±0.52	0.04±0.01
				Prolixibacter	1.33±0.24	0.00±0.00	0.05±0.06	0.23±0.03
				WCHB1-32	4.03±0.50	10.96±1.11	2.31±0.03	0.05±0.04
				Uncultured	3.22±0.66	0.00±0.00	0.11±0.09	0.26±0.12
				Unassigned	1.05±0.22	0.00±0.01	0.12±0.09	0.35±0.05
			Unassigned	Unassigned	0.95±0.19	0.08±0.02	0.28±0.20	0.47±0.11
		Cytophagales	Cyclobacteriaceae	*Ekhidna*	0.13±0.07	0.00±0.00	0.01±0.01	0.05±0.05
				Unassigned	1.25±0.11	0.00±0.00	0.02±0.02	0.05±0.04
			Microscillaceae	Uncultured	0.55±0.34	0.00±0.00	0.03±0.01	0.06±0.01
		Flavobacteriales	Unassigned	Unassigned	0.13±0.04	0.01±0.01	0.02±0.02	0.01±0.02
	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	*Ignavibacterium*	1.43±0.23	0.00±0.00	0.03±0.02	0.18±0.09
Chloroflexi	KD4-96	Uncultured Chloroflexi bacterium			0.81±0.11	0.00±0.00	0.10±0.06	0.13±0.07
	SJA-15	Ambigous taxa	Unassigned		0.19±0.07	0.00±0.00	0.02±0.02	0.02±0.00
Cyanobacteria	Oxyphotobacteria	Chloroplast	Coelastrella sp. M60	Unassigned	0.18±0.04	0.00±0.00	0.03±0.04	0.00±0.00
Nitrospinae	P9X2b3D02	Uncultured bacterium			0.28±0.12	0.00±0.00	0.00±0.00	0.03±0.03
Proteobacteria	α-Proteobacteria	Acetobacterales	Acetobacteraceae	*Roseomonas*	0.53±0.10	0.01±0.01	0.15±0.10	0.26±0.06
				Rhodovastum	0.14±0.01	0.00±0.00	0.01±0.02	0.05±0.01
	δ-Proteobacteria	Desulfuromonadales	Geobacteraceae	*Geobacter*	9.88±1.75	0.01±0.01	9.35±1.69	1.39±0.66
				Geothermobacter	0.96±0.32	0.00±0.00	0.02±0.02	0.09±0.06
		Myxococcales	Archangiaceae	*Anaeromyxobacter*	1.46±0.12	0.00±0.00	0.12±0.03	0.47±0.21
			MidBa8	Uncultured deltaprotebacterium	0.47±0.10	0.00±0.00	0.02±0.02	0.01±0.00
	γ-Proteobacteria	Betaproteobacteriales	Burkholderiaceae	*Rhodoferax*	9.40±1.18	2.73±0.09	8.95±1.32	3.74±1.24
			Unassigned	Unassigned	4.22±0.63	1.19±0.05	0.97±0.65	1.11±0.80
		Steroidobacterales	Steroidobacteraceae	Uncultured	3.44±0.82	0.00±0.00	0.22±0.19	2.25±0.25
		Xanthomonadales	Xanthomonadaceae	*Luteimonas*	1.53±0.27	0.26±0.02	0.96±0.74	0.76±0.29
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Sphaerochaeta*	1.96±0.63	0.00±0.00	0.16±0.08	0.41±0.19

Lokacije uzorkovanja: UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta; WW, otpadna voda.

Tablica 19. Relativna zastupljenost rodova (izražena u postotku) koji su se značajno povećali na nizvodnim lokacijama (DW) u odnosu na uzvodnu lokaciju (UP) tijekom zimske sezone. Značajna razlika u relativnoj zastupljenosti rodova između nizvodnih lokacija (DW) i uzvodne lokacije (UP) je označena podebljano ($p < 0.05$). Oznaka tablice u radu **Table S7**.

Phylum	Class	Order	Family	Genus	Sampling site			
					UP	WW	DW0	DW3000
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae (Subgroup 1)	Uncultured	0.00±0.00	0.00±0.00	0.00±0.00	**0.22±0.17**
	Blastocatellia (Subgroup 4)	Blastocatellales	Blastocatellaceae	JGI 0001001-H03	0.09±0.05	0.00±0.00	0.05±0.06	**0.51±0.20**
				Uncultured	0.02±0.01	0.00±0.00	0.05±0.05	**0.51±0.30**
		DS-100	Uncultured bacterium	Unassigned	0.03±0.02	0.00±0.00	**0.24±0.15**	0.00±0.00
		Pyrinomonadales	Pyrinomonadaceae	RB41	0.05±0.02	0.00±0.00	**0.85±0.43**	0.07±0.09
	Holophagae	Holophagales	Holophagaceae	Marine group	0.06±0.02	0.00±0.00	**0.48±0.14**	**0.25±0.29**
		Subgroup 7	Uncultured bacterium	Uncultured	0.15±0.09	0.00±0.00	0.00±0.00	**0.58±0.41**
	Subgroup 6	Uncultured bacterium			1.35±0.31	0.00±0.00	0.11±0.10	**1.72±0.41**
		Unassigned	Unassigned	Unassigned	0.06±0.05	0.00±0.00	0.04±0.04	**0.66±0.44**
	Subgroup 18	Uncultured bacterium			0.05±0.02	0.00±0.00	0.00±0.01	**0.35±0.21**
	Subgroup 22	Uncultured bacterium			0.92±0.39	0.00±0.00	0.12±0.10	**1.96±1.01**
	Unassigned	Unassigned	Unassigned	Unassigned	0.07±0.04	0.00±0.00	0.01±0.01	**0.52±0.25**
Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.09±0.04	0.00±0.00	0.02±0.02	**0.24±0.16**
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.01±0.01	0.33±0.12	**0.91±1.06**	0.00±0.00
			Barnesiellaceae	Uncultured	0.16±0.09	0.05±0.03	**3.42±2.27**	0.02±0.04
			Paludibacteraceae	*Paludibacter*	0.03±0.04	0.01±0.01	**0.35±0.19**	0.00±0.00
			Prolixibacteraceae	*Roseimarinus*	0.63±0.29	1.07±0.26	**2.60±0.72**	0.30±0.10
			Rikenellaceae	Blvii 28 wastewater-sludge group	0.02±0.02	0.00±0.00	**0.54±0.39**	0.01±0.02
		Chitinophagales	Chitinophagaceae	*Ferruginibacter*	0.04±0.03	0.00±0.00	**0.15±0.21**	**0.23±0.11**
				Terrimonas	0.03±0.01	0.00±0.00	**0.23±0.32**	**0.51±0.08**
				Unassigned	0.10±0.03	0.00±0.00	0.17±0.22	**0.47±0.20**
			Saprospiraceae	*Haliscomenobacter*	0.10±0.06	0.00±0.00	**0.26±0.24**	**0.42±0.12**
				Saprospira	0.00±0.00	0.00±0.00	**0.16±0.16**	0.00±0.00
		Cytophagales	Microscillaceae	Uncultured	0.23±0.08	0.00±0.00	0.17±0.16	**0.49±0.17**
			Unassigned	Unassigned	0.03±0.02	0.06±0.00	**0.15±0.17**	**0.25±0.13**
		Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	1.29±0.31	21.67±1.27	**5.47±1.78**	0.47±0.09
				Unassigned	0.10±0.03	0.46±0.14	**0.35±0.11**	0.03±0.03
Calditrichaeota	Calditrichia	Calditrichales	Calditrichaceae	*Calorithrix*	0.00±0.00	0.00±0.00	0.00±0.00	**0.19±0.30**
Cloacimonetes	Cloacimonadia	Cloacimonadales	Uncultured bacterium		0.00±0.00	0.00±0.00	**0.30±0.33**	0.00±0.00
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Uncultured	0.23±0.11	0.00±0.00	0.05±0.04	**0.56±0.08**
		RBG-13-54-9	Uncultured bacterium		0.01±0.01	0.00±0.00	0.00±0.00	**0.39±0.49**
		SBR1031	A4b	Uncultured bacterium	0.10±0.01	0.00±0.00	0.06±0.08	**0.36±0.14**
			Uncultured bacterium		0.14±0.06	0.00±0.00	0.15±0.03	**1.19±0.51**
	KD4-96	Uncultured Chloroflexi bacterium			0.95±0.26	0.00±0.00	0.06±0.05	**1.15±0.51**
	Unassigned	Unassigned	Unassigned	Unassigned	0.03±0.03	0.00±0.00	0.00±0.01	**0.61±0.22**
Defferibacteres	Defferibacteres	Defferibacteriales	Defferibacteriaceae	Uncultured	0.12±0.03	0.12±0.07	**0.76±0.57**	0.03±.05
Epsilonbacteraeota	Campylobacteria	Campylobacteriales	Arcobacteraceae	*Arcobacter*	0.11±0.07	5.68±1.96	**2.36±1.07**	0.01±0.02
			Sulfurospirillaceae	*Sulfurospirillum*	0.01±0.01	0.03±0.01	**0.16±0.07**	0.00±0.00
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Carnobacterium*	0.02±0.02	0.05±0.04	**0.41±0.20**	0.00±0.00
	Clostridia	Clostridiales	Christesenellaceae	*Christesenellaceae* R7 Group	0.02±0.01	0.01±0.01	**0.42±0.22**	0.01±0.01
			Family XII	Uncultured	0.04±0.04	0.00±0.00	**0.29±0.13**	0.00±0.00

			Family XIII	Uncultured	0.05±0.03	0.00±0.00	**0.31±0.20**	0.01±0.02
			Lachnospiraceae	*Lachnoclostridium* 10	0.01±0.01	0.00±0.00	**0.17±0.19**	0.01±0.02
				Uncultured	0.02±0.01	0.15±0.04	**0.39±0.43**	0.00±0.00
			Ruminococcaceae	Unassigned	0.04±0.03	0.43±0.11	**0.27±0.16**	0.06±0.07
		Unassigned	Unassigned	Unassigned	0.17±.06	0.17±0.07	**0.30±0.13**	0.02±0.02
	Erysipelotrichia	Elysipelotrichiales	Erysipelotrichiaceae	*Solobacterium*	0.06±0.04	0.01±0.01	**0.75±0.15**	0.02±0.04
	Negativicutes	Selenomonadales	Acidaminococcaceae	Uncultured	0.00±0.00	0.09±0.03	**0.33±0.34**	0.00±0.00
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*Gemmatimonas*	0.03±0.02	0.00±0.00	0.01±0.01	**0.21±0.19**
				Uncultured	0.06±0.05	0.00±0.00	0.16±0.15	**1.81±0.78**
				Unassigned	0.01±0.01	0.00±0.00	0.07±0.08	**0.62±0.22**
Latescibacteria	Uncultured bacterium				0.26±0.11	0.00±0.00	0.15±0.12	**1.52±0.41**
	Uncultured Latescibacteria bacterium				0.00±0.01	0.00±0.00	0.02±0.03	**0.31±0.13**
	Unassigned	Unassigned	Unassigned	Unassigned	0.01±0.02	0.00±0.00	0.01±0.01	**0.34±0.16**
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	1.51±0.65	0.00±0.00	0.38±0.32	**3.53±2.41**
Proteobacteria	α-Proteobacteria	Caulobacterales	Hyphomonadaceae	UKL3-1	0.46±0.14	0.00±0.00	0.34±0.15	**1.41±0.54**
		Dongiales	Dongiaceae	*Dongia*	0.27±0.11	0.00±0.00	0.12±0.11	**0.86±0.34**
		Rhizobiales	A0389	Uncultured bacterium	0.00±0.01	0.00±0.00	0.02±0.02	**0.20±0.13**
			Xanthobacteraceae	Uncultured	0.35±0.19	0.00±0.00	0.05±0.06	**1.51±0.89**
		Sphingomonadales	Sphingomonadaceae	Unassigned	0.10±0.04	0.24±0.02	**0.67±0.31**	**0.53±0.15**
		Tistrellales	Tistrellaceae	*Tistrella*	0.04±0.04	0.00±0.00	0.04±0.04	**0.20±0.12**
		Uncultured	Uncultured Green bay ferromanganous micronodule bacterium MNH4		0.00±0.00	0.00±0.00	0.00±0.00	**0.22±0.12**
	δ-Proteobacteria	Desulfarculales	Desulfarculaceae	Uncultured	0.20±0.12	0.00±0.00	0.03±0.03	**0.32±0.17**
		Desulfobacterales	Desulfobacteraceae	SEEP-SRB1	0.26±0.06	0.00±0.00	0.02±0.02	**0.81±0.40**
			Desulfobulbaceae	*Desulfobulbus*	1.18±0.15	0.00±0.00	**1.56±0.78**	0.23±0.18
		Myxococcales	Phaselicystidaceae	*Phaselyscistis*	0.13±0.06	0.00±0.00	**0.24±0.20**	**0.58±0.24**
		MBNT15	Uncultured bacterium		0.05±0.01	0.00±0.00	0.01±0.01	**0.41±0.40**
		NB1-j	Uncultured bacterium		0.37±0.18	0.00±0.00	0.09±0.08	**1.37±0.72**
		Syntrophobacterales	Syntrophaceae	*Smithella*	0.42±0.14	0.00±0.00	0.07±0.03	**0.56±0.52**
		Unassigned	Unassigned	Unassigned	0.52±0.11	0.01±0.01	**1.72±1.40**	**2.92±0.43**
	γ-Proteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	0.13±0.07	9.89±0.77	**3.82±1.02**	0.01±0.03
		Alteromonadales	Pseudoalteromonadaceae	*Pseudoalteromonas*	0.00±0.00	0.32±0.20	**0.13±0.08**	0.00±0.00
		CCD24	metagenome		0.58±0.19	0.00±0.00	0.17±0.14	**0.94±0.29**
		Betaproteobacteriales	Burkholderiaceae	*Acidovorax*	0.17±0.06	9.61±1.33	**8.25±1.09**	0.25±0.13
				Caenimonas	0.01±0.01	0.00±0.00	0.04±0.03	**0.27±0.25**
				Massilia	0.05±0.01	0.64±0.04	**0.22±0.34**	**0.16±0.27**
				Polaromonas	0.03±0.01	0.05±0.02	**0.65±0.55**	0.00±0.00
				Rhodoferax	10.86±0.86	3.46±0.36	**13.33±2.35**	3.82±1.49
				Uncultured	2.32±0.57	2.49±0.28	0.69±0.56	**3.08±0.63**
			Gaiellaceae	*Sideroxydans*	0.20±0.10	0.07±0.02	**1.23±0.47**	**1.70±0.49**
			Hydrogenophilaceae	*Thiobacillus*	0.28±0.10	0.00±0.00	0.03±0.05	**0.41±0.43**
			Nitrosomonadaceae	Ellin6067	1.90±0.58	0.01±0.01	0.62±0.41	**3.97±0.51**
			Rhodocyclaceae	*Azoarcus*	0.04±0.02	0.01±0.01	**0.99±0.43**	**0.12±0.10**
				Thauera	0.00±0.00	0.00±0.00	**0.50±0.20**	0.00±0.00
				Unassigned	0.02±0.03	0.00±0.00	0.00±0.01	**0.39±0.73**
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.63±0.49	15.05±3.29	**3.00±0.78**	0.02±0.02
			Pseudomonadaceae	*Pseudomonas*	0.81±0.07	14.10±1.45	**4.94±1.04**	0.42±0.20
		Xanthomonadales	Xanthomonadaceae	*Luteimonas*	0.84±0.18	0.04±0.03	**1.96±1.34**	0.46±0.19

		Unassigned	Unassigned	Unassigned	2.79±1.05	0.35±0.05	2.20±0.21	**3.59±0.64**
Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Spirochaeta 2*	0.02±0.01	0.00±0.00	0.02±0.03	**3.74±1.44**
				Treponema 2	0.20±0.10	0.01±0.01	**0.82±0.28**	0.07±0.66
				Unassigned	0.16±0.03	0.00±0.00	0.18±0.19	**0.73±0.03**
Synergistetes	Synergistia	Synergistales	Synergistaceae	*Aminomonas*	0.00±0.00	0.00±0.00	**0.47±0.3**	0.00±0.00

Lokacije uzorkovanja: UP, uzvodno; DW0, ispust; DW3000, 3000 m nizvodno od ispusta; WW, otpadna voda.

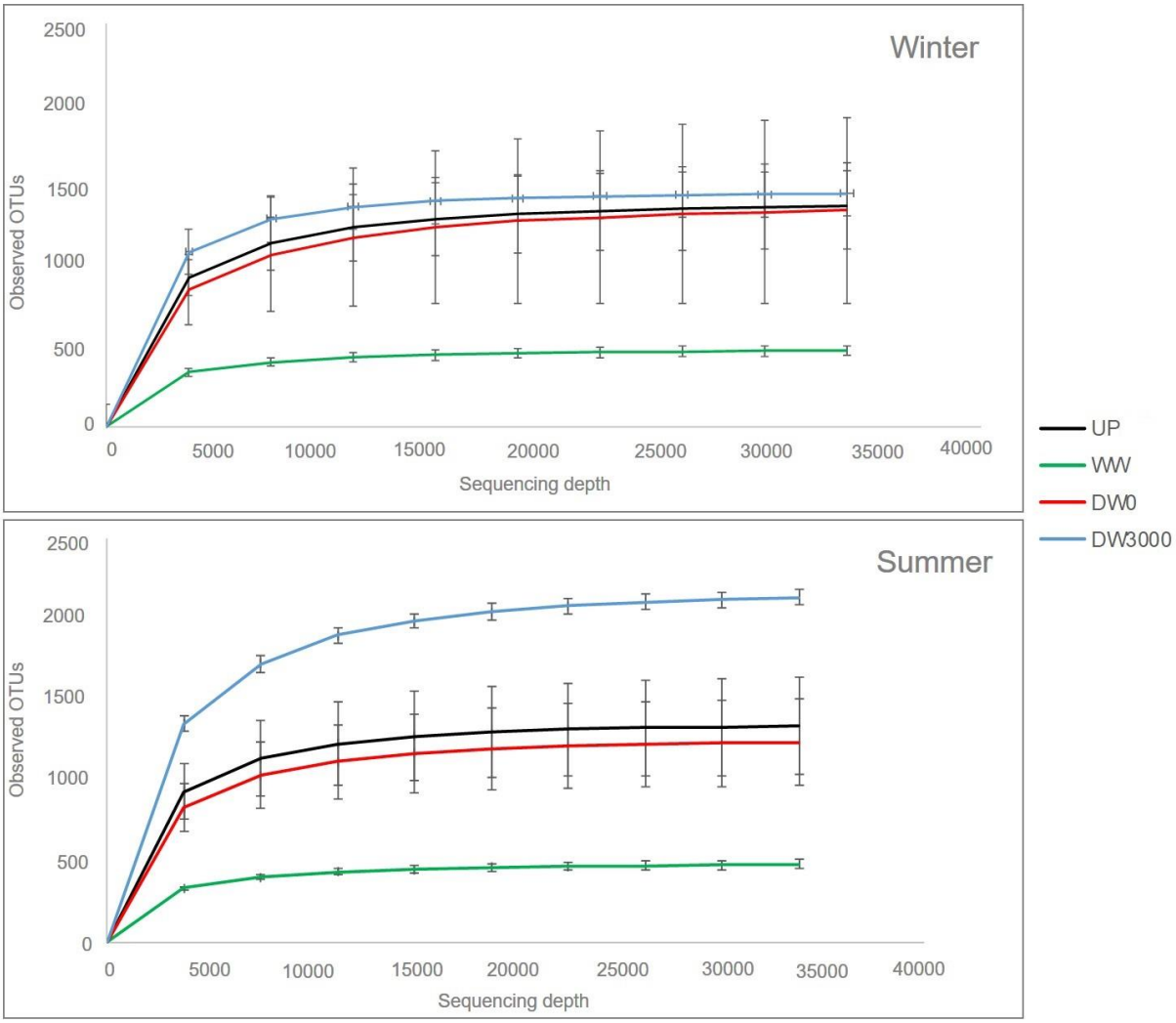
Tablica 20. Relativna zastupljenost rodova (izražena u postotku) koji su se značajno obogatili/povećali na nizvodnim lokacijama (DW) u odnosu na uzvodnu lokaciju (UP) tijekom ljetne sezone. Značajna razlika u relativnoj zastupljenosti rodova između nizvodnih lokacija (DW) i uzvodne lokacije (UP) je označena podebljano ($p < 0.05$). Oznaka tablice u radu **Table S8**.

Phylum	Class	Order	Family	Genus	Sampling sites			
					UP	WW	DW0	DW3000
Acidobacteria	Blastocatellia (Subgroup 4)	Blastocatellales	Blastocatellaceae	Unassigned	0.04±0.04	0.00±0.00	0.05±0.03	**0.34±0.23**
	Holophagae	Holophagales	Holophagaceae	Marine group	0.15±0.14	0.04±0.01	**0.90±0.51**	0.09±0.04
		Subgroup 7	Uncultured bacterium SJA-36		2.05±0.67	0.00±0.00	**2.96±0.68**	0.68±0.19
	Subgroup 5	Uncultured Acidobacterium sp.			0.09±0.03	0.00±0.00	0.00±0.00	**0.15±0.08**
	Subgroup 6	metagenome			0.16±0.06	0.00±0.00	0.01±0.01	**0.26±0.04**
	Subgroup 18	Unassigned	Unassigned	Unassigned	0.03±0.03	0.00±0.00	0.00±0.00	**0.52±0.23**
	Subgroup 22	Uncultured bacterium			0.00±0.00	0.00±0.00	0.01±0.00	**0.36±0.18**
	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	Subgroup 10	0.19±0.14	0.00±0.00	0.00±0.00	**0.65±0.14**
	Unassigned	Unassigned	Unassigned	Unassigned	0.06±0.01	0.00±0.00	0.07±0.05	**0.67±0.11**
Actinobacteria	Coriobacteriia	OPB41	Uncultured bacterium		0.20±0.07	0.00±0.00	**0.33±0.08**	0.03±0.02
	Unassigned	Unassigned	Unassigned	Unassigned	0.19±0.04	0.00±0.00	**0.45±0.27**	**0.21±0.10**
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.00±0.01	4.06±0.14	**0.60±0.42**	0.00±0.00
			Bacteroidetes vadinHA17	Uncultured bacterium	0.00±0.00	0.00±0.00	**0.30±0.18**	0.01±0.01
			Barnesiellaceae	Uncultured	0.10±0.07	1.72±0.13	**2.45±0.58**	0.01±0.01
			Paludibacteraceae	*Paludibacter*	0.04±.03	0.01±0.01	**0.15±0.11**	0.01±0.01
			Prolixibacteraceae	*Roseimarinus*	0.28±0.15	1.99±0.04	**1.97±0.67**	0.29±0.09
			Rikenellaceae	Blvii28 wastewater-sludge group	0.03±0.03	0.00±0.00	**0.63±0.36**	0.01±0.02
				Uncultured	0.02±0.01	0.11±0.01	**0.17±0.11**	0.00±0.00
		Chitinophagales	Chitinophagaceae	*Terrimonas*	0.08±0.10	0.00±0.00	0.06±0.05	**0.47±0.05**
			Saprospiraceae	*Haliscomenobacter*	0.12±0.11	0.00±0.00	0.05±0.04	**0.30±0.14**
		Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.48±0.18	8.00±0.46	**0.66±0.18**	0.13±0.09
	Ignavibacteria	Kryptoniales	BSV26	Uncultured bacterium	0.00±0.00	0.00±0.00	0.00±0.00	**0.14±0.06**
Calditrichaeota	Calditrichia	Calditrichales	Calditrichaceae	*Calorithrix*	0.01±0.01	0.00±0.00	0.03±0.01	**0.44±0.30**
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	Uncultured	0.20±0.05	0.00±0.00	0.04±0.02	**0.64±0.07**
		RBG-13-54-9	Uncultured bacterium		0.01±0.01	0.00±0.00	0.02±0.04	**0.56±0.39**
		SBR1031	A4b	Ambiguous taxa	0.03±0.05	0.00±0.00	0.00±0.01	**0.32±0.07**
				Uncultured bacterium	0.09±0.02	0.00±0.00	0.01±0.01	**0.47±0.05**
			Uncultured bacterium		0.41±0.13	0.00±0.00	**0.95±0.29**	**2.08±0.36**
	Dehalococcoidia	GIF9	Uncultured bacterium		0.00±0.00	0.00±0.00	0.02±0.01	**0.54±.25**
	Unassigned	Unassigned	Unassigned	Unassigned	0.01±0.01	0.00±0.00	0.01±0.01	**0.56±0.06**
Cloacimonetes	Cloacimonadia	Cloacimonadales	PBS-18	Uncultured bacterium	0.00±0.00	0.00±0.00	**0.18±0.16**	**0.22±0.12**
			Uncultured bacterium		0.00±0.00	0.00±0.00	**0.60±0.25**	0.00±0.00
			Unassigned	Unassigned	0.00±0.00	0.00±0.00	**0.27±0.12**	0.00±0.00
Epsilonbacteraeota	Campylobacteria	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.02±0.02	14.28±0.60	**1.25±0.92**	0.02±0.02
			Thiovulaceae	*Sulfuricurvum*	0.22±0.13	0.06±0.01	**0.98±0.18**	0.07±0.02
Firmicutes	Bacilli	Bacillales	Bacillaceae	Unassigned	0.00±0.00	0.04±0.00	**0.22±0.09**	0.01±0.02
		Lactobacillales	Carnobacteriaceae	*Carnobacterium*	0.00±0.00	0.11±0.01	**0.14±0.07**	0.00±0.00
	Clostridia	Clostridiales	Christesenellaceae	*Christesenellaceae* R7 Group	0.06±0.05	0.04±0.01	**0.76±0.34**	0.01±0.01
			Family XIII	Uncultured	0.05±0.03	0.03±0.02	**0.39±0.08**	0.00±0.00
			Ruminococcaceae	Unassigned	0.14±0.03	0.13±0.04	**0.56±0.47**	0.06±0.05
			Unassigned	Unassigned	0.12±0.05	0.11±0.01	**0.44±0.19**	0.03±0.01

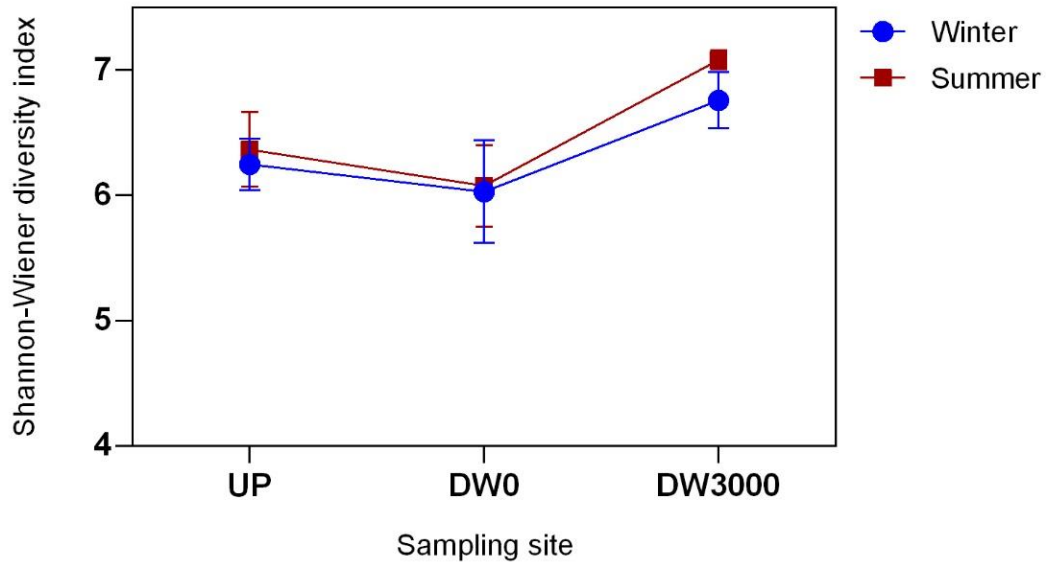
	Erysipelotrichia	Erysipelotrichiales	Erysipelotrichiaceae	*Solobacterium*	0.04±0.04	0.09±0.01	**1.21±0.61**	0.01±0.01
	Unassigned	Unassigned	Unassigned	Unassigned	0.26±0.04	0.07±0.02	**1.47±0.53**	0.07±0.02
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadaceae	Uncultured	0.31±0.36	0.00±0.00	0.13±0.13	**1.69±0.43**
				Unassigned	0.02±0.04	0.00±0.00	0.02±0.01	**0.55±0.14**
Latescibacteria	Latescibacteria	Latescibacteriales	Latescibacteriaceae	Uncultured bacterium	0.00±0.00	0.00±0.00	0.00±0.00	**0.25±0.17**
	Uncultured bacterium				0.30±0.17	0.00±0.00	**0.54±0.18**	**1.63±0.10**
	Unassigned	Unassigned	Unassigned	Unassigned	0.01±0.01	0.00±0.00	0.03±0.02	**0.31±0.05**
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	1.18±0.44	0.00±0.00	0.13±0.10	**3.59±1.67**
	Thermodesulfovibrionia	Uncultured	Uncultured Nitrospirae bacterium		0.00±0.00	0.00±0.00	0.00±0.00	**0.23±0.14**
	4-29-1	Uncultured bacterium			0.08±0.05	0.00±0.00	0.03±0.02	**0.50±0.07**
Proteobacteria	α-Proteobacteria	Caulobacterales	Hyphomonadaceae	UKL13-1	0.51±0.27	0.26±0.04	0.35±0.05	**1.53±0.07**
		Rhizobiales	A0839	Uncultured bacterium	0.03±0.05	0.00±0.00	0.00±0.00	**0.30±0.12**
			Beijerinckiaceae	*Psychroglaciecola*	0.00±0.00	0.10±0.03	**0.13±0.06**	0.04±0.04
			KF-JG30-B3	Metagenome	0.02±0.03	0.00±0.00	0.00±0.01	**0.24±0.11**
			Rhizobiales Inc. Sedis	Uncultured	0.03±0.03	0.00±0.00	0.01±0.01	**0.21±0.03**
			Xanthobacteraceae	Uncultured	0.27±0.24	0.00±0.00	0.08±0.03	**1.41±0.50**
			Unassigned	Unassigned	0.19±0.09	0.27±0.03	0.08±0.02	**0.62±0.06**
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	0.01±0.02	0.00±0.00	**0.23±0.10**	0.08±0.01
				Unassigned	0.04±0.07	0.13±0.01	**0.35±0.03**	**0.52±0.22**
		Unassigned	Unassigned	Unassigned	0.69±0.35	0.52±0.03	0.67±0.07	**1.82±0.54**
	δ-Proteobacteria	Desulfarculales	Desulfarculaceae	*Desulfatiglans*	0.01±0.01	0.00±0.00	0.01±0.00	**0.65±0.36**
		Desulfobacterales	Desulfobulbaceae	*Desulfobulbus*	1.40±0.33	0.00±0.00	**2.50±0.80**	0.18±0.08
				Desulfurivibrio	0.00±0.00	0.00±0.00	**0.80±1.36**	0.08±0.02
				MSBL7	0.00±0.00	0.00±0.00	**0.38±0.41**	0.01±0.01
				SEEP-SRB1	0.17±0.07	0.00±0.00	0.11±0.07	**0.72±0.16**
		Desulfuromonadales	Sva1033	Unassigned	0.00±0.00	0.00±0.00	**0.32±0.12**	0.00±0.00
			Unassigned	Unassigned	0.07±0.07	0.00±0.00	**0.24±0.25**	0.08±0.01
		Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*	0.00±0.00	0.02±0.02	**0.17±0.18**	0.00±0.01
			Phaselicystidaceae	*Phaselicystis*	0.35±0.15	0.00±0.00	0.37±0.31	**0.48±0.19**
		NB1-j	Uncultured bacterium		0.39±0.27	0.00±0.00	0.03±0.03	**1.20±0.24**
		Syntrophobacterales	Syntrophaceae	*Smithella*	0.45±0.14	0.00±0.00	**1.47±1.39**	**1.36±0.46**
			Syntrophobacteraceae	*Syntrophobacter*	0.03±0.02	0.00±0.00	**0.23±0.27**	0.05±0.02
		Unassigned	Unassigned	Unassigned	0.46±0.04	0.02±0.02	**1.84±0.47**	**2.66±0.50**
	γ-Proteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	0.13±0.03	9.71±1.25	**2.22±1.48**	0.02±0.03
			Succinivibrionaceae	Uncultured	0.01±0.01	0.02±0.00	**0.28±0.12**	0.00±0.00
		Alteromonadales	Shewanellaceae	*Shewanella*	0.00±0.00	0.19±0.03	**0.11±0.08**	0.00±0.00
		CCD24	metagenome		0.68±0.07	0.00±0.00	0.03±0.04	**1.09±0.18**
		Betaproteobacteriales	Burkholderiaceae	*Acidovorax*	0.11±0.02	13.30±0.24	**5.05±2.96**	**0.23±0.13**
				Uncultured	1.81±0.27	0.06±0.00	0.16±0.13	**3.51±0.65**
			Galliionellaceae	*Sideroxydans*	0.14±0.09	0.15±0.03	**0.65±0.28**	**1.53±0.53**
			Hydrogenophilaceae	*Thiobacillus*	0.42±0.01	0.00±0.00	0.05±0.04	**0.58±0.31**
			Nitrosomonadaceae	Ellin6067	1.98±0.22	0.00±0.01	0.66±0.20	**3.54±0.33**
			Rhodocyclaceae	*Azoarcus*	0.04±0.03	0.18±0.01	**1.00±0.43**	0.06±0.05
				Thauera	0.00±0.00	0.05±0.02	**1.24±0.45**	0.00±0.00
				Unassigned	0.05±0.02	0.02±0.00	0.00±0.00	**0.51±0.44**
			Unassigned		2.43±0.54	0.68±0.04	**2.68±0.72**	**3.43±1.51**
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.01±0.01	16.55±1.32	**1.97±1.35**	0.02±0.03
			Pseudomonadaceae	*Pseudomonas*	0.89±0.11	1.66±0.07	**1.20±0.81**	0.19±0.04
		Unassigned	Unassigned	Unassigned	2.92±0.31	4.51±0.13	**3.16±1.47**	**3.59±0.50**

Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	*Spirochaeta 2*	0.08±0.04	0.00±0.00	**0.69±0.10**	**3.89±1.58**
				Treponema 2	0.48±0.13	0.01±0.01	**1.07±0.64**	0.09±0.01
				Uncultured	0.96±0.43	0.00±0.00	**7.38±2.07**	**1.24±0.23**
				Unassigned	0.28±0.12	0.00±0.00	**0.37±0.17**	**0.71±0.22**
Synergistetes	Synergistia	Synergistales	Synergistaceae	*Aminomonas*	0.00±0.00	0.02±0.01	**0.57±0.06**	0.00±0.00

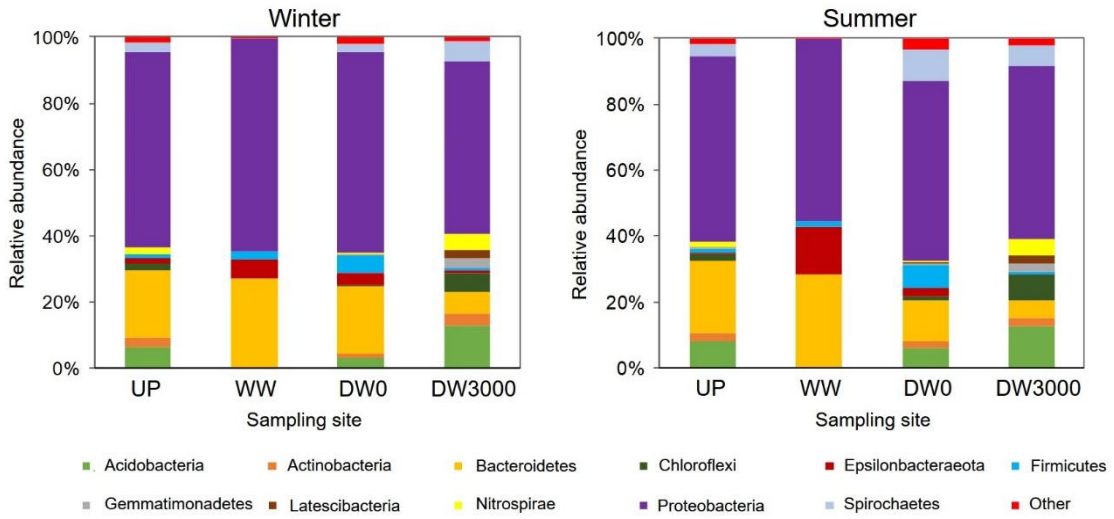
Lokacije uzorkovanja: UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta; WW, otpadna voda.



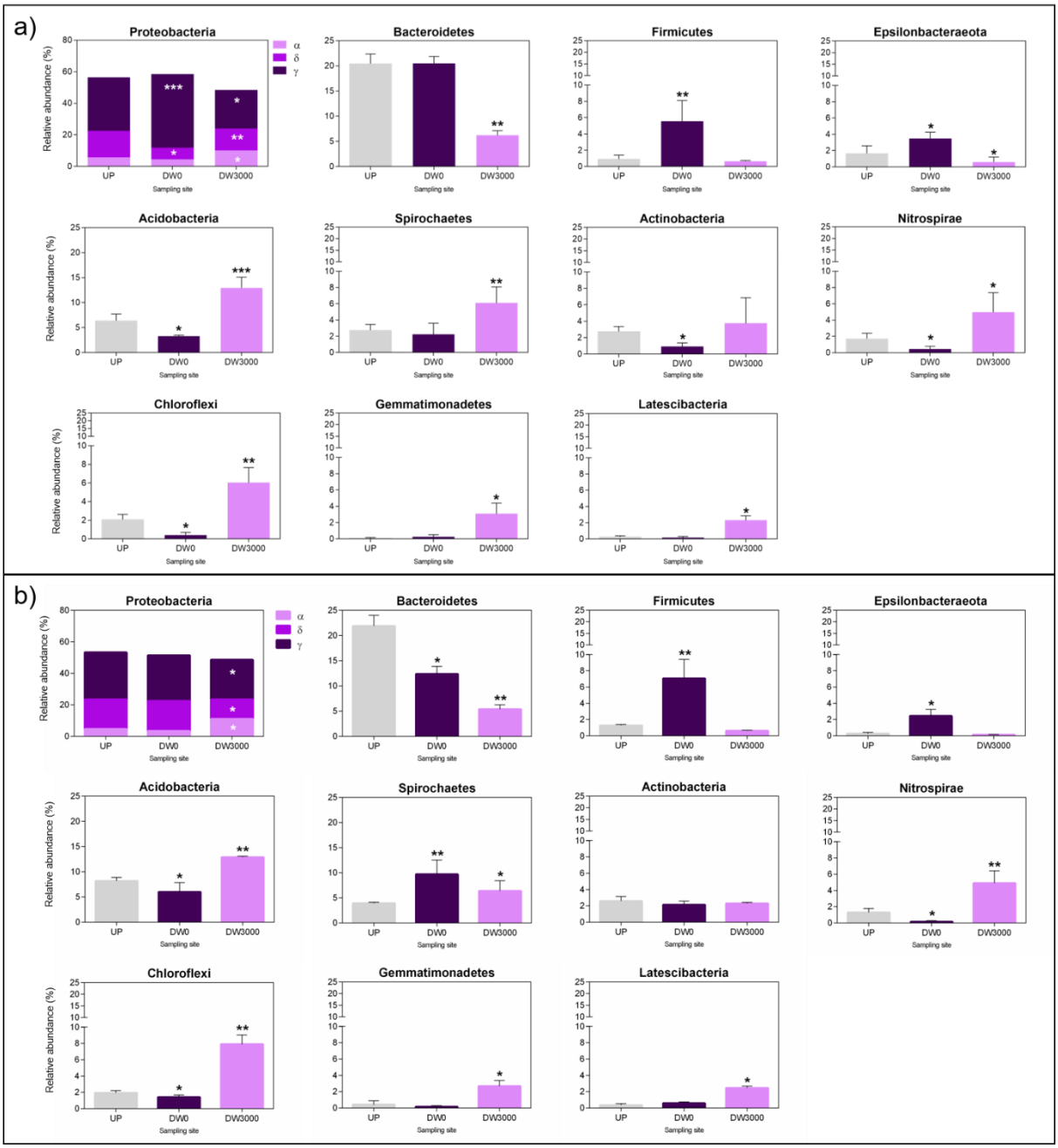
Slika 11. Rarefakcijske krivulje koje prikazuju alfa raznolikost otpadne vode iz formulacije antibiotika (WW) i sedimenata potoka Kalinovica dobivenih pomoću metrike „Observed ASVs (amplicon sequencing variants)“. Lokacije uzorkovanja: UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta. Oznaka slike u radu **Figure S1**.



Slika 12. Shannon-Wiener indeks raznolikosti sedimenata potoka Kalinovica u koji se ispuštaju otpadne vode iz formulacije antibiotika tijekom zimske i ljetne sezone. Lokacije uzorkovanja: UP, uzvodno; DW0, ispust; DW3000, 3000 m nizvodno od ispusta. Oznaka slike u radu **Figure S2**.



Slika 13. Taksonomska strukture bakterijske zajednice na razini koljena otpadne vode iz formulacije antibiotika (WW) i sedimenata potoka Kalinovica na različitim lokacijama tijekom zimske i ljetne sezone uzorkovanja. Bakterijska koljena sa relativnom zastupljenošću manjom od 1% u svim uzorcima su grupirana u „Other“. Lokacije uzorkovanja: UP, uzvodno; DW0, ispust; DW3000, 3000 m nizvodno od ispusta. Oznaka slike u radu **Figure S3**.



Slika 14. Promjene u sastavu bakterijske zajednice na razini koljena u nizvodnim sedimentima (DW) u odnosu na uzvodni sediment (UP) tijekom a) zimske i b) ljetne sezone. Prikazana relativna zastupljenost bakterijskih koljena temelji se na 99 % sličnosti sa podacima iz SILVA baze podataka. Zvjezdice prikazuju statistički značajnu razliku (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; DESeq2) između svake nizvodne lokacije (DW) i uzvodne (UP) lokacije. Lokacije uzorkovanja: UP, uzvodno od ispusta; DW0, ispust; DW3000, 3000 m nizvodno od ispusta. Oznaka slike u radu **Figure S4**.

7.6. Dodatne informacije uz rad:

Milaković M, Petrić I, Križanović S, Šimatović A, Gonzalez Plaza JJ, Gužvinec M, Pole L, Mrkonjić Fuka M, Udiković-Kolić N: Characterization of macrolide resistance in clinical and environmental isolates from macrolide-polluted and unpolluted river sediments. U postupku u časopisu Science of the Total Environment.

Tablica 21. Zastupljenost okolišnih kultivabilnih bakterijskih rodova otpornih na azitromicin.

Phylum	**Genus**	**Abundance (%)**	
		Upstream	**Discharge**
Actinobacteria (Gram-positive)	*Microbacterium*	42.31	29.84
	Arthrobacter	4.81	0.81
	Agromyces	3.85	0.81
	Rhodococcus	3.85	4.03
	Chryseoglobus	0.96	0.00
	Cellulosimicrobium	0.00	8.87
	Dietzia	0.00	4.03
	Lysinibacillus	0.00	1.61
	Tessaracoccus	0.00	0.81
	Sanguibacter	0.00	0.81
	Micrococcus	0.00	0.81
	Mycobacterium	0.96	0.00
Firmicutes (Gram-positive)	*Bacillus*	38.46	4.84
	Staphylococcus	1.92	0.81
	Paenibacillus	0.96	0.00
	Fictibacillus	0.96	0.00
	Enterococcus	0.00	5.65
	Streptococcus	0.00	4.84
	Trichococcus	0.00	1.61
	Aerococcus	0.00	1.61
	Lactococcus	0.00	0.81
Bacteroidetes (Gram-negative)	*Chryseobacterium*	0.00	4.84
	Flavihumibacter	0.00	0.81
γ-Proteobacteria (Gram-negative)	*Acinetobacter*	0.96	0.81
	Citrobacter	0.00	2.42
	Thermomonas	0.00	1.61
	Pseudoxanthomonas	0.00	1.61
	Pseudomonas	0.00	0.81
	Stenotrophomonas	0.00	0.81
α-Proteobacteria (Gram-negative)	*Brevundimonas*	0.00	7.26
	Rhodobacter	0.00	0.81
β-Proteobacteria (Gram-negative)	*Acidovorax*	0.00	3.23
	Comamonas	0.00	1.61
	Neisseria	0.00	0.81

Tablica 22. Fenotipska i genotiska karakterizacija okolišnih bakterijskih izolata sa uzvodne lokacije i lokacije ispusta. Profil otpornosti bakterijskih izolata na antibiotike temelji se na CLSI standardima.

Source	Phylum	Genus/Species	N	MRGs	Resistance profile
Upstream	***Actinobacteria***	*Arthrobacter* sp.	1	*erm*F	ERY; AZM; CLA
		Microbacterium sp.	1	*msr*D	ERY; AZM; CLA
			1	*mef*E	ERY; AZM; CLA; TET
			1	*mph*E	ERY; AZM; CLA; CIP
			1	*erm*F	ERY; AZM; CLA; CIP
			1	*msr*D	ERY; AZM; CLA; CIP
	Firmicutes	*Bacillus licheniformis*	1	*msr*A+*hfl*X	ERY; AZM; CLA
			2	*erm*F	
			1	*msr*E	
			1	*msr*D	
			3	*hfl*X	
	Proteobacteria	*Acinetobacter* sp.	1	*msr*E+*mph*E	**
Discharge	***Actinobacteria***	*Arthrobacter* sp.	1	*erm*F	ERY; AZM; CLA; TET
		Cellulosimicrobium cellulans	1	*erm*B	ERY; AZM; CLA; CIP
		Microbacterium sp.	1	*erm*B	ERY; AZM; CLA; TET; CIP
			1	*mef*E	ERY; AZM; CLA
			1	*mph*E	ERY; AZM; CLA; CIP
		Microbacterium estearomaticum	1	*erm*B	ERY; AZM; CLA; TET
			1	*erm*B	ERY; AZM; CLA; CIP
		Microbacterium keratanolyticum	1	*hfl*X	ERY; AZM; CLA
		Microbacterium saccharophilum	2	*erm*F	ERY; AZM; CLA
		Microbacterium paraoxydans	1	*hfl*X	ERY; AZM; CLA
			1	*erm*F+*msr*E	
			1	*erm*F	

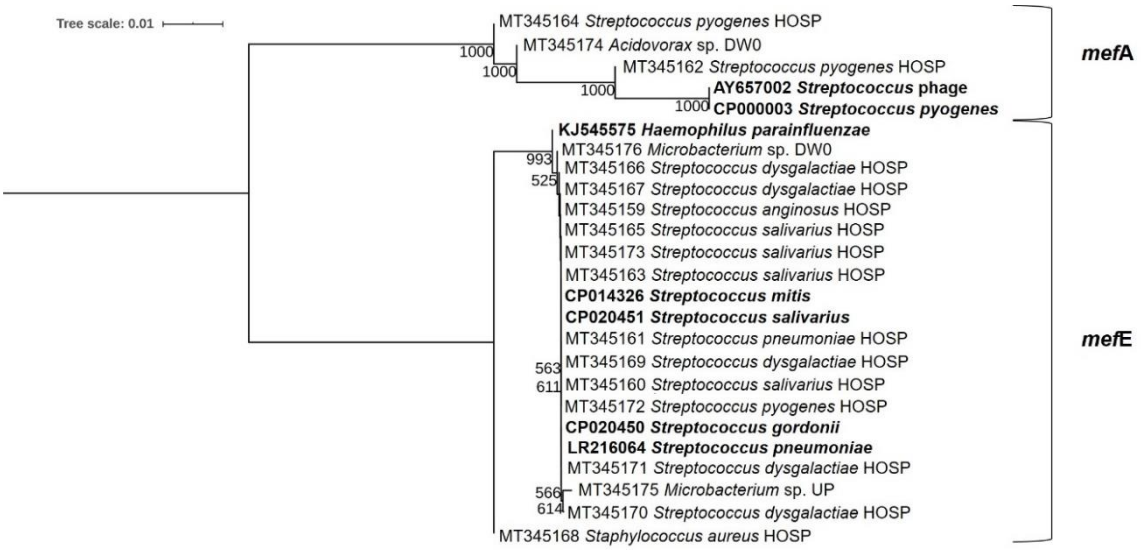
		Micrococcus aloeverae	1	*msr*E	ERY
		Lysinibacillus fusiformis	1	*erm*B	ERY; AZM; CLA; TET; AMP; CIP
			1	*erm*B	ERY; AZM; CLA; AMP
		Tessaracoccus flavescens	1	*mph*E	*
	Bacteroidetes	*Chryseobacterium* sp.	1	*erm*F+*msr*D	*
	Firmicutes	*Aerococcus viridans*	1	*erm*B	TET; CIPRO
		Bacillus sp.	1	*erm*B+*msr*E	ERY; AZM; CLA
		Bacillus cereus	1	*erm*B	ERY; AZM; CLA
		Enterococcus saccharolyticus	1	*erm*B+*erm*F	ERY; AZM; CLA
			1	*erm*B	ERY; AZM; CLA; TET; AMP; CIP
		Enterococcus sp.	1	*erm*B+*mef*C	ERY; AZM; CLA; TET; AMP
		Enterococcus asini	1	*erm*B	ERY; AZM; CLA; AMP; CIP
		Enterococcus avium	1	*erm*B	ERY; AZM; CLA; AMP; CIP
		Lactococcus lactis	1	*erm*B	ERY; AZM; CLA
		Staphylococcus epidermidis	1	*msr*A+*msr*E	ERY
		Streptococcus sp.	5	*erm*B	ERY; AZM; CLA; AMP
		Trichococcus sp.	1	*erm*B	*
		Trichococcus flocculiformis	1	*erm*F	
	Proteobacteria	*Acinetobacter johnsonii*	1	*msr*E+*mph*E	**
		Brevundimonas bullata	1	*erm*F	*
			1	*msr*E	
		Brevundimonas diminuta	1	*erm*B	
		Comamonas sp.	1	*msr*E+*mph*E	
		Comamonas denitrificans	1	*msr*E+*mph*E	
		Citrobacter freundii	2	*msr*E+*mph*E	
			1	*erm*F+*msr*E+*mph*E	
		Acidovorax sp.	1	*mef*A+*msr*E+*mph*E+*hfl*X	
			1	*msr*E+*mph*E	
		Neisseria sp.	1	*mph*E	
		Pseudomonas sp.	1	*msr*E	
		Pseudoxanthomonas sp.	1	*mph*E	

		Rhodobacter sp.	1	*msr*E+*mph*E	
		Stenotrophomonas sp.	1	*msr*E+*mph*E	
		Thermomonas brevis	1	*mph*E	

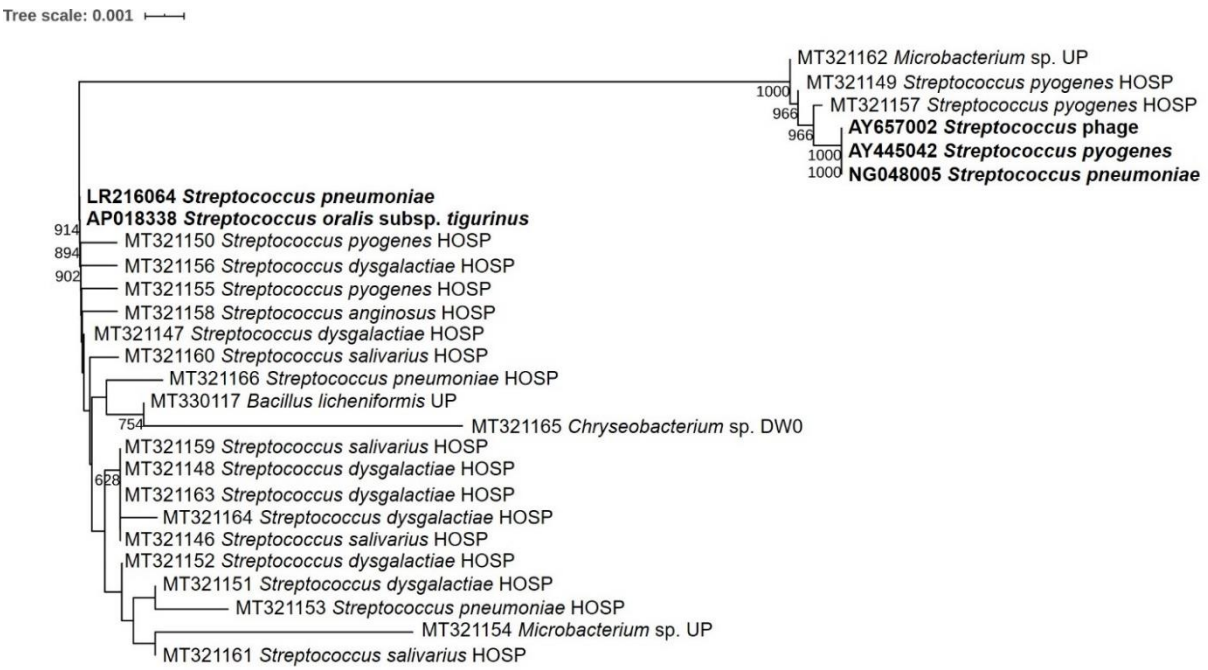
***** Ne postoje CLSI smjernice za određivanje razine otpornosti na tu vrstu antibiotika (priručnik M45 *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria*) (2016).

** Ne postoje CLSI smjernice za određivanje razine otpornosti na makrolidne antibiotike (priručnik M45 *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria*) (2016).

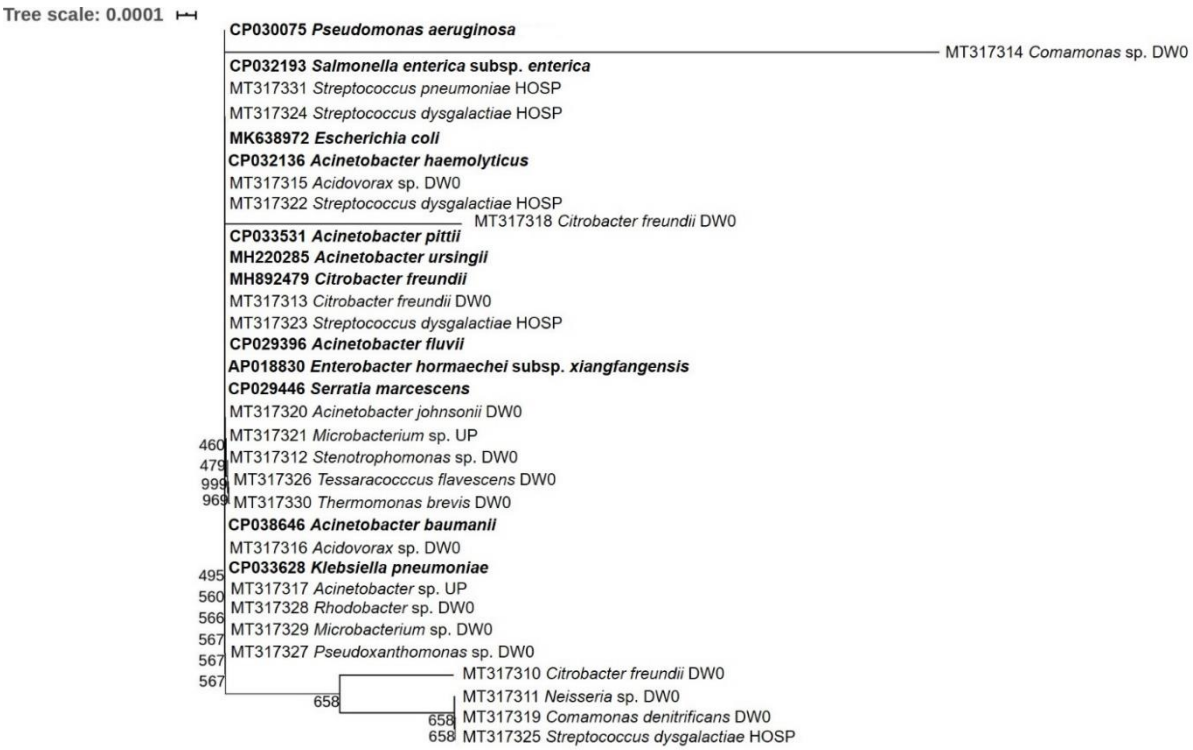
N-broj izolata; MRGs – geni za otpornost na makrolide. ERY – eritromicin; AZI – azitromicin; CLA – klaritromicin; TET – tetraciklin; AMP – ampicilin; CIP – ciprofloksacin. CLSI smjernice za AZI i CLA su temeljene na smjernicama za ERY za sve rodove osim *Streptococcus* spp. i *Staphylococcus* spp. Za rodove *Streptococcus* spp. i *Lactococcus* spp. smjernice za određivanje otpornosti na CIPRO su temeljene na smjrnicama za levofloksacin i ofloksacin.



Slika 15. Filogenetsko stablo dobiveno usporedbom sekvenci gena *mef*A/E iz kliničkih (HOSP) i okolišnih izolata sa uzvodne lokacije (UP) i lokacije ispusta (DW0) te reprezentativnih sekvenci (prikazane podebljano) iz NCBI baze. Stablo je izgrađeno metodom povezivanja susjeda (engl. *Neighbour joining*) pomoću računalnog programa MEGA X. Podrška za grananja unutar stabla određena je na temelju 1.000 ponavljanja.

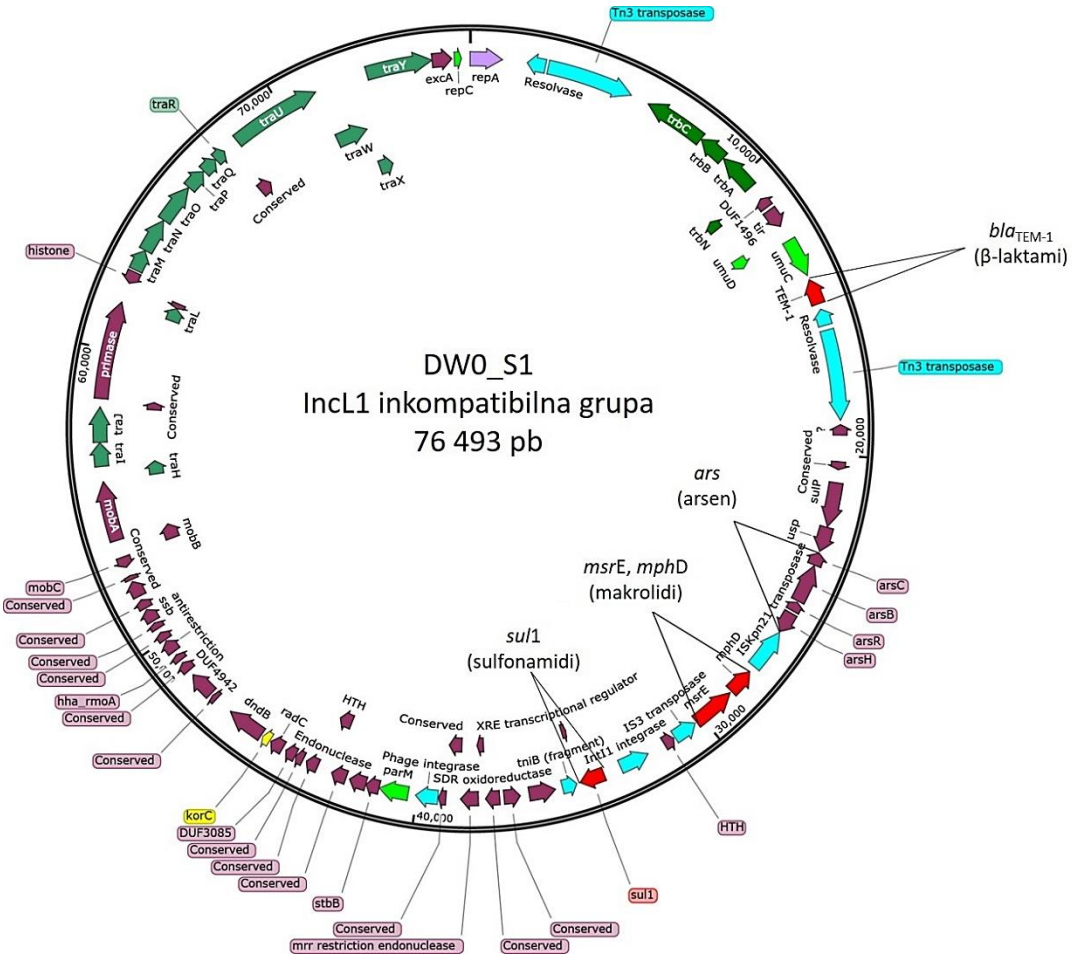


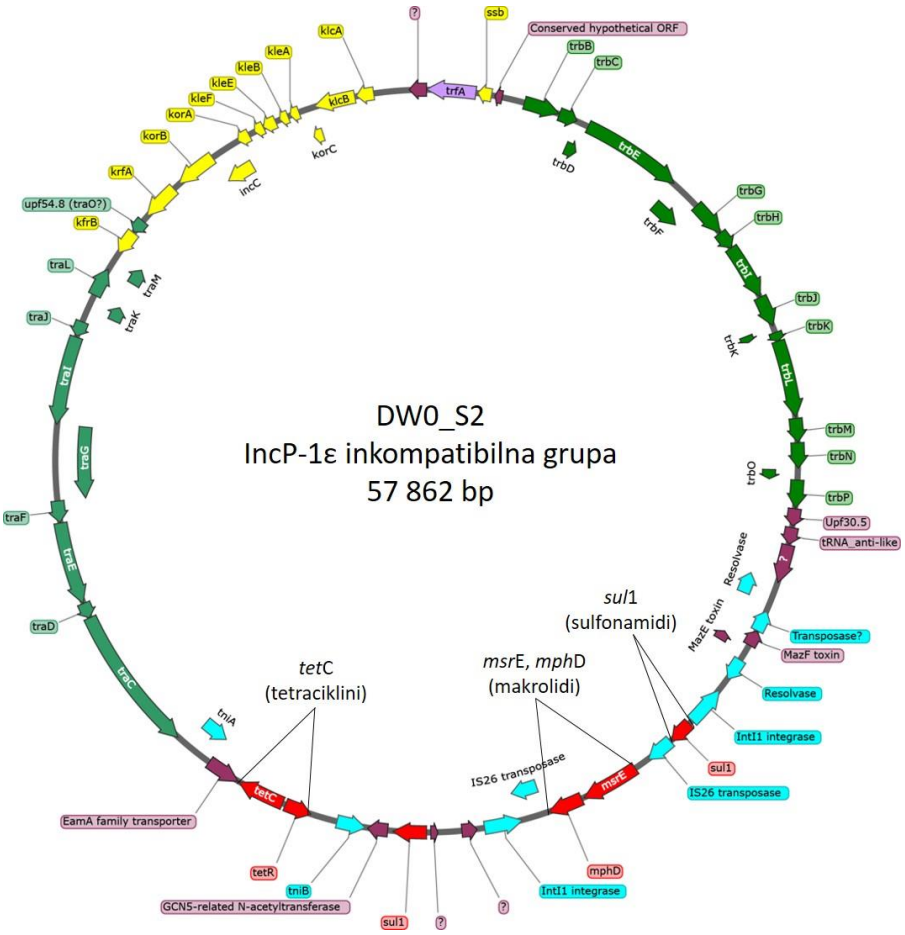
Slika 16. Filogenetsko stablo dobiveno usporedbom sekvenci gena *msr*D iz kliničkih (HOSP) i okolišnih izolata sa uzvodne lokacije (UP) i lokacije ispusta (DW0) te reprezentativnih sekvenci (prikazane podebljano) iz NCBI baze. Stablo je izgrađeno metodom povezivanja susjeda (engl. *Neighbour joining*) pomoću računalnog programa MEGA X. Podrška za grananja unutar stabla određena je na temelju 1.000 ponavljanja.

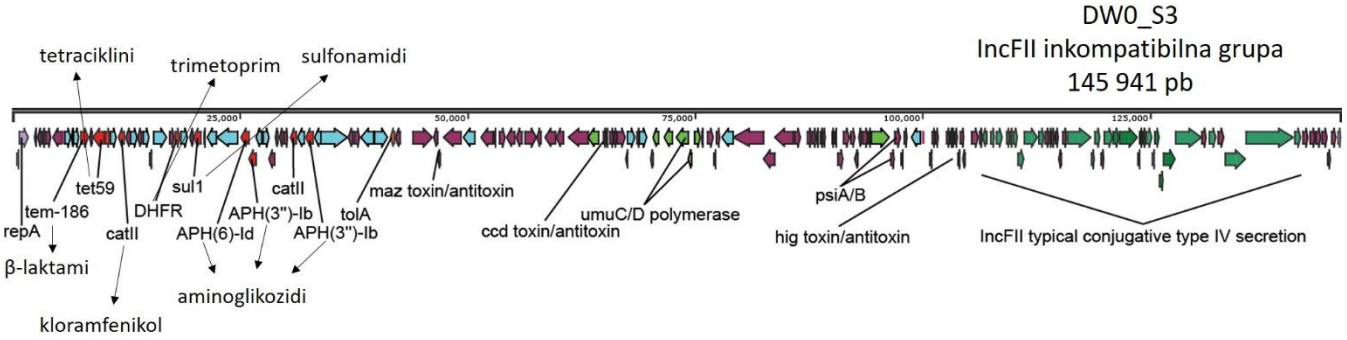


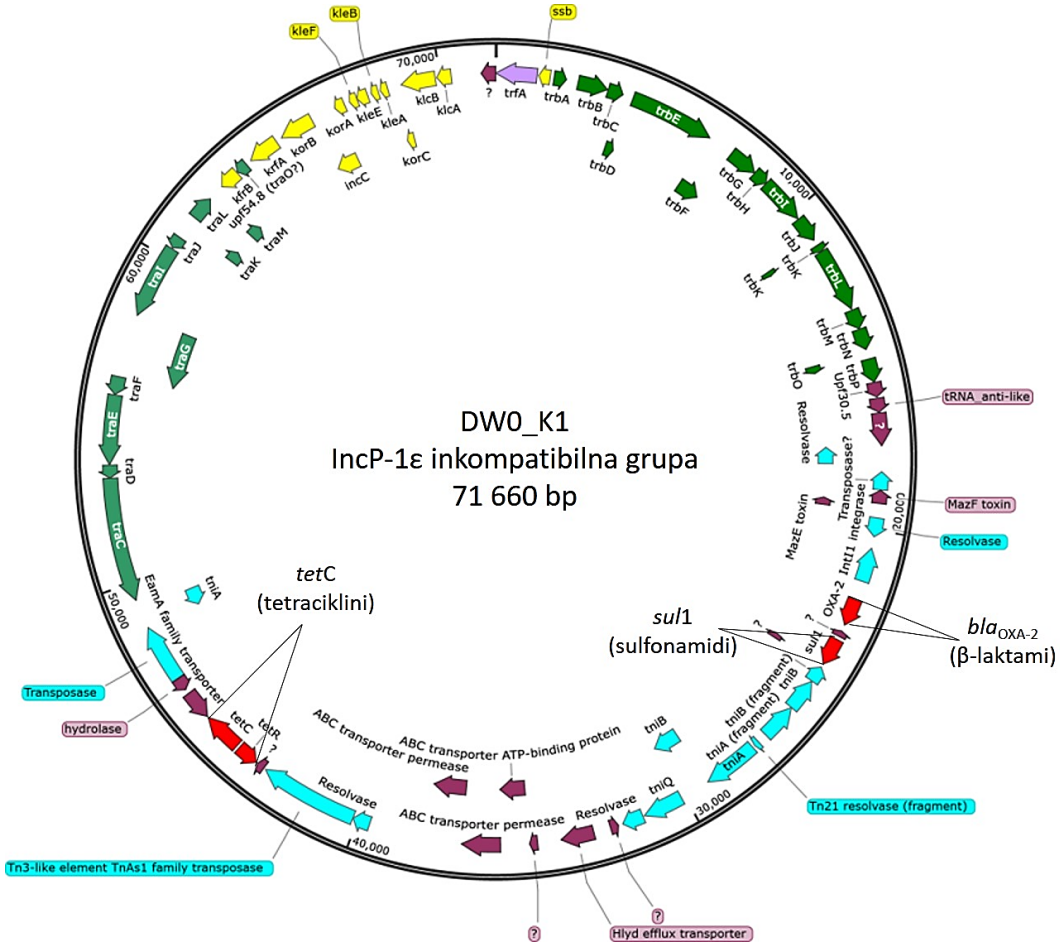
Slika 17. Filogenetsko stablo dobiveno usporedbom sekvenci gena *mph*E iz kliničkih (HOSP) i okolišnih izolata sa uzvodne lokacije (UP) i lokacije ispusta (DW0) te reprezentativnih sekvenci (prikazane podebljano) iz NCBI baze. Stablo je izgrađeno metodom povezivanja susjeda (engl. *Neighbour joining*) pomoću računalnog programa MEGA X. Podrška za grananja unutar stabla određena je na temelju 1.000 ponavljanja.

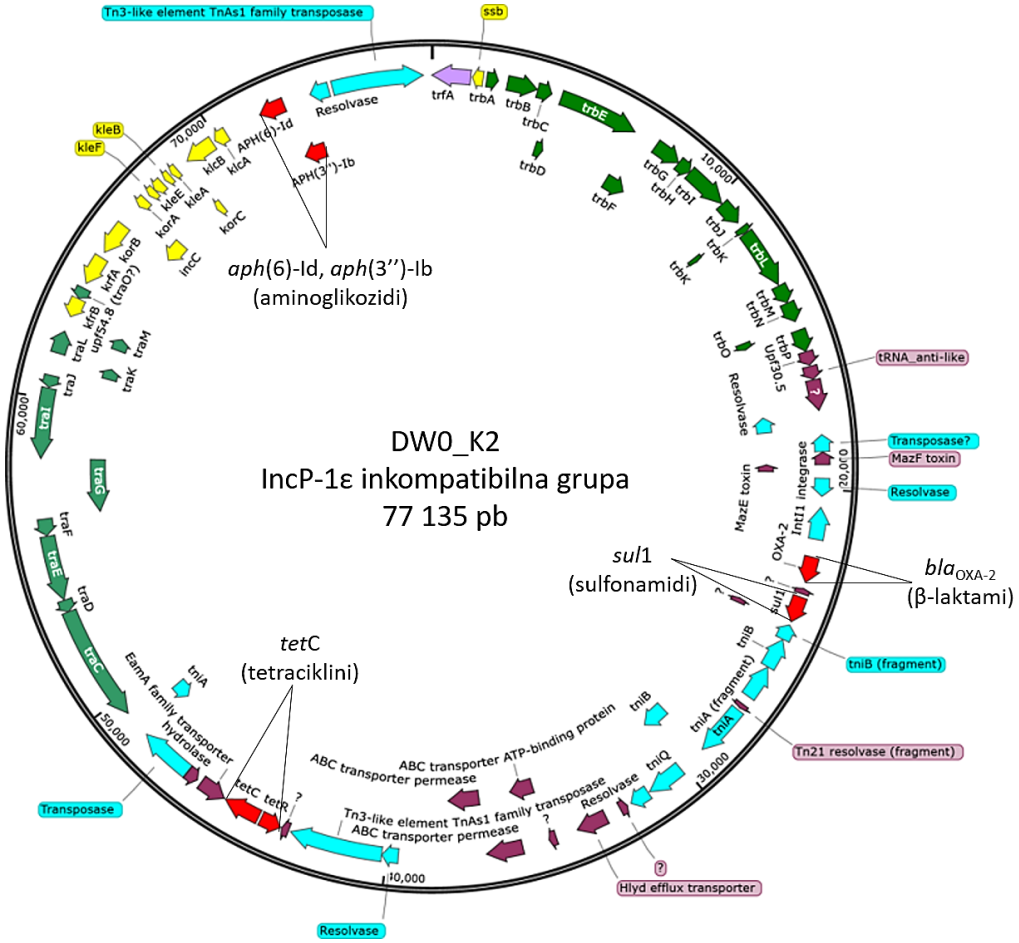
7.7. Sekvence plazmida izoliranih iz sedimenta s lokacije ispusta rijeke Save (DW0_S1-3) i potoka Kalinovica (DW0_K1-3).

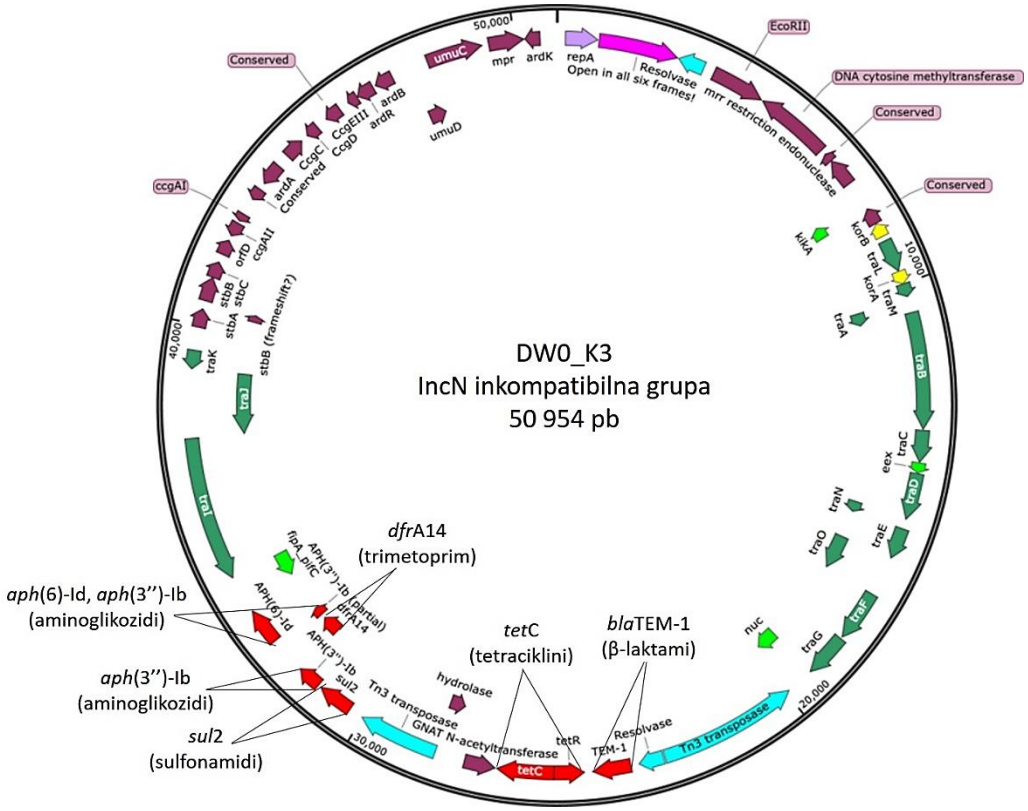



DW0_S2
IncP-1ε inkompatibilna grupa
57 862 bp
sul1
(sulfonamidi)
msrE, mphD
(makrolidi)
tetC
(tetraciklini)
?
ssb
Conserved hypothetical ORF
trbB
trbC
trfA
trbE
trbD
trbF
trbG
trbH
trbI
trbJ
trbK
trbL
trbM
trbN
trbO
trbP
Upf30.5
tRNA_anti-like
Resolvase
MazE toxin
Transposase?
MazF toxin
Resolvase
IntI1 integrase
sul1
IS26 transposase
msrE
IS26 transposase
mphD
IntI1 integrase
?
?
sul1
GCN5-related N-acetyltransferase
tniB
tetR
tetC
EamA family transporter
tniA
traC
traD
traF
traG
traI
traJ
traK
traM
traL
kfrB
upf54.8 (traO?)
krfA
korB
korA
kleF
kleE
kleB
kleA
klcA
klcB
korC
incC


DW0_S3
IncFII inkompatibilna grupa
145 941 pb
tetraciklini
trimetoprim
sulfonamidi
25,000
50,000
75,000
100,000
125,000
repA
tem-186
tet59
catII
DHFR
sul1
APH(6)-Id
APH(3")-Ib
catII
APH(3")-Ib
tolA
maz toxin/antitoxin
ccd toxin/antitoxin
umuC/D polymerase
psiA/B
hig toxin/antitoxin
IncFII typical conjugative type IV secretion
β-laktami
aminoglikozidi
kloramfenikol


DW0_K1
IncP-1ε inkompatibilna grupa
71 660 bp
tetC
(tetraciklini)
sul1
(sulfonamidi)
bla OXA-2
(β-laktami)
kleF
kleB
ssb
70,000
10,000
60,000
50,000
40,000
30,000
20,000
trfA
trbA
trbB
trbC
trbD
trbE
trbF
trbG
trbH
trbI
trbJ
trbK
trbL
trbM
trbN
trbO
trbP
Upf30.5
tRNA_anti-like
Resolvase
Transposase?
Int11 integrase
MazF toxin
MazE toxin
OXA-2
sul1
tniB (fragment)
tniA (fragment)
tniB
tniA
tniQ
Tn21 resolvase (fragment)
ABC transporter ATP-binding protein
ABC transporter permease
ABC transporter permease
Resolvase
Resolvase
Hlyd efflux transporter
tetC
tetR
EamA family transporter
Transposase
hydrolase
Tn3-like element TnAs1 family transposase
tniA
traC
traD
traE
traF
traG
traI
traJ
traL
traK
traM
upf54.8 (traO?)
krfA
krfB
korB
korA
kleE
kleA
klcB
klcA
korC
IncC


DW0_K2
IncP-1ε inkompatibilna grupa
77 135 pb
aph(6)-Id, aph(3'')-Ib
(aminoglikozidi)
sul1
(sulfonamidi)
bla OXA-2
(β-laktami)
tetC
(tetraciklini)
Tn3-like element TnAs1 family transposase
ssb
Resolvase
trfA
trbA
trbB
trbC
trbD
trbE
trbF
trbG
trbH
trbI
trbJ
trbK
trbL
trbM
trbN
trbO
trbP
Upf30.5
tRNA_anti-like
Transposase?
MazF toxin
Resolvase
MazE toxin
IntI1 integrase
OXA-2
sul1
tniB (fragment)
tniA (fragment)
tniA
Tn21 resolvase (fragment)
tniB
tniQ
Resolvase
Hlyd efflux transporter
ABC transporter ATP-binding protein
ABC transporter permease
Tn3-like element TnAs1 family transposase
ABC transporter permease
tetC
tetR
EamA family transporter
hydrolase
tniA
Transposase
Resolvase
traC
traD
traE
traF
traG
traI
traJ
traK
traL
traM
upf54.8 (traO?)
krfA
korB
korA
kleE
kleA
kleB
kleF
kleB
kleA
korC
incC
APH(6)-Id
APH(3'')-Ib
70,000
60,000
50,000
40,000
30,000
20,000
10,000


DW0_K3
IncN inkompatibilna grupa
50 954 pb
dfrA14
(trimetoprim)
aph(6)-Id, aph(3'')-Ib
(aminoglikozidi)
aph(3'')-Ib
(aminoglikozidi)
sul2
(sulfonamidi)
tetC
(tetraciklini)
blaTEM-1
(β-laktami)
Conserved
ccgAI
EcoRII
DNA cytosine methyltransferase
Conserved
Conserved
50,000
10,000
20,000
30,000
40,000
umuC
umuD
mpr
ardK
repA
Open in all six frames!
Resolvase
mrr restriction endonuclease
ardB
ardR
CcgEIII
CcgD
CcgC
ardA
Conserved
ccgAII
orfD
stbB
stbC
stbA
stbB (frameshift?)
traK
traJ
traI
fipA_pifC
APH(3'')-Ib (partial)
dfrA14
APH(6)-Id
APH(3'')-Ib
sul2
Tn3 transposase
hydrolase
GNAT N-acetyltransferase
tetR
tetC
TEM-1
Resolvase
Tn3 transposase
nuc
traG
traF
traE
traO
traN
traD
eex
traC
traB
traA
traM
korA
traL
korB
kikA

POPIS KORIŠTENIH KRATICA

AMP	ampicilin
AZI	azitromicin
BPK_5	biokemijska potrošnja kisika
CEFO	cefotaksim
DHFR	dihidrofolat reduktaza
DHPS	dihidropteroat sintaza
DNA	deoksiribonukleinska kiselina
ENR	enrofloksacin
ERY-H_2O	dehidrirani eritromicin
ESBL	β-laktamaza proširenog spektra
HGT	horizontalni prijenos gena
ICP-MS	masena spektrometrija s induktivno spregnutom plazmom
kb	kilobaza
KPK	kemijska potrošnja kisika
LC-MS/MS	vezani sustav tekućinska kromatografija - tandemna spektrometrija masa
MIK	minimalna inhibitorna koncentracija
NADPH	nikotinamid adenin dinukleotid fosfat
N-DMA	n-desmetil azitromicin
NMDS	nemetrijsko višedimenzijsko grupiranje
NGS	metode sekvenciranja sljedeće generacije
OTC	oksitetraciklin
PABA	*p*-aminobenzojeva kiselina
pb	parova baza
PBPs	penicilin-vezujući proteini
PBRT	tipizacija replikona lančanom reakcijom polimerazom
PCR	lančana reakcija polimerazom
PNEC	granična koncentracija iznad koje se može očekivati selekcija antibiotičke otpornosti
RNA	ribonukleinska kiselina
rrn	16S rRNA gen
r-RNA	ribosomska RNA
t-RNA	transportna RNA

SDZ	sulfadiazin
SMZ	sulfametazin
SZO	Svjetska zdravstvena organizacija
TET	tetraciklin
TMP	trimetoprim
TOC	ukupni organski ugljik
TSS	ukupne suspendirane tvari
TYMS	timidilat sintaza
qPCR	kvantitativna lančana reakcija polimerazom

ŽIVOTOPIS

8. ŽIVOTOPIS

Milena Milaković rođena je 27. listopada 1991. godine u Bjelovaru, gdje je završila osnovnu i srednju školu. Integrirani preddiplomski i diplomski studij Biologije i Kemije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu završila je 2016. godine čime je stekla akademski naziv magistre edukacije biologije i kemije (*mag. educ. biol. et chem.*). Od 2016. godine do danas je zaposlena na mjestu doktorandice u Laboratoriju za okolišnu mikrobiologiju i biotehnologiju Instituta Ruđer Bošković u Zagrebu. Njezin znanstveni rad obuhvaća istraživanje razvoja i širenja otpornosti bakterija na antibiotike kao i sastava i strukture bakterijskih zajednica u vodenom okolišu izloženom ispustu otpadnih voda iz farmaceutskih industrija. U akademskoj godini 2016./2017. upisala je Poslijediplomski sveučilišni doktorski studij biologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu. Dobitnica je stipendije COST akcije NEREUS (ES1403) za dvomjesečno znanstveno usavršavanje u Njemačkoj na institutu HelmholtzZentrum Munchen u grupi prof. dr. Michaela Schlotera. Dobitnica je i nekoliko stipendija Federacije europskih mikrobioloških društava (FEMS) za sudjelovanje na znanstvenim skupovima u inozemstvu i u državi. Pored toga, dobitnica je i Godišnje nagrade mladim znanstvenicima „Jasenka Pigac“ Hrvatskog mikrobiološkog društva 2020. godine kao i nagrade za drugi najbolji poster predstavljen na kongresu XENOWAC II održanom u Limassolu, Cipar 2018. godine.

Do sada je autorica/suautorica 6 znanstvenih radova, od čega je prva autorica na dva znanstvena rada, u jednima od najprestižnijih časopisa u području okolišnih znanosti citiranih bazom Web of Science (WoS), te 12 sažetaka u zbornicima znanstvenih skupova.

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