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Eliminacja i detoksykacja wybranych związków fenolowych oraz jonów metali ciężkich z wykorzystaniem grzyba strzępkowego *Umbelopsis isabellina*

Elimination and detoxification of selected phenolic compounds and heavy metal ions by the filamentous fungus *Umbelopsis isabellina*

Praca doktorska

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Lista używanych skrótów

4-*t*-OP	–	4-*tert*-oktylofenol (*ang.* 4-*tert*-octylphenol)
CAS	–	oznaczenie numeryczne przypisane substancji chemicznej przez amerykańską organizację Chemical Abstracts Service
ChZT	–	chemiczne zapotrzebowanie tlenu (*ang.* chemical oxygen demand, COD)
CP	–	4-kumylofenol (*ang.* 4-cumylphenol)
CWO	–	całkowity węgiel organiczny (*ang.* total organic carbon, TOC)
EDCs	–	modulatory endokrynne (*ang.* endocrine disrupting compounds)
GC-MS	–	chromatograf gazowy sprzężony z detektorem masowym (*ang.* gas chromatography-mass spectrometry)
NP	–	4-nonylofenol (*ang.* 4-nonylphenol)
VPs	–	fenole lotne (*ang.* volatile phenols)
WWA	–	wielopierścieniowe węglowodory aromatyczne (*ang*. polycyclic aromatic hydrocarbons, PAH)
YAS	–	drożdżowy test androgenowy (*ang.* yeast androgen screen)
YES	–	drożdżowy test estrogenowy (*ang.* yeast estrogen screen)

Spis treści

Źródła finansowania badań prowadzonych w ramach pracy doktorskiej

1. Grant NCN (Opus 1) UMO-2011/01/B/NZ9/02898: „Mikrobiologiczna degradacja ksenoestrogenów i estrogenów w obecności metali ciężkich oraz NaCl" (2011 – 2014)
   Kierownik projektu: prof. dr hab. Jerzy Długoński

2. Dotacja celowa na działalność związaną z prowadzeniem badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich w latach 2014, 2015 i 2016 (kody projektów: B1411000000743.02; B1511000001018.02; B1611000001199.02)

3. Grant NCN (Sonata) UMO-2014/13/D/NZ9/04743: ,,Mikrobiologiczna degradacja wybranych ksenobiotyków o właściwościach endokrynnych obecnych w produktach codziennego użytku i chemii gospodarczej'' (2015-2021)
   Kierownik projektu: dr Mariusz Krupiński

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Prace opublikowane:

P1 - **Janicki T**., Krupiński M., Długoński J. 2016. Degradation and toxicity reduction of the endocrine disruptors nonylphenol, 4-*tert*-octylphenol and 4-cumylphenol by the non-ligninolytic fungus *Umbelopsis isabellina.* Bioresource Technology, 200, 223-229; doi: 10.1016/j.biortech.2015.10.034

IF = 9,642; 33 cytowania; 140 punktów MEiN

P2 - **Janicki T.**, Długoński J., Krupiński M. 2018. Detoxification and simultaneous removal of phenolic xenobiotics and heavy metals with endocrine-disrupting activity by the non-ligninolytic fungus *Umbelopsis isabellina.* Journal of Hazardous Materials, 360, 661-669; doi: 10.1016/j.jhazmat.2018.08.047

IF = 10,588; 13 cytowań; 200 punktów MEiN

Manuskrypt pracy wysłany do czasopisma:

P3 – **Janicki T**., Długoński A., Felczak A., Długoński J., Mariusz Krupiński. 2021. Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol and volatile leachate phenol degradation by the microscopic fungus *Umbelopsis isabellina* using a battery of biotests. Manuskrypt przyjęty do recenzji w czasopiśmie Chemosphere, 20 grudnia 2021 r.

Na rozprawę doktorską składają się:

- 2 opublikowane prace doświadczalne o łącznym IF = 20,23 oraz sumą punktów MEiN 340, cytowane łącznie 46 razy;
- 1 manuskrypt przyjęty do recenzji w czasopiśmie Chemosphere o wartości IF = 7,086 i 140 pkt MEiN.

Wartości IF oraz punktacja MEiN podana na rok 2021

I. Wprowadzenie

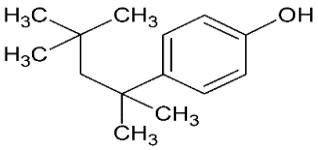
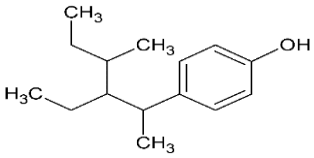
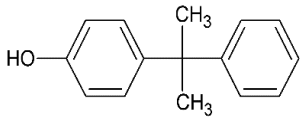
Uwalnianie do otoczenia ksenobiotyków ze źródeł antropogenicznych generuje wiele niepożądanych efektów środowiskowych. Związki te charakteryzują się przeważnie wysoką opornością na degradację, tendencją do bioakumulacji oraz znaczną szkodliwością względem różnych gatunków organizmów, przez co mogą powodować negatywne zmiany zarówno na poziomie osobniczym, jak i wśród całych ekosystemów. Wiele ksenobiotyków wykazuje działanie kancerogenne, mutagenne, teratogenne, stanowi przyczynę uogólnionej reakcji alergicznej organizmu jak również zaburza prawidłowe funkcjonowanie narządów.

Szczególną grupę zanieczyszczeń stanowią związki zaliczane do zewnątrz środowiskowych modulatorów hormonalnych - EDCs (*ang.* endocrine disrupting compounds) wywołujące negatywne zmiany w funkcjonowaniu układu endokrynnego poprzez zaburzanie transportu, syntezy lub bezpośrednie oddziaływanie z hormonami lub ich komórkowymi receptorami. W wyniku zakłócenia homeostazy układu wewnątrzwydzielniczego EDCs wywołują liczne zaburzenia endokrynologiczne w tym dysfunkcje reprodukcyjne, rozwojowe i behawioralne (Długoński, 2016, 2021). Właściwości destabilizowania układu hormonalnego wykazują m.in. niektóre związki fenolowe (w tym alkilofenole jak: 4-nonylofenol, 4-*tert*-oktylofenol) (Tabela 1) oraz jony metali ciężkich (np. Zn, Mn, Ni, Cd, Pb) szeroko wykorzystywane na skalę przemysłową w wielu gałęziach gospodarki (Georgescu i in., 2011; Iavicoli i in., 2009; Rana, 2014; Soares i in., 2008). Coraz wyższy stopień zanieczyszczenia tymi ksenobiotykami środowiska naturalnego wynika z ich ciągłego uwalniania przede wszystkim wraz ze ściekami komunalnymi i przemysłowymi. Obecność tych związków wykazano w wielu elementach biosfery m.in. wodach gruntowych, osadach dennych, glebie, rzekach i jeziorach. Ksenobiotyki te zostały również wykryte w ściekach uznanych za oczyszczone, wskazując tym samym na niepełny ich rozkład w trakcie zastosowanych procesów technologicznych (Lu i Gan, 2014a; Soares i in., 2008; Zuo i Zhu, 2014). Obszary środowiska, w których zidentyfikowano modulatory hormonalne, będące niejednokrotnie w znacznych odległościach od potencjalnych źródeł ich emisji, pokazują, iż mają one zdolność do transgranicznej migracji, zwiększając tym samym terytorium bezpośredniego wpływu na organizmy. Narażenie na kontakt z EDCs jest zatem zjawiskiem ciągłym i dotyczy różnych gatunków bez względu na środowisko ich bytowania.

W efekcie wciąż rosnącej skali produkcji substancji wykazujących właściwości endokrynne, powiększającym się stopniem kontaminacji środowiska tymi związkami oraz ich negatywnym oddziaływaniem na organizmy, obserwuje się intensywny wzrost zainteresowania poszukiwaniem wydajnych sposobów eliminacji tych zanieczyszczeń w ekologicznie rozsądny i ekonomicznie korzystny sposób. Fizykochemiczne metody usuwania i rozkładu ksenobiotyków charakteryzują się przeważnie wysokimi kosztami, generują toksyczne produkty uboczne oraz często prowadzą do powstawania intermediatów charakteryzujących się wyższą toksycznością od związków macierzystych (Oluwasanu, 2018). Dlatego też, w poszukiwaniu efektywnych sposobów ich rozkładu szczególną uwagę zwraca się na metody biologiczne wykorzystujące drobnoustroje (Krupiński, 2021). Obszary intensywnych badań i analiz naukowych nad modulatorami hormonalnymi dotyczą przede wszystkim ich oddziaływania na organizmy oraz skali występowania i zanieczyszczenia środowiska tymi ksenobiotykami. Procesy ich eliminacji i degradacji, zwłaszcza na drodze biologicznej, zostały dotychczas słabo poznane i opisane. Innowacje ekologiczne w ochronie środowiska zmierzają zatem do opracowania bezpiecznych dla środowiska technik mikrobiologicznych, które zmniejszają i eliminują zagrożenie związane z emisją tych zanieczyszczeń. W świetle aktualnego stanu wiedzy, uzasadnione wydaje się zatem podjęcie badań, których istotą jest poszukiwanie skutecznych i wydajnych metod mikrobiologicznej eliminacji opisywanych ksenobiotyków, poznanie mechanizmów regulujących przebieg ich rozkładu i oszacowanie toksycznych właściwości produktów pośrednich i końcowych zachodzących przemian.

Głównym obszarem badawczym niniejszej rozprawy była mikrobiologiczna eliminacja degradacja wybranych związków fenolowych (4-nonylofenolu, 4-kumylofenolu i 4-*tert*-oktylofenolu) oraz jonów metali ciężkich (Zn, Mn, Ni, Cd, Pb) należących do grupy EDCs (Zhao i in., 2021), występujących powszechnie w środkach codziennego użytku, kosmetykach oraz chemii gospodarczej, jak również szeroko wykorzystywanych jako surowce do syntezy innych substancji chemicznych o cennych właściwościach technologicznych i przemysłowych, które używane są m. in. w wyrobach plastikowych, materiałach kompozytowych, lakierach, smarach oraz przy produkcji sprzętu elektronicznego, tekstyliów i wyrobów papierniczych. Przeprowadzone prace skupiały się także nad oceną zdolności użytego w pracy grzyba strzępkowego do detoksykacji środowiska w trakcie procesów eliminacji testowanych toksykantów.

Tabela 1. Podstawowe właściwości fizykochemiczne badanych związków chemicznych.

Nazwa	Wzór chemiczny		Masa cząsteczkowa [g/mol]	Temperatura topnienia/wrzenia [°C]	Gęstość [g/cm^3]
	strukturalny	sumaryczny			
4-*tert*-oktylofenol CAS: 140-66-9		$C_{14}H_{22}O$	206,32	85/279	0,89 w 90 °C
4-nonylofenol CAS: 84852-15-3		$C_{15}H_{24}O$	220,35	-8/290-300	0,95 w 20 °C
4-kumylofenol CAS: 599-64-4		$C_{15}H_{16}O$	212,29	74,5/335	1,12 w 25 °C

Wartości podane za PubChem (data dostępu: 20.12.2021)

II. Cele pracy

Prace badawcze realizowane w ramach prezentowanej rozprawy wpisują się w nurt badań obejmujący identyfikację i charakterystykę układów biologicznych pozwalających na zmniejszenie toksycznego oddziaływania ksenobiotyków poprzez ich eliminację i biodegradację.

Głównymi celami badań wykonanych podczas realizacji rozprawy doktorskiej były:

1. Określenie zdolności grzyba strzępkowego *U. isabellina* do degradacji ksenobiotyków fenolowych o właściwościach endokrynnych: 4-nonylofenolu, 4-*tert*-oktylofenolu oraz 4-kumylofenolu.
2. Analiza potencjału *U. isabellina* do jednoczesnej eliminacji 4-nonylofenolu, 4-*t*-oktylofenolu, 4-kumylofenolu oraz wybranych jonów metali ciężkich destabilizujących funkcjonowanie układu endokrynnego.
3. Ekotoksykologiczna ocena procesów eliminacji 4-nonylofenolu, 4-*tert*-oktylofenolu, 4-kumylofenolu oraz jonów metali ciężkich przez szczep *U. isabellina* z wykorzystaniem baterii biotestów toksyczności.
4. Określenie efektywności *U. isabellina* do usuwania i detoksykacji lotnych fenoli zawartych w odciekach ze składowiska odpadów niebezpiecznych.

III. Techniki wykorzystane w czasie realizacji pracy doktorskiej

- Mikroskopia – określenie wpływu 4-nonylofernolu, 4-kumylofenolu oraz 4-*tert*-oktylofenolu na wzrost i morfologię *Umbelopsis isabellina.*
- Chromatografia gazowa sprzężona ze spektrometrią mas – wykorzystana w celu scharakteryzowania grup izomerów w NP oraz oznaczenia ilościowego oraz jakościowego (poszukiwanie związków pochodnych badanych ksenobiotyków).
- Spektrofluorymetria – ocena toksykologiczna badanych filtratów pohodowlanych.
- Absorpcyjna spektrometria atomowa – ocena zdolności *U. isabellina* do biosorpcji i bioakumulacji metali ciężkich.

Wszystkie wyniki zamieszczone w publikowanych pracach zostały podane jako wartości uśrednione o odchylenie standardowe. Analizy statystyczne wykonano za pomocą pakietu statystycznego SPSS (Windows ver. 17), przeprowadzono analizę wariacji ANOVA. Testy wykonano przy poziomie istotności statystycznej wynoszącym $P \leq 0,05$.

IV. Omówienie wyników przedstawionych w cyklu publikacji wchodzących w skład rozprawy doktorskiej

Poszukiwanie nowych metod efektywnego usuwania i rozkładu toksycznych zanieczyszczeń z użyciem mikroorganizmów, których aktywność degradacyjna jest zazwyczaj specyficzna w stosunku do określonej grupy związków, stanowi jeden z istotnych elementów strategii zmierzających do remediacji środowisk skażonych ksenobiotykami. W celu pozyskania drobnoustrojów cechujących się zdolnościami rozkładu badanych w pracy związków fenolowych o właściwościach endokrynnych, w początkowym etapie analiz oszacowano potencjał pięćdziesięciu szczepów grzybów mikroskopowych do eliminacji NP, 4-CP oraz 4-*t*-OP z grzybowego podłoża wzrostowego. Testowane mikroorganizmy pochodziły z kolekcji drobnoustrojów Katedry Mikrobiologii Przemysłowej i Biotechnologii UŁ i były pierwotnie wyizolowane z matryc środowiskowych (gleba, ścieki, hałdy kopalniane) zanieczyszczonych wieloma różnymi ksenobiotykami organicznymi i metalami ciężkimi. Na podstawie otrzymanych wyników wykazano, że szczep *Umbelopsis isabellina* IM 833 charakteryzował się największą opornością na toksyczne oddziaływanie badanych związków fenolowych (najniższe zahamowanie wzrostu w obecności ksenobiotyków) jak również najwyższą efektywnością w eliminacji NP, 4-CP oraz 4-*t*-OP z podłoża hodowlanego po 72 godzinach inkubacji. Z tego względu, ww. szczep grzybowy został wybrany jako model badawczy w pracach eksperymentalnych realizowanych w ramach prezentowanej rozprawy.

IV.1 Publikacja P1

Rezultaty doświadczeń będące wynikiem realizacji pierwszego celu badawczego prezentowanej dysertacji zostały opisane w pracy eksperymentalnej **(P1)** – Janicki T., Krupiński M., Długoński J. 2016. Degradation and toxicity reduction of the endocrine disruptors nonylphenol, 4-*tert*-octylphenol and 4-cumylphenol by the non-ligninolytic fungus *Umbelopsis isabellina*. Bioresource Technology; 200, 223-229, tworzącej cykl publikacji wchodzących w skład rozprawy doktorskiej. Analiza danych dynamiki eliminacji NP, 4-*t*-OP oraz CP z podłoża wzrostowego przez szczep *U. isabellina* uwidoczniła całkowity ubytek testowanych ksenobiotyków, w stężeniu wyjściowym 25 mg/l, po 24 godzinach prowadzenia hodowli. W porównaniu do zawartości toksykantów w układach kontrolnych, zaobserwowano ponad 90 procentową redukcję NP oraz 4-*t*-OP w hodowlach grzybowych po 12 godzinach inkubacji. Tę samą wartość eliminacji wykazano po 6 godzinach hodowli *U. isabellina* z dodatkiem CP. Okresy półtrwania testowanych związków w fazie analizowanej mikrobiologicznej eliminacji wynosiły około 4 godziny dla NP i 4-*t*-OP oraz 3 godziny dla CP. Większość danych literaturowych dotyczących mikrobiologicznej degradacji NP, 4-*t*-OP oraz CP odnosi się do procesów ich biotransformacji przez bakterie lub konsorcja różnych gatunków drobnoustrojów (Gabriel i in., 2008; Lu i Gan, 2014b; Toyama i in., 2011). Ilość badań opisujących eliminację tych ksenobiotyków przez grzyby, zwłaszcza nieligninolityczne, jest ograniczona. W świetle dostępnych danych literaturowych, porównanie dynamiki mikrobiologicznej eliminacji NP, 4-*t*-OP oraz CP wskazuje, że *U. isabellina* charakteryzuje się kilkukrotnie wyższą efektywnością w usuwaniu tych zanieczyszczeń ze środowiska wzrostu niż inne scharakteryzowane grzyby mikroskopowe (*Trametes versicolor*, *Bjerkandera* sp. BOL13) (Girlanda i in., 2009; Soares i in., 2005).

Nonylofenol wykorzystywany w skali przemysłowej głównie jako półprodukt do syntezy związków o bardziej pożądanych właściwościach technologicznych, występuje w postaci mieszaniny kilkunastu izomerów różniących się stopniem rozgałęzienia łańcucha alifatycznego. W kolejnym etapie badań postanowiono określić czy struktura podstawnika alifatycznego warunkuje stopień eliminacji poszczególnych izomerów przez badany drobnoustrój. Na podstawie analizy rozkładu masy jonów fragmentacyjnych izomerów NP otrzymanych metodą chromatografii gazowej sprzężonej ze spektrometrią mas (GC-MS),

w zależności od ich budowy względem podstawników w pozycji α- oraz β-węgla łańcucha alifatycznego, zostały podzielone na pięć grup. Otrzymane wyniki wykazały brak istotnej korelacji między szybkością usuwania izomerów ksenobiotyku a stopniem rozgałęzienia podstawnika alkilowego w ich strukturze. W oparciu o posiadaną wiedzę, uzyskane dane są pierwszymi opisującymi szczep grzyba mikroskopowego zdolnego do całkowitej eliminacji nonylofenolu wskazującymi jednocześnie, iż szybkość usuwania różnych grup izomerów związku przez ten drobnoustrój nie zależy od wzorca substytucji α- oraz β-węgla łańcucha alifatycznego. Analiza chromatograficzna (GC-MS) ekstraktów z hodowli *U. isabellina* traktowanych NP, 4-*t*-OP lub CP pozwoliła ponadto zidentyfikować kilka pośrednich produktów degradacji testowanych ksenobiotyków. Na ich podstawie sugeruje się, że rozkład NP i 4-*t*-OP przez badany szczep grzybowy inicjowany jest przez hydroksylację końcowego węgla w ugrupowaniu alkilowym, po której następuje utlenienie grupy hydroksylowej powstałego kwasu karboksylowego i stopniowe skracanie łańcucha alifatycznego poprzez kolejne utlenianie subterminalnych lub terminalnych atomów węgla. Mechanizm degradacji CP opiera się prawdopodobnie na hydroksylacji atomu C grupy metylowej (C-8 lub C-9) a następnie utlenieniu odpowiedniej grupy karboksylowej. Dalsza transformacja przebiega poprzez kilka przegrupowań w obrębie struktury cząsteczki prowadząc do rozszczepienia wiązania C-C między ugrupowaniami aromatycznymi. Ze względu na podobieństwo strukturalne między kumylofenolem a cząsteczką bisfenolu – ksenoestrogenu, którego mikrobiologiczna degradacja została udokumentowana w wielu pacach naukowych, sugeruje się, że procesy biotransformacji tych związków mogą zachodzić w podobnych szlakach metabolicznych (Zhang i in., 2013).

Podczas mikrobiologicznych przemian ksenobiotyków może dochodzić do wytworzenia intermediatów charakteryzujących się bardziej szkodliwym wpływem na organizmy od substratów macierzystych. Z tego względu, kolejny etap badań realizowanych w ramach prezentowanej pracy, obejmował ocenę zmiany toksyczności hodowli *U. isabellina* w trakcie procesów rozkładu NP, 4-*t*-OP i CP. Ekotoksyczność filtratów pohodowlanych analizowano z zastosowaniem biotestów wykorzystujących w roli organizmów wskaźnikowych dwa gatunki bezkręgowców: słonowodne skorupiaki *Artemia franciscana* oraz słodkowodne rozwielitki *Daphnia magna*, reprezentujące różne ekosystemy. Wyniki badań toksykologicznych wykazały, iż w przebiegu procesów degradacji wszystkich testowanych zanieczyszczeń

dochodziło do powstawania mniej szkodliwych pochodnych. Zmniejszenie toksyczności sześcio- i siedmiokrotne dla hodowli z CP, około siedmio- i ośmiokrotne dla kultur grzybowych traktowanych NP oraz cztero- i sześciokrotne dla przesączy otrzymanych z hodowli z dodatkiem 4-*t*-OP, po 24 godzinach inkubacji, zaobserwowano odpowiednio w badaniach z użyciem *A. franciscana* i *D. magna*. Odnotowane w testach toksykologicznych procesy detoksykacji ksenobiotyków, zostały dodatkowo potwierdzone poprzez analizy mikroskopowe. Obecność NP, 4-*t*-OP i CP w środowisku wzrostu *U. isabellina* generowała zmiany morfologiczne w strzępkach grzybni takie jak: wypukłości na powierzchni strzępek oraz pojawianie się wewnątrzkomórkowych pęcherzyków. Po sześciu godzinach inkubacji, we wszystkich analizowanych układach hodowlanych z ksenobiotykami, zauważono zanik wspomnianych wyżej zaburzeń morfologicznych. Zależność między szybkością eliminacji testowanych zanieczyszczeń przez szczep grzybowy a obserwowanymi w czasie zmianami w strukturze jego strzępek wskazuje, że procesy biodegradacji NP, 4-*t*-OP i CP prowadziły do zmniejszenia toksyczności podłoża wzrostowego.

IV.2 Publikacja P2

W kolejnych doświadczeniach realizowanych w ramach wyznaczonych celów badawczych prezentowanej rozprawy, analizowano potencjał szczepu *U. isabellina* do jednoczesnej eliminacji z podłoża hodowlanego: NP, 4-*t*-OP i CP oraz wybranych jonów metali ciężkich wykazujących właściwości endokrynne. Wyniki przeprowadzonych ksperymentów zostały przedstawione w publikacji **(P2)**: Janicki T., Długoński J., Krupiński M. 2018. Detoxification and simultaneous removal of phenolic xenobiotics and heavy metals with endocrine-disrupting activity by the non-ligninolytic fungus *Umbelopsis isabellina.* Journal of Hazardous Materials; 360, 661-669. Ekspozycja na metale ciężkie, wśród których Mn, Zn, Ni, Pb i Cd uznawane są jako jedne z najbardziej toksycznych metali występujących w zanieczyszczonych środowiskach. wywołuje szereg niekorzystnych skutków dla organizmów. W wielu badaniach wykazano, że narażenie na wysokie poziomy tych metali może powodować zaburzenia układu hormonalnego, w szczególności poprzez modulowanie aktywności estrogenowej hormonów endogennych (Georgescu i in., 2011; Iavicoli i in., 2009; Rana, 2014).

W pierwszym etapie badań oszacowano toksyczny wpływ Zn (II), Mn (II), Ni (II), Cd (II), oraz Pb (II) na badany szczep *U. isabellina* w oparciu o analizę zahamowania wzrostu drobnoustroju w hodowlach suplementowanych wspomnianymi metalami ciężkimi. Na podstawie wyznaczonych krzywych zależności „dawka – odpowiedź" pomiędzy wielkością wytworzonej biomasy grzyba a zawartością poszczególnych jonów metali w podłożu wzrostowym zdefiniowano stężenia testowanych inhibitorów ograniczające w 50 % wzrost szczepu (IC_{50}), które posłużyły do oceny stopnia toksyczności badanych metali manifestującego się według schematu Cd > Pb = Ni > Mn = Zn. Przeprowadzone badania wykazały, że *U. isabellina* charakteryzował się wysoką tolerancją na obecność jonów Pb, Ni, Mn i Zn w środowisku wzrostu. Dodatkowo, w świetle danych naukowych, analizowany drobnoustrój wykazywał silniejsze reakcje fizjologiczne oraz mechanizmy obronne warunkujące jego oporność na Pb i Ni w porównaniu do wielu szczepów grzybowych opisywanych w literaturze jako wysoce tolerancyjne na stres wywołany przez te metale ciężkie. Ze względu na wysoką toksyczność Cd na przyrost biomasy *U. isabellina* (zahamowanie wzrostu o 87 % przy zawartości Cd w pożywce 2,5 mM) pominięto dalsze prace eksperymentalne z wykorzystaniem tego metalu.

W strategii tolerowania stresu środowiskowego wywołanego toksycznym działaniem metali ciężkich, grzyby mikroskopowe rozwinęły wiele mechanizmów oporności z czego do najważniejszych z nich należą: biosorpcja - niezależne od metabolizmu wiązanie z powierzchnią komórek i bioakumulacja – aktywny proces transportu metalu do wnętrza komórki (Deng i in., 2011; Vargas-Garcia i in., 2012). W dalszych etapach badań postanowiono zatem ocenić zdolność *U. isabellina* do eliminacji Pb, Ni, Mn i Zn z podłoża hodowlanego analizując wspomniane procesy pobierania jonów metali ze środowiska. Wyniki przeprowadzonych badań ukazują, że we wszystkich testowanych układach badawczych dominującym mechanizmem usuwania metalu była sorpcja do powierzchni komórek grzyba. Jednym z głównych składników ściany komórkowej grzybów są lipidy zawierające określone grupy funkcyjne, które bezpośrednio uczestniczą w wiązaniu metali. Wiele publikacji wskazuje, że *U. isabellina* należy do tzw. „grzybów oleistych", które pod wpływem stresu mogą wytwarzać duże ilości lipidów (Hussain i in., 2014; Ruan i in. 2015). Obserwacje te sugerują, że oddziaływania fizykochemiczne między metalami i ligandami, takimi jak grupy estrowe i karboksylowe obecne na powierzchni komórki, mogą być zaangażowane w mechanizmy biosorpcji badanych metali przez *U. isabellina*. W procesach pobierania testowanych metali z pożywki wzrostowej przez drobnoustrój, najwyższą wydajność eliminacji otrzymano dla hodowli suplementowanych jonami Pb wynoszącą 74,3 mg metalu na gram suchej masy, z czego około 91 % (67,8 mg) było chelatowanych na powierzchni komórki. Efektywność wychwytywania jonów Zn, Mn i Ni przez grzybnię *U. isabellina* wynosiła odpowiednio 23,4; 7,4 i 6,8 mg metalu na gram suchej masy a około 96 % tych ilości ulegała wiązaniu do struktur zewnątrzkomórkowych. Otrzymane wyniki wskazują na możliwości zastosowania *U. isabellina* jako efektywnego biosorbentu do eliminacji Pb, Zn, Mn i Ni z zanieczyszczonych matryc środowiskowych.

Badania nad oceną potencjału *U. isabellina* do usuwania metali ciężkich ze środowiska, zostały następnie poszerzone o analizy procesów biosorpcji i bioakumulacji testowanych metali z układów hodowlanych traktowanych równolegle NP, 4-*t*-OP lub CP. Obecność dodatkowego zanieczyszczenia w pożywce skutkowała zróżnicowanym oddziaływaniem na stopień wychwytywania metali przez drobnoustrój zależnie od użytego ksenobiotyku. W hodowlach suplementowanych NP lub 4-*t*-OP zaobserwowano hamujący wpływ alkilofenoli na zdolność *U. isabellina* do usuwania wszystkich testowanych metali z podłoża wzrostowego w porównaniu do układów kontrolnych. Do wskazywanych procesów mogły przyczynić się

zmiany w składzie i właściwościach fizykochemicznych ściany komórkowej szczepu będące efektem działania zastosowanych ksenobiotyków. W przeciwieństwie do hodowli suplementowanych NP lub 4-*t*-OP, ekspozycja *U. isabellina* na CP nie generowała istotnych zmian w aktywności tego szczepu do biosorpcji i bioakumulacji Zn, Mn i Ni w odniesieniu do układów nie zawierających ksenobiotyków w pożywce wzrostowej. Zaobserwowano natomiast wyraźny wzrost poziomu eliminacji Pb w hodowlach grzybowych traktowanych CP o około 25 % w stosunku do układu kontrolnego. Poprawę zdolności sorpcyjnych *U. isabellina* w odniesieniu do jonów Pb można tłumaczyć wpływem związku na procesy metaboliczne determinujące budowę i funkcjonowanie ściany komórkowej grzyba. Jeden z tych mechanizmów mógł sprzyjać zwiększeniu ilości anionowych grup funkcyjnych osadzonych na powierzchni komórki, które uczestniczyły w wiązaniu metalu. Dodatkowo, kumylofenol mógł przyczyniać się do zaburzenia integralności ściany komórkowej prowadząc do ekspozycji ukrytych wcześniej ligandów wiążących Pb.

W układach hodowlach „metal + związek fenolowy" analizowano ponadto stopień usuwania zanieczyszczenia organicznego przez szczep *U. isabellina* ze środowiska wzrostu. Jednoczesne wprowadzenie do pożywki Pb, Ni, Mn lub Zn oraz jednego z testowanych substratów fenolowych pociągało za sobą ograniczenie szybkości eliminacji NP, 4-*t*-OP oraz CP w większości testowanych wariantach badawczych w porównaniu do hodowli bez dodatku jonów metalu. W stosunku do poziomów wydajności eliminacji substratów organicznych odnotowanych w hodowlach bez obecności metalu, najwyższe zahamowanie aktywności biodegradacyjnej *U. isabellina* zaobserwowano dla NP wynoszące od 62 do 23 % odpowiednio dla układów z Pb i Zn w 24 godzinie inkubacji. Obserwowany spadek efektywności drobnoustroju w biotransformacji NP, 4-*t*-OP i CP mógł wynikać z niekorzystnego wpływu metali ciężkich na wiele procesów metabolicznych zaangażowanych w transport i degradację tych zanieczyszczeń takich jak: zahamowanie aktywności kluczowych enzymów katalizujących reakcje rozkładu związków czy ograniczenie syntezy ATP (Chen i in., 2011; Liu i in., 2017). Pomimo ograniczenia szybkości eliminacji większości testowanych związków fenolowych przez *U. isabellina* w odpowiedzi na stres spowodowany ekspozycją na metale ciężkie, wyniki badań wykazały także przyspieszenie niektórych procesów degradacyjnych jak np. rozkładu CP oraz 4-*t*-OP w obecności Mn, czy biotransformacji 4-*t*-OP w hodowlach z Zn. Sugeruje się, iż procesy te mogą być wynikiem zwiększenia adsorpcji substratów organicznych do powierzchniowych struktur komórkowych. Niektóre metale ciężkie mogą wykazywać silniejsze

przyciąganie elektrostatyczne do powierzchni grzybni niż związki fenolowe, czyniąc zewnętrzną przestrzeń komórki mniej hydrofilową, a tym samym promując adsorpcję związków hydrofobowych, takich jak 4-*t*-OP czy CP (Liu i in., 2017). Dodatkowo, intensyfikacji degradacji związków organicznych można również przypisać tworzenie się kompleksów enzym-metal-ksenobiotyk fenolowy, w którym metale działają jako kofaktory regulując w ten sposób funkcję enzymów biorących udział w biotransformacji zanieczyszczeń organicznych.

Ze względu na powszechne współwystępowanie w skażonych ekosystemach wodnych ksenobiotyków fenolowych, takich jak NP, 4-*t*-OP i CP oraz metali ciężkich o właściwościach endokrynnych, w kolejnym etapie badań oszacowano zdolność *U. isabellina* do usuwania wyżej wymienionych toksykantów z hodowli zawierających wszystkie testowane w pracy zanieczyszczenia organiczne oraz jony metali. Wyniki badań ukazały, że równoczesna suplementacja pożywki ksenobiotykami fenolowymi oraz Pb, Ni, Mn i Zn hamowała zarówno wydajność wychwytywania przez drobnoustrój jonów metali jak i efektywność degradacyjną NP, 4-*t*-OP i CP w porównaniu z poziomem ich eliminacji w mieszaninach dwuskładnikowych jak i hodowlach traktowanych osobno poszczególnymi zanieczyszczeniami. Wartości pobierania przez grzybnię metali ciężkich oscylowały od 62,01 do 3,43 mg na gram suchej masy, odpowiednio dla jonów Pb i Ni, natomiast szybkość rozkładu testowanych związków fenolowych zmniejszyła się od 77 do 33 % po 24 godzinach inkubacji odpowiednio w hodowlach z NP i 4-*t*-OP w odniesieniu do kultur traktowanych jednym ksenobiotykiem. Chociaż współwystępowanie metali i ksenobiotyków organicznych w pożywce hodowlanej negatywnie wpłynęło na skuteczność ich eliminacji przez *U. isabellina*, szczep nadal efektywnie usuwał zanieczyszczenia ze środowiska wzrostu, co czyni go obiecującym modelem w opracowywaniu metod bioremediacji skażonych ekosystemów wodnych.

W ostatnim etapie realizacji założonych celów badawczych tej części pracy, przeprowadzono ocenę zmiany stopnia toksyczności hodowli *U. isabellina* inkubowanych w układach „metal + związek fenolowy". Do analiz ekotoksykologicznych ponownie zastosowano testy wykorzystujące w roli bioindykatorów skorupiaki *A. franciscana* oraz *D. magna*. Otrzymane wyniki jednoznacznie wykazały redukcję szkodliwego potencjału filtratów pohodowlanych po 24 godzinach inkubacji dla wszystkich badanych układów eksperymentalnych w stosunku do testowanych organizmów wskaźnikowych. Najszybszy spadek toksyczności odnotowano w trakcie pierwszych sześciu godzin prowadzenia hodowli, co może wskazywać na zachodzące wówczas intensywne procesy adsorpcji metali do struktur

powierzchniowych grzybni. Obserwowany wzrost toksyczności dla niektórych układów hodowlanych z NP i 4-*t*-OP w 12 godzinie inkubacji mógł wynikać z pojawienia się w pożywce produktów biotransformacji ksenobiotyków wykazujących silniejsze szkodliwe właściwości od substratów macierzystych (Dave i in., 2012). Podsumowując, analizy ekotoksykologiczne przedstawione w tej pracy wykazały, że jednoczesnemu usuwaniu testowanych zanieczyszczeń organicznych i nieorganicznych przez *U. isabellina* towarzyszyła detoksykacja środowiska.

IV.3 Publikacja P3 (manuskrypt pracy wysłany do recenzji)

Zakres eksperymentów wchodzących w skład trzeciej publikacji **(P3)**: Janicki T., Długoński A., Felczak A., Długoński J., Krupiński M. Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol and volatile leachate phenol degradation by the microscopic fungus Umbelopsis isabellina using a battery of biotests, (manuskrypt na etapie recenzji w czasopiśmie Chemosphere), stanowiącego spójny tematycznie zbiór publikacji, obejmował przede wszystkim poszerzenie analiz nad oceną zmiany toksyczności środowiska wzrostu szczepu *U. isabellina* w hodowlach traktowanych NP, 4-*t*-OP lub CP, stanowiących uzupełnienie badań ekotoksykologicznych podjętych we wcześniejszej pracy doświadczalnej (P1). Szeroka ocena ekotoksyczności zanieczyszczeń stanowi podstawę oceny ryzyka negatywnego oddziaływania danego czynnika na organizmy. Dlatego też, w przeprowadzonych badaniach zastosowano wielogatunkowe podejście toksykologiczne wykorzystując do analiz tzw. baterię biotestów zawierających jako bioindykatory organizmy pełniące kluczowe funkcje w ekosystemach oraz reprezentujące szeroki zakres taksonów wszystkich poziomów łańcucha troficznego (konsumenci, producenci, destruenci). Umożliwiło to kompleksową ocenę całkowitego ryzyka stwarzanego przez wszystkie związki zawarte w kulturach grzybowych inkubowanych z zanieczyszczeniami, w tym wykrywanie toksyczności wykazywanej przez intermediaty powstające podczas biodegradacji (Kar i in., 2020; Manfra i in., 2015). Analiza wielokierunkowych efektów biologicznych narażenia na substancje toksyczne (m.in. aktywność bioluminescencyjna u bakterii *Aliivibrio fischeri*, skala pobierania pokarmu przez skorupiaki *Thamnocephalus platyurus* czy stopień kiełkowania nasion i zahamowanie wzrostu korzeni u *Lepidium sativum, Sinapis alba* i *Sorghum saccharatum*) u wszystkich testowanych organizmów wskaźnikowych wykazała zmniejszenie szkodliwości filtratów otrzymanych w trakcie prowadzenia hodowli grzybowych traktowanych NP, 4-*t*-OP lub CP. Wskazuje to, iż rozkład związków fenolowych przez *U. isabellina* prowadził do powstawania metabolitów o niższej toksyczności od substratów macierzystych.

Poza standardowymi biotestami ekotoksykologicznymi, w pracy przeprowadzono także testy wykorzystujące zmodyfikowane genetycznie drożdże *Sacharomyces cerevisiae:* YES (yeast estrogen screen) oraz YAS (yeast androgen screen) umożliwiające ocenę potencjału estrogennego i androgennego zarówno analizowanych ksenobiotyków jak i hodowli

grzybowych suplementowanych tymi zanieczyszczeniami. Wyniki badań wykazały właściwości estrogenne NP i CP oraz aktywność antyandrogenną CP i 4-*t*-OP. W oparciu o otrzymane dane, w kolejnych doświadczeniach, poddano analizie na aktywność endokrynną filtraty pohodowlane *U. isabellina* z układów suplementowanych NP, CP i 4-*t*-OP. Rezultaty doświadczeń pokazały, że żaden z badanych przesączy uzyskanych w trakcie inkubacji drobnoustroju z NP lub CP nie wykazywał potencjału estrogennego W testach określających właściwości antyandrogenne, zarówno w pożywkach z dodatkiem NP jak i CP obserwowano spadek testowanej aktywności wraz z czasem prowadzenia hodowli. Wyniki testów jednoznacznie zatem wskazują, że procesy biodegradacji związków fenolowych skutkowały redukcją potencjału endokrynnego środowiska.

W obliczu narastającego zanieczyszczenia środowiska odciekami generowanymi z poprzemysłowych składowisk i zawierającymi różne toksyczne związki fenolowe, w prezentowanej pracy skoncentrowano się również na określeniu możliwości wykorzystania szczepu *U. isabellina* do usuwania i detoksykacji lotnych fenoli (VPs) z odcieków obciążonych złożonymi zanieczyszczeniami organicznymi. W tym celu jako matrycę wykorzystano odcieki ze składowiska odpadów przemysłowych dawnych Zakładów Produkcji Barwników ,,Boruta'' w Zgierzu. Substancje zaliczane do VPs to przede wszystkim hydroksylowe pochodne benzenu i innych związków aromatycznych, które mogą powstawać w procesach biotransformacji i biodegradacji zarówno związków pochodzenia naturalnego jak i ksenobiotyków np.: wielopierścieniowych węglowodorów aromatycznych (WWA) czy polifenoli (Kennes i in., 2016). Wiele lotnych fenoli charakteryzuje się wysoką toksycznością, w tym także zdolnością do zaburzania funkcjonowania układu endokrynnego (Oluwasanu, 2018). Przeprowadzone analizy fizykochemiczne badanych odcieków wykazały nie tylko znaczną zawartość VPs w testowanych matrycach, ale również wysokie wartości ChZT, CWO, przewodnictwa czy metali świadczące o istotnym obciążeniu testowanych prób środowiskowych toksycznymi substancjami organicznymi i nieorganicznymi.

Wyniki uzyskane z analizy zdolności *U. isabellina* do wzrostu w hodowlach zawierających 20 lub 40 % odcieku składowiskowego wykazały, że użyty drobnoustrój jest w stanie przystosować się do niekorzystnych warunków środowiskowych nawet w przypadku jego ekspozycji na wyższe stężenia toksykantów obecnych w testowanych matrycach. Pomimo obserwowanego zahamowania przyrostu biomasy grzyba w pożywkach suplementowanych

próbkami odcieków, wykonane badania ilościowe na zawartość w hodowlach lotnych fenoli uwidoczniły spadek ich stężenia w trakcie inkubacji analizowanych układów. Przyspieszenie procesu eliminacji VPs z podłoża wzrostowego po 24 i 48 godzinie inkubacji odpowiednio dla hodowli traktowanych 20 i 40 % odcieku, sugeruje, że lotne fenole wykorzystywane są przez *U. isabellina* zawłaszcza w fazie stacjonarnej wzrostu, kiedy wyczerpuje się pula łatwo metabolizowanych składników odżywczych pożywki. Przeprowadzone testy ekotoksykologiczne wykazały istotną korelację między redukcją toksyczności filtratów otrzymanych z hodowli *U. isabellina* w obecności odcieków a poziomem eliminacji VPs w tych układach. Wartości 3,8; 4,9 i 1,9-krotnego zmniejszenia toksyczności dla 20 % objętości odcieku w hodowlach oraz 3,7; 4,6 i 2,1-krotnego dla 40 % jego zawartości w kulturach grzybowych po 96 godzinach inkubacji, zaobserwowano w badaniach odpowiednio z użyciem *A. franciscana*, *D. magna* oraz *A. fischeri* pełniących rolę organizmów wskaźnikowych. Eliminacja zanieczyszczeń obecnych w odciekach przez szczep *U. isabellina* prowadzi zatem do redukcji toksyczności środowiska.

V. Podsumowanie oraz wnioski i stwierdzenia końcowe

W prezentowanej rozprawie doktorskiej po raz pierwszy scharakteryzowano procesy mikrobiologicznej degradacji i detoksykacji 4-nonylofenolu, 4-*tert*-oktylofenolu, 4-kumylofenolu oraz fenoli lotnych przez nieligninolityczny grzyb strzępkowy *Umbelopsis isabellina* Wykazano także zdolność analizowanego drobnoustroju do usuwania metali ciężkich: Pb, Ni, Mn i Zn z pożywki wzrostowej przy jednoczesnym zmniejszeniu toksyczności środowiska. Wyniki badań otrzymanych w niniejszej pracy uprawniają do sformułowania następujących wniosków i stwierdzeń końcowych:

1. Szczep *U. isabellina* wykazuje wysoką aktywność w eliminacji 4-nonylofenolu, 4-*tert*-oktylofenolu oraz 4-kumylofenolu ze środowiska wzrostu.
2. Badane procesy mikrobiologicznej eliminacji związków fenolowych zachodzą poprzez ich degradację inicjowaną procesami hydroksylacji, prowadząc do wytworzenia mniej toksycznych metabolitów pośrednich.
3. Wysoka tolerancja *U. isabellina* na obecność Pb, Ni, Mn i Zn w podłożu wzrostowym jest związana głównie z biosorpcją jonów metali w obrębie struktur powierzchniowych grzyba.
4. Uwidoczniona zależność między szybkością rozkładu NP, 4-*t*-OP oraz CP i zmianami w strukturze strzępek *U. isabellina* wskazuje, że biotransformacja ksenobiotyków fenolowych ogranicza szkodliwy wpływ tych związków na morfologię grzyba.
5. Jednoczesne usuwanie z pożywki wzrostowej związków fenolowych i metali ciężkich przez *U. isabellina* prowadzi do redukcji toksycznego wpływu zanieczyszczeń na reakcje biologiczne organizmów wskaźnikowych reprezentujących szeroki zakres taksonów różnych poziomów troficznych.
6. Grzyb *U. isabellina* charakteryzuje się zdolnością do eliminacji lotnych fenoli i detoksykacji zanieczyszczeń obecnych w odciekach pochodzących ze składowiska odpadów przemysłowych dawnych Zakładów Produkcji Barwników ,,Boruta'' w Zgierzu.
7. Rezultaty badań przygotowanych w ramach prezentowanej pracy doktorskiej uwidaczniają przydatność badanego grzyba do oczyszczania skażonych matryc, w tym ścieków przemysłowych, co może również stanowić interesującą alternatywę dla

często kosztownych i nieprzyjaznych dla środowiska fizykochemicznych metod eliminacji substancji toksycznych.

VI. Streszczenie

4-nonylofenol, 4-*t*-oktylofenol, 4-kumylofenol, liczne fenole lotne, a także wybrane metale ciężkie są zaliczane do związków toksycznych zaburzających prawidłowe funkcjonowanie układu hormonalnego ludzi i zwierząt.

Prezentowana rozprawa doktorska miała na celu charakterystykę procesów degradacji i detoksykacji 4-nonylofenolu, 4-*tert*-oktylofenolu 4-kumylofenolu przez grzyb mikroskopowy *Umbelopsis isabellina*. Realizowane badania obejmowały ponadto ocenę zdolności grzyba do usuwania wybranych jonów metali ciężkich ze środowiska wzrostu. W pracy podjęto także ekotoksykologiczną analizę procesów eliminacji 4-nonylofenolu, 4-*tert*-oktylofenolu, 4-kumylofenolu oraz metali ciężkich przez szczep *U. isabellina* z użyciem testów wykorzystujących bioindykatory reprezentujące różne poziomy łańcucha troficznego. Poddano również ocenie efektywność *U. isabellina* do usuwania i detoksykacji lotnych fenoli zawartych w odciekach ze składowiska odpadów niebezpiecznych.

W pierwszym etapie badań, których wyniki zostały przedstawione w publikacji P1, wykazano zdolność *U. isabellina* do eliminacji NP, 4-*t*-OP i CP z podłoża wzrostowego. Ubytek około 96 % początkowej zawartości ksenobiotyków w pożywce (25 mg/l) zaobserwowano po 24 godzinach inkubacji. Analizy chromatograficzne ekstraktów pohodowlanych, umożliwiły zidentyfikowanie kilku pośrednich produktów biotransformacji NP, 4-*t*-OP i CP, wskazując na potencjał grzyba do degradacji badanych związków fenolowych. Rozkład NP i 4-*t*-OP przez *U. isabellina* inicjowany był procesami hydroksylacji końcowego atomu węgla we fragmentach alkilowych ksenobiotyków, po których następowało utlenienie grupy hydroksylowej i stopniowe skracanie łańcucha alifatycznego cząsteczki. Biotransformacja CP zachodziła prawdopodobnie poprzez hydroksylację atomu C grupy metylowej (C-8 lub C-9) a następnie utlenienie odpowiednio powstałej grupy karboksylowej. W ramach prowadzonych badań analizowano ponadto wpływ testowanych związków fenolowych oraz powstających w trakcie ich rozkładu intermediatów na szybkość wzrostu i morfologię grzybni *U. isabellina*. Zaobserwowano, że ekspozycja grzyba na działanie NP, 4-*t*-OP i CP powodowała powstawanie zmian w strukturze strzępek (wewnątrzkomórkowe ziarnistości) , które zanikały w czasie prowadzenia hodowli co wskazywało na zmniejszenie toksyczności środowiska wzrostu drobnoustroju w trakcie badanych procesów degradacyjnych. Zastosowanie testów

ekotoksyczności potwierdziło wcześniejsze obserwacje, iż procesy biotransformacji substratów fenolowych prowadziły do spadku toksyczności hodowli względem zastosowanych bioindykatorów.

Zakres badań, opisanych w publikacji P2, obejmował przede wszystkim ocenę zdolności *U. isabellina* do biosorpcji i akumulacji metali ciężkich: Ni, Pb, Zn, Mn. Efektywność wychwytywania testowanych jonów metali przez grzybnię drobnoustroju wynosiła od 74,3 mg dla PB do 6,8 mg dla Ni na gram suchej masy. Otrzymane w trackie doświadczeń wyniki wskazały ponadto, że dominującym mechanizmem pobierania jonów metali przez grzyba jest sorpcja do powierzchniowych struktur komórkowych. Rezultaty przeprowadzonych doświadczeń pozwoliły także wykazać aktywność *U. isabellina* do jednoczesnej eliminacji ksenobiotyków fenolowych oraz metali ciężkich z podłoża hodowlanego. Równoczesna suplementacja pożywki wzrostowej NP, 4-*t*-OP i CP oraz Pb, Ni, Mn i Zn hamowała zarówno efektywność wychwytywania przez drobnoustrój jonów metali jak i eliminację NP, 4-*t*-OP i CP w porównaniu z poziomem ich ubytku w hodowlach traktowanych osobno badanymi zanieczyszczeniami.

Ostatnim realizowanym w niniejszej rozprawie etapem badań opisanych w manuskrypcie P3 była przede wszystkim ocena całkowitego ryzyka przez wszystkie związki zawarte w kulturach grzybowych, powstające podczas biodegradacji NP, 4-*t*-OP i CP, poprzez zastosowanie wielogatunkowych testów toksyczności. Analizy ekotoksykologiczne uwidoczniły zmniejszenie szkodliwego wpływu filtratów pohodowlanych w trakcie inkubacji drobnoustroju z zanieczyszczeniami wobec wszystkich testowanych bioindykatorów. Przeprowadzone w pracy doświadczenia ukazały ponadto potencjału *U. isabellina* do redukcji fenoli lotnych z odcieków przemysłowych dawnych Zakładów Produkcji Barwników ,,Boruta'' w Zgierzu. Wykonane analizy wykazały zarówno spadek ilości VPs w trakcie hodowli grzyba suplementowanych odciekami jak i zmniejszenie toksycznego wpływu przesączy na organizmy wskaźnikowe.

Przeprowadzone badania dostarczają dowodów skuteczności redukcji zagrożeń generowanych przez toksyczne ksenobiotyki fenolowe oraz metale ciężkie w wyniku procesów ich mikrobiologicznej eliminacji i degradacji, wskazując na potencjał wykorzystania grzyba *U. isabellina* jako efektywnego narzędzia do bioremediacji środowisk skażonych organicznymi i nieorganicznymi zanieczyszczeniami.

VII. Abstract

4-nonylphenol, 4-t-octylphenol, 4-cumylphenol, numerous volatile phenols, and selected heavy metals are classified as toxic compounds that disrupt the proper functioning of the endocrine system of humans and animals.

The presented dissertation was aimed at the characterization of the degradation and detoxification processes of 4-nonylphenol (NP), 4-*tert*-octylphenol (4-*t*-OP) and 4-cumylphenol (4-CP) by the microscopic fungus *Umbelopsis isabellina*. The conducted research also included the assessment of the fungus' ability to remove selected heavy metal ions from the growth environment. The study also undertook an ecotoxicological analysis of the elimination processes of 4-nonylphenol, 4-*tert*-octylphenol, 4-cumylphenol and heavy metals by the *U. isabellina* strain by performing tests using bioindicators representing different levels of the trophic chain. The effectiveness of *U. isabellina* for the removal and detoxification of volatile phenols contained in the leachate from a hazardous waste landfill was also assessed.

In the first stage of the research, the results of which were presented in the publication P1, the ability of *U. isabellina* to eliminate NP, 4-*t*-OP and CP from the growth medium was demonstrated. A loss of about 96% of the initial content of xenobiotics in the medium (25 mg/l) was observed after 24 hours of incubation. Chromatographic analyzes of post-culture extracts allowed the identification of several intermediate products of NP, 4-*t*-OP and CP biotransformation, indicating the fungus' potential to degrade the phenolic compounds tested. The decomposition of NP and 4-*t*-OP by *U. isabellina* was initiated by the processes of hydroxylation of the terminal carbon atom in alkyl fragments of xenobiotics, followed by oxidation of the hydroxyl group and gradual shortening of the aliphatic chain of the molecule. CP biotransformation probably took place by hydroxylation of the C atom of the methyl group (C-8 or C-9) and then oxidation of the correspondingly formed carboxyl group. As part of the research, the influence of the tested phenolic compounds and the intermediates formed during their decomposition on the growth rate and morphology of the mycelium of *U. isabellina* was also analyzed. It was observed that the fungal exposure to NP, 4-*t*-OP and CP caused changes in the structure of the hyphae (intracellular granules), which disappeared during cultivation, indicating a reduction in the toxicity of the microorganism growth

environment during the degradation processes studied. The use of ecotoxicity tests confirmed the earlier observations that the processes of biotransformation of phenolic substrates led to a decrease in the toxicity of the culture in relation to the bioindicators used.

The scope of the research described in publication P2 included the assessment of *U. isabellina's* ability to biosorb and accumulate heavy metals: Ni, Pb, Zn, Mn. The efficiency of capturing the tested metal ions by the microbial mycelium ranged from 74,3 mg for PB to 6,8 mg for Ni per gram of dry fungal mass. The results obtained during the experiments also indicated that the dominant mechanism of metal ion uptake by the fungus is sorption to the surface cell structures. The results of the conducted experiments also allowed to demonstrate the activity of *U. isabellina* for the simultaneous elimination of phenolic xenobiotics and heavy metals from the culture medium. Simultaneous supplementation of the growth medium with NP, 4-*t*-OP and CP as well as Pb, Ni, Mn and Zn inhibited both the efficiency of metal ion capture by the organism and the elimination of NP, 4-*t*-OP and CP compared to the level of their depletion in the cultures treated separately tested pollutants.

The last stage of the research described in the P3 manuscript was assessment of the total risk by all compounds contained in fungal cultures, formed during the biodegradation of NP, 4-*t*-OP and CP, through the use of multispecies toxicity tests. Ecotoxicological analyzes showed a reduction in the harmful effect of post-culture filtrates during the incubation of the microorganism with contaminants against all tested bioindicators. The experiments carried out in the study also showed the potential of *U. isabellina* to reduce volatile phenols from industrial leachate of the former "Boruta" Dye Production Plant in Zgierz. The performed analyzes showed both a decrease in the amount of VPs during the cultivation of the fungus supplemented with leachate and a decrease in the toxic effect of filtrates on the indicator organisms.

The conducted studies provide evidence of the effectiveness of reducing the risks generated by toxic phenolic xenobiotics and heavy metals as a result of the processes of their microbiological elimination and degradation, indicating the potential of using the *U. isabellina* fungus as an effective tool for the bioremediation of environments contaminated with organic and inorganic pollutants.

VIII. Całkowity dorobek naukowy

VIII.1 Publikacje

1. **Janicki T**., Krupiński M., Długoński J. 2016. Degradation and toxicity reduction of the endocrine disruptors nonylphenol, 4-*tert*-octylphenol and 4-cumylphenol by the non-ligninolytic fungus *Umbelopsis isabellina*. Bioresource Technology, 200, 223-229; doi: 10.1016/j.biortech.2015.10.034

   IF = 9,642; 34 cytowania; punkty MEiN = 140

2. **Janicki T**., Długoński J., Krupiński M. 2018. Detoxification and simultaneous removal of phenolic xenobiotics and heavy metals with endocrine-disrupting activity by the non-ligninolytic fungus *Umbelopsis isabellina.* Journal of Hazardous Materials, 360, 661-669; doi: 10.1016/j.jhazmat.2018.08.047

   IF = 10,588; 14 cytowań; punkty MEiN = 200

3. **Janicki T**., Długoński A., Felczak A., Długoński J., Mariusz Krupiński. 2021. Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol and volatile leachate phenol degradation by the microscopic fungus *Umbelopsis isabellina* using a battery of biotests. Manuskrypt przyjęty do recenzji w czasopiśmie Chemosphere, 20 grudnia 2021 r.

4. Krupiński M., **Janicki T**., Pałecz B., Długonski J. 2014. Biodegradation and utilization of 4-*n*-nonylphenol by *Aspergillus versicolor* as a sole carbon and energy source. Journal of Hazardous Materials, 280, 678-684; doi: 10.1016/j.jhazmat.2014.08.060

   IF = 10,588; 23 cytowania; punkty MEiN = 200

Sumaryczny IF = 30,818

Łączna liczba punktów MEiN = 540

Liczba cytowań = 71

Index H = 3

VIII.2 Doniesienia konferencyjne

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VIII.3 Kursy i szkolenia

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Degradation and toxicity reduction of the endocrine disruptors nonylphenol, 4-*tert*-octylphenol and 4-cumylphenol by the non-ligninolytic fungus *Umbelopsis isabellina*

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HIGHLIGHTS

- Non-ligninolytic fungus *U. isabellina* was able to degrade NP, 4-*t*-OP and 4-CP.
- Xenobiotics removal was accompanied by the formation of hydroxylated metabolites.
- Decrease of toxicity was observed during the biodegradation of NP, 4-*t*-OP and 4-CP.
- Non-isomer-specific degradation of NP by *U. isabellina* was noticed.

GRAPHICAL ABSTRACT

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ABSTRACT

Nonylphenol (NP), 4-*tert*-octylphenol (4-*t*-OP) and 4-cumylphenol (4-CP) are pollutants that are known as endocrine disruptors mainly due to their estrogen-mimicking activity. These phenolic substances are used in a wide range of industrial and commercial applications. In the present study, biodegradation of tNP, 4-*t*-OP and 4-CP using the non-ligninolytic fungus *Umbelopsis isabellina* was investigated. After 12 h of incubation, more than 90% of initially applied tNP, 4-*t*-OP and 4-CP (25 mg L^{-1}) were eliminated. GC–MS analysis revealed several derivatives mainly (hydroxyalkyl)phenols. Moreover, xenobiotic biotransformation led to the formation of intermediates with less harmful effects than the parent compounds. For all xenobiotics, a decrease in growth medium toxicity was observed, using *Artemia franciscana* and *Daphnia magna* as bioindicators. The results indicate that *U. isabellina* has potential in the degradation and detoxification of contaminants with endocrine activity. Moreover, this is the first report demonstrating that a microorganism is capable of effective 4-CP elimination.

1. Introduction

The environmental release and fate of numerous organic chemicals have attracted increasing attention because of their potential adverse health effects. Numerous environmental pollutants are known to disturb endocrine functions by mimicking natural hormones or by interfering with hormone receptors and biosignalling pathways. These are defined as EDCs, or endocrine disrupting compounds. 4-*tert*-octylphenol (4-*t*-OP), 4-cumylphenol (4-CP) and technical nonylphenol (tNP) are among the most frequently detected EDCs in different environmental matrices (Soares et al., 2008; Ying et al., 2002). 4-*t*-OP and tNP occur mainly in the environment as incomplete microbial transformation products of nonyl- and octylphenol polyethoxylates, which are widely used as non-ionic surfactants in household products and in many industries as detergents, emulsifiers, plasticizers or preservatives (Ying

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et al., 2002). 4-CP is commonly used in the production of effective stabilizers for fuels, oils, polymers, and rubbers. These compounds are discharged into the environment mainly due to the insufficient effectiveness of conventional wastewater treatment facilities. As a consequence, they are ubiquitously found in various environmental compartments such as surface water and groundwater, sediments, soil and the atmosphere (Soares et al., 2008; Zuo and Zhu, 2014; Lu and Gan, 2014a). Moreover, 4-CP, 4-*t*-OP and tNP tend to adsorb onto surface water particles and accumulate in aquatic organisms, which causes their transfer and accumulation with increasing trophic level and thereby poses a serious ecotoxicological risk (Junghanns et al., 2005). Numerous papers have demonstrated that the exposure to 4-CP, 4-*t*-OP and tNP results in different adverse effects in various organisms, e.g., fish and invertebrates. For instance, these compounds have been shown to interfere with sexual development and reproductive function (Soares et al., 2008; Zuo and Zhu, 2014). Additionally, several reports have suggested that they affect the human body, for example, inducing hormone-dependent cancers and causing immune dysfunction (Inadera, 2006; Lee and Choi, 2013). Due to the widespread presence of 4-CP, 4-*t*-OP and tNP in the environment and their high toxicity and persistence, it is important to investigate biodegradation processes that can reduce the risks posed by these xenobiotics. Furthermore, because the current conventional treatment techniques cannot effectively remove 4-CP, 4-*t*-OP and tNP, the development of effective methods to eliminate these compounds from various environments, and especially methods that use microorganisms, is strongly recommended (Girlanda et al., 2009).

The information about pollutant biodegradation and detoxification by microorganisms enables not only new potential applications in bioremediation but also improves the understanding of the fate of these compounds in the environment and allows the assessment of the ecotoxicological risk caused by their derivatives (Cajthaml et al., 2009; Chen et al., 2015; Lu and Gan, 2014a; Zeng et al., 2013; Zhang et al., 2013). Therefore, the results presented in this work on 4CP, 4-*t*-OP and tNP biodegradation by *Umbelopsis isabellina* can be used to develop effective methods for removing these xenobiotics from contaminated environments such as water, sediment or soil.

Many studies have focused on the microbial degradation of NP and 4-*t*-OP (Cajthaml et al., 2009; Gao et al., 2011; Junghanns et al., 2005; Kolvenbach and Corvini, 2012; Różalska et al., 2015; Toyama et al., 2011; Moon and Song, 2012). In contrast, the mechanisms of their biotransformation have not been investigated sufficiently (Lu and Gan, 2014b; Girlanda et al., 2009). The reports concerning potential derivatives and the biotoxic effects of these processes are very limited. To the best of our knowledge, there is no information available about the microbial biotransformation of 4-CP. Therefore, in this study, the biodegradation of 4-CP, 4-*t*-OP and tNP by the non-ligninolytic filamentous fungus *U. isabellina* was examined. The biotransformation processes were characterized by identifying several intermediates. Special attention was also paid to the evaluation of changes in the toxicity of fungal cultures during cultivation.

2. Methods

2.1. Chemicals

Technical nonylphenol (purity > 84%), 4-*tert*-octylphenol (purity > 97%) and 4-cumylphenol (purity 99%) were purchased from Sigma–Aldrich (Germany). *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Merck (Germany). Other solvents and high-purity analytical reagents were supplied by POCH (Poland) and J.T. Baker (Holland).

2.2. Microorganisms and growth conditions

The filamentous fungus *U. isabellina* originated from the Culture Collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz, Poland. Fungal spores from 10 days old cultures on ZT slants (Różalska et al., 2010) were used to inoculate of 20 mL Sabouraud medium (Difco) and precultured for 24 h at 28 °C on a rotary shaker (150 rpm). Then, precultures were transferred to fresh medium (at a 1:9 dilution) containing xenobiotics. In each experiment, tNP, 4-*t*-OP or 4-CP was added to a final concentration of 25 mg L^{-1} (stock solution 5 mg mL^{-1}, dissolved in 96% ethanol). Fungal cultures were incubated with agitation (150 rpm) at 28 °C in the dark. Biotic cultures and abiotic controls were also prepared. Furthermore, inactivated fungal cultures were prepared by adding sodium azide (500 mg L^{-1}) (Junghanns et al., 2005).

2.3. Biomass estimation

All the tested fungal cultures were filtrated through a preweighed filter membrane (Sartorius, 0.45 μm pore size) and air-dried in an oven at 105 °C to obtain a constant weight.

2.4. Sample extraction and GC–MS analysis

Whole fungal cultures were homogenized using a FastPrep-24 bead mill homogenizer (MP Biomedicals, Santa Ana, CA, USA) and then acidified to pH 2. Each sample was then extracted three times with 1:1 ratio of ethyl acetate, dried over anhydrous sodium sulfate and evaporated to dryness by nitrogen gas stream. After extraction, the organic phase was redissolved in ultra pure ethyl acetate and derivatized with BSTFA according to a previously described method (Różalska et al., 2010). The concentrations of xenobiotics and metabolites were analysed using a 7890A gas chromatograph equipped with a 5975C mass spectrometer (Agilent Technologies, USA). The samples (1.6 μL) were introduced into a split injector (split ratio 10:1) and heated to 280 °C, and separation was achieved on a HP-5 MS capillary column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness; Agilent Technologies, USA). The helium carrier gas flow was held constant at 1.2 mL min^{-1}. The column temperatures applied were: 80 °C for 2 min, ramped to 210 °C at 20 °C min^{-1}, increased to 225 °C at 2 °C min^{-1} and further increased to 300 °C at 20 °C min^{-1} and held at 300 °C for 3 min. Analysis was conducted in a full-scan mode over an m/z range of 40–450 amu. Identification of tNP, 4-*t*-OP and 4-CP metabolites was carried out on the basis of retention times and mass spectra analysis as described previously (Krupiński et al., 2013; Różalska et al., 2010), completed with an Isotope Calculator (NIST) and AMDIS software, and confirmed using the NIST08 MS library. Quantitative analysis was performed using standard curves, which showed linearity in the range of 0–100 μg mL^{-1} for all tested xenobiotics.

2.5. Toxicity bioassays

Toxicity levels of filtrates from fungal cultures with tested xenobiotics were assessed using the Artoxkit M and Daphtoxkit F toxicity bioassays (Microbiotests, Inc., Mariakerke-Gent, Belgium). Changes in the toxicity of the samples were determined using larvae of the crustaceans *Artemia franciscana* and *Daphnia magna* in accordance with standard operational procedures. The results of the tests were expressed as $LC_{50/24}$ (in % dilution), i.e., the concentration of compound leading to 50% mortality of the tested bioindicators after 24 h, and converted into Toxic Units

(TU = 1/LC_{50} * 100). Lethal concentrations were determined from the linear portion of each curve using regression analysis.

2.6. Statistical analyses

All experiments were performed in triplicate, and the mean and standard deviations were calculated. The data were tested by standard variance ANOVA and followed by Student's *t*-test to determine significant differences. The statistical significance level was set at $P \leqslant 0.05$.

3. Results and discussion

3.1. Elimination and degradation of t-NP, 4-t-OP and 4-CP by fungal cultures

Study of the tested EDCs showed complete degradation of xenobiotics by *U. isabellina* by the end of cultivation i.e., 24 h. More than 90% of tNP and 4-*t*-OP was removed after 12 h of incubation compared with the abiotic controls (Fig. 1A and C). The same percentage of 4-CP elimination was observed as early as after 6 h in *U. isabellina* cultures (Fig. 1B). In the experiments employing abiotic controls and inactivated fungal cultures treated with sodium azide, all xenobiotic recoveries ranged from 92% to 96%, which indicates their metabolic biotransformation in fungal cultures.

The half-life of the described compounds depends on both environmental and physicochemical factors and ranges from 1 to 300 days for tNP, from 38 to more than 340 days for 4-CP and from 8 to more than 150 days in the case of 4-*t*-OP in different environmental compartments. Elimination and degradation of tNP or its individual isomers and 4-*t*-OP were reported both in pure microbial cultures and in environmental samples (soil, sediments and wastewater) (Cajthaml et al., 2009; Di Gioia et al., 2008; Girlanda et al., 2009; Kolvenbach and Corvini, 2012; Moon and Song, 2012; Tamagawa et al., 2007; Toyama et al., 2011). Most of the available data on microbial degradation of tNP and 4-*t*-OP refer to processes of biotransformation by bacteria or microbial consortia (Gabriel et al., 2008; Lu and Gan, 2014b; Toyama et al., 2011). In contrast, the number of studies describing the elimination of these xenobiotics by fungi, especially non-ligninolytic fungi, is limited. Nevertheless, filamentous fungi exhibit several advantages over bacteria: they have a particularly high metabolic versatility and soil colonization efficiency, tolerate considerable amounts of toxicants, and produce enzymes that can reach contaminants with poor bioavailability (Girlanda et al., 2009; Soares et al., 2005). In this work, the elimination half-life values of the tested compounds (initially added to the culture at 25 mg L^{-1}) were approximately 4 h for tNP and 4-*t*-OP and 3 h for 4-CP. A comparison of the tNP and 4-*t*-OP disappearance rates obtained in this work and in other studies indicates that *U. isabellina* has a higher degradation efficiency than other fungi. In a study by Soares et al. (2005) that used NP at a concentration of 100 mg L^{-1}, a 50% reduction of the xenobiotic was observed in ligninolytic *Bjerkandera* sp. BOL13 and *Trametes versicolor* fungal cultures after 5 days of cultivation. Another paper reported that the half-life of nonylphenol was approximately 15 and 17 days in UHH 1-6-18-4 and *Clavariopsis aquatica* fungal cultures, respectively, when applying almost the same initial concentration of tNP (22 mg L^{-1}) used in the present study (Junghanns et al., 2005). Removal of more than 50% of the applied xenobiotic by white rot fungi was also documented to occur at 14 days for *Pleurotus ostreatus* and *Irpex lacteus* and at 7 days for *T. versicolor* and *Stereum hirsutum* with the initial amount of tNP lower than that applied in *U. isabellina* cultures (2, 5 and 1 mg L^{-1}, respectively) (Cajthaml et al., 2009; Castellana and Loffredo, 2014). To the best of our knowledge, only a few studies reported tNP elimination rates by fungal cultures higher than those obtained in this work (Moon and Song, 2012; Tamagawa et al., 2007). Unfortunately, the authors did not structurally identify tNP isomers or examine toxicity changes during the analysed processes. Potential intermediates that are direct evidence of xenobiotic biotransformation were also not identified.

Based on the analysis of the mass distribution of fragment ions, isomers of NP were divided into 5 groups (Supplementary Fig. 1), depending on their structure relative to α- and β-carbons of the alkyl chain (Gabriel et al., 2008; Lu and Gan, 2014b). Fragment ions *m/z* 107, 121, 135, 149, 163 and 191 were selected to structurally classify different types of the 17 separated NP isomers (comprising approximately 94% of total tNP), and the identification process was performed as proposed previously (Katase et al., 2008; Wu et al., 2010). Four types of fragment ions, *m/z* 121, 135, 149 and 163, were used for quantification calculations of individual NP isomers.

The results obtained in this work showed that there was no significant correlation between the removal rate of tNP isomers and the degree of branching in the alkyl substituent in their structure. The data presented in Fig. 2 indicate that during incubation of the fungus with tNP, all analysed groups of isomers were transformed simultaneously. These data suggested a non-isomer-specific degradation of tNP by *U. isabellina*. These findings are in contrast to the results described for some other NP microbiological degradation processes; however, significant differences in the susceptibility of NP isomers to biodegradation have been documented in numerous papers (Das and Xia, 2008; Gabriel et al., 2008; Ikunaga et al., 2004; Lu and Gan, 2014b). Biotransformation of different single NP isomers by the bacteria *Sphingomonas* sp. TTNP3 showed that the degradation was more efficient for α-methyl-α-ethyl isomers than for isomers with the α-dimethyl structure (Kolvenbach and

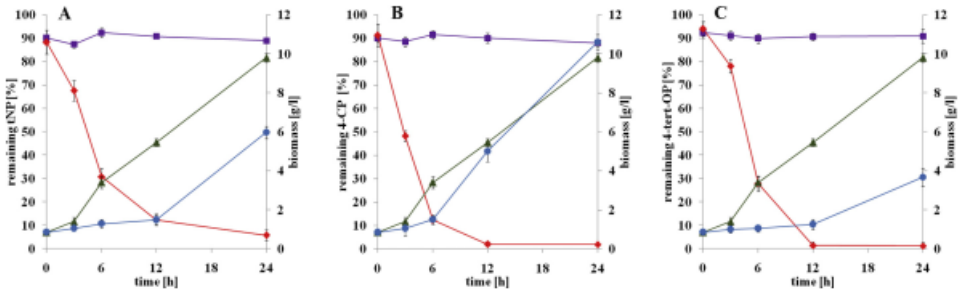

Fig. 1. Elimination of tNP (A), 4-CP (B) and 4-*tert*-OP (C) by *U. isabellina*: xenobiotic concentration in fungal cultures (◆) and in abiotic controls (■); growth of fungus with xenobiotics (●) and without xenobiotics in culture medium (▲).

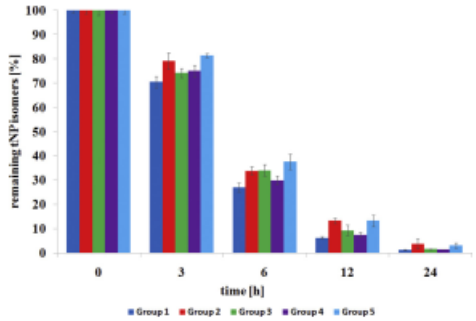

Fig. 2. Elimination of tNP isomer groups [%] by *U. isabellina* after 24 h of cultivation.

Corvini, 2012). Gabriel et al. (2008) demonstrated that the bacterium *Sphingomonas xenophaga* Bayram preferentially degraded NP isomers when the α-position was less bulky, whereas Das and Xia (2008) reported that degradation of NP isomers with the α-methyl-α-propyl structure were transformed more slowly than the other tested isomers during biosolid composting. Biodegradation of tNP by *Sphingobium amiense* also showed isomer selectivity. Isomers with the α-dimethyl and α-ethyl-α-methyl substituents were removed by these bacteria more quickly than were isomers with the α-methyl-α-propyl structure (Ikunaga et al., 2004). Another study showed that the biodegradability of tNP by microbial consortia in river sediments also strongly depended on the structures of the isomers. NP isomers with a short side chain and bulky α-substituents were more recalcitrant to biotransformation (Lu and Gan, 2014b). Therefore, the authors concluded that biodegradation of different tNP isomers was significantly influenced by the length of the main alkyl chain and the substitution pattern at the α- and β-carbon, which relate to steric constraints. However, only one paper documented non-isomer-specific microbial biotransformation of tNP, that is, removal by the bacterium *Sphingomonas cloacae* (Ikunaga et al., 2004). To the best of our knowledge, the results obtained in this work are the first to describe a filamentous fungus capable of eliminating total tNP during 12 h of incubation, concurrently indicating that the removal rates of different isomers of tNP do not depend on the substitution pattern of the α- and β-carbons.

While the processes of tNP biodegradation by microorganisms have been described by several studies, less information is available about the microbial removal of 4-*t*-OP. Moreover, only a few reports have focused on the biodegradation of octylphenols by pure fungal cultures or microbial communities, and 4-*t*-OP degradation by filamentous fungi has not been investigated sufficiently. The half-life values for this xenobiotic in the ligninolytic fungus *Phanerochaete sordida* YK-624 cultures were approximately 12 times higher than the results obtained in this study; they were also at a lower initial concentration of 4-*t*-OP (20 mg L^{-1}) (Tamagawa et al., 2007). The potential for the white-rot fungus *Marasmius quercophilus* to degrade 4-*t*-OP has also been reported (Farnet et al., 2011). Although that study used a higher initial amount of xenobiotic than was used in this study, they found that *M. quercophilus* cultures almost completely removed the 4-*t*-OP by 20 days. Degradation of 4-*t*-OP was also tested in cultures of *I. lacteus* and *T. versicolor* (Moon and Song, 2012). The authors who demonstrated a significant reduction of tNP by these ligninolytic fungi also indicated their ability to effectively remove 4-*t*-OP. The observed 4-*t*-OP elimination rates in *I. lacteus* and *T. versicolor* cultures were higher than those reported in this work and ranged from 46% to 100% after 3 h of cultivation, respectively. One caveat of that work, however, is that in contrast to our research, and as was the case in the studies with tNP, no intermediates of 4-*t*-OP transformation were shown, and no toxicity changes during the elimination processes were detected.

In contrast to NP and 4-*t*-OP, there are currently no studies on the microbiological degradation of cumylphenol. Therefore, this is the first report demonstrating a microorganism capable of biotransforming this xenobiotic. Only a few studies have focused on the decomposition of 4-CP through physical or chemical pathways (Xiao et al., 2015). Nevertheless, 4-CP removal by fungi maybe a safer, less invasive and more cost-effective alternative for bioremediation processes than applied conventional physicochemical treatment techniques.

As mentioned above, most papers address the removal of tNP and 4-*t*-OP by ligninolytic fungi. However, it should be noted that non-ligninolytic fungi offer certain advantages over white-rot fungi because of their rapid colonization of different environmental compartments, higher competitive abilities, ecological plasticity and faster metabolic processes (Krupiński et al., 2014). For these reasons, the use of *U. isabellina* could be a convenient tool for the decontamination of different xenobiotic-polluted areas. Only a few studies have reported non-ligninolytic fungi capable of effectively degrading individual NP isomers, especially of those with non-branched aliphatic chains (4-*n*-NP), which are not present in the NP technical mixture (Krupiński et al., 2013; Lu and Gan, 2014b; Różalska et al., 2010). However, these data should not be compared with the results obtained in the present study because it is more appropriate to use total tNP than to use single isomers in environmental studies. This is because tNP has practical applications in industry and agriculture and is characterized by higher toxicity and estrogenic activity compared with single NP isomers (Gabriel et al., 2008).

GC–MS analysis identified several potential intermediates in fungal cultures treated with tNP, 4-*t*-OP and 4-CP. The identified products obtained from the metabolism of xenobiotics are shown in Supplementary Fig. 2 and Table 1. Intracellular extracts of *U. isabellina* grown on tNP and 4-*t*-OP contained mainly (hydroxyalkyl)phenols. Mass spectrometric analysis revealed the presence of 2-hydroxy-2-(3-hydroxyphenyl)acetic acid and 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid in the cultures with 4-*t*-OP and 4-hydroxybenzoic acid and benzene-1,4-diol in the extracts from cultures treated with 4-*t*-OP and tNP. This implies that the degradation of alkylphenols by *U. isabellina* was most likely initiated by hydroxylation of the terminal carbon in the alkyl moiety. Subsequently, oxidation of the hydroxyl group of the corresponding carboxylic acid and further shortening of the aliphatic chain through subterminal or terminal carbon oxidation occurred, and *p*-quinone was formed as the final intermediate. This suggests that tNP and 4-*t*-OP were metabolized by the fungus via similar pathways. Alkyl-chain oxidation intermediates of NP were also found in other fungal cultures. In our previous study, we identified 4-hydroxybenzoic acid and several alkoxyphenol derivatives in *Aspergillus versicolor* cultures with 4-*n*-NP (Krupiński et al., 2013). 4-hydroxybenzoic acid was also identified as a product of tNP degradation by the aquatic fungus UHH 1-6-18-4 (Junghanns et al., 2005). Moreover, the alkylphenols formed as degradation products of *U. isabellina* were similar to those proposed in *Gliocephalotrichum simplex* (Różalska et al., 2010). The metabolism of 4-*n*-NP by this fungus led to the detection of two intermediates identical to those found in this study: 2-hydroxy-2-(3-hydroxyphenyl)acetic acid and 4-hydroxybenzoic acid. Based on the identified derivatives, the authors of the above studies concluded that NP degradation pathways proceeded via terminal hydroxylation of the alkyl chain followed by subterminal oxidation processes.

Table 1
Mass spectral data of 4-*t*-OP, tNP and 4-CP biodegradation products in *U. isabellina* cultures.

Metabolite (TMS derivatives)	Substance	Retention time [min]	Chemical formula	Molecular weight	Mass spectrum *m/z* (10 largest ions, relative intensity)
2-Hydroxy-2-(3-hydroxyphenyl) acetic acid, tri-TMS	4-*t*-OP	9.27	$C_{17}H_{32}O_4Si_3$	384.69	267(99.9), 73(57.2), 268(24.8), 147(16.3), 269(14.2), 341(10.1), 75 (8.2), 74(7.3), 45(7.2), 342(4.2)
2-Hydroxy-3-(4-hydroxyphenyl) propanoic acid, tri-TMS	4-*t*-OP	11.44	$C_{18}H_{34}O_4Si_3$	398.72	179(99.9), 73(80.3), 147(24.3), 308(18.1), 180(17.3), 75(12.3), 45 (8.3), 74(6.9), 148(3.9), 281(3.1)
Benzene-1,4-diol, di TMS	4-*t*-OP tNP	6.15	$C_{12}H_{22}O_2Si_2$	254.48	239(99.9), 256(77.9), 73(48.7), 240(29.5), 112(22.8), 257(21.5), 241 (16.2), 258(14.8), 133(9.7), 75(9.2)
4-Hydroxybenzoic acid, di-TMS	4-*t*-OP tNP 4-CP	6.44	$C_{13}H_{22}O_3Si_2$	282.49	267(99.9), 223(72.4), 73(68.1), 193(60.8), 282(23.7), 268(22.9), 224 (16.4), 126(15.5), 194(11.2), 45(10.2)
2-(4-Hydroxyphenyl) acetic acid, di-TMS	4-CP	6.69	$C_{14}H_{24}O_3Si_2$	296.50	73(99.9), 179(19.5), 75(18.2), 164(11.1), 74(9.9), 45(9.5), 252(7.1), 281(6.8), 296(6.6), 224(5.5)
2-(4-Hydroxyphenyl)-2-phenylpropanoic acid, di-TMS	4-CP	12.37	$C_{21}H_{30}O_3Si_2$	386.63	357(99.9), 372(47.8), 73(45.4), 358(32.9), 373(16.0), 103(13.3), 359 (12.1), 119(7.7), 269(6.6), 75(6.3)

Therefore, a similar mechanism is proposed for NP biotransformation by *U. isabellina*. The mechanism for 4-*t*-OP degradation starts with the shortening of its alkyl moiety, which most likely occurred simultaneously with the detachment of methyl groups from the α-C. Although the cleavage of the alkylphenols aromatic ring was not confirmed in this study, the detection of *p*-quinone and 4-hydroxybenzoic acid implied their subsequent conversion into organic acid and CO_2 due to their lower resistance to biodegradation and higher bioavailability than 4-*t*-OP and tNP (Junghanns et al., 2005; Kolvenbach and Corvini, 2012).

There are no studies addressing the possible metabolic pathways for the microbial decomposition 4-CP. In our experiments with the *U. isabellina* strain, the presence of three metabolites identified as 2-(4-hydroxyphenyl)-2-phenylpropanoic acid, 2-(4-hydroxyphenyl) acetic acid and 4-hydroxybenzoic acid were detected in cultures supplemented with 4-CP. This is the first study reporting the intermediates formed during the biodegradation of this xenobiotic. The results of the qualitative analysis suggest that 4-CP decomposition was initiated by the hydroxylation of the C atom of a methyl group (C-8 or C-9) and subsequent oxidation of the corresponding carboxyl group. Further degradation most likely occurred via several rearrangements, and cleavage of the C–C bond between the aromatic moieties in two possible routes resulted in the formation of 2-(4-hydroxyphenyl) acetic acid and 4-hydroxybenzoic acid. 4-CP is structurally very similar to bisphenol A (BPA), whose mechanisms of biodegradation have been proposed in several papers. BPA transformation by *Pseudomonas* sp. strain MV1 showed metabolites with a chemical structure corresponding to the 4-CP derivatives produced by *U. isabellina* (Zhang et al., 2013). Thus, it is hypothesized that metabolism of BPA by *Pseudomonas* sp. strain MV1 and 4-CP by *U. isabellina* may follow a similar pattern.

3.2. Toxicity assessment

Biodegradation results in the complete or partial decomposition of xenobiotics. These metabolic processes may lead to the formation of intermediates with more harmful effects than the original compound. To evaluate the toxicity changes during the biotransformation of 4-CP, 4-*t*-OP and tNP by *U. isabellina*, Artoxkit M and Daphtoxkit F bioassays were applied. The ecotoxic effects of filtrates from fungal cultures were assessed using bioindicator species from different ecosystems, i.e., *A. franciscana* as a marine organism and *D. magna* as a freshwater species. These water crustaceans have been used to test the toxicity of different environmental matrices, microbial culture samples and many pollutants such as pesticides, dyes, mycotoxins and other xenobiotics (Bernat et al., 2013; Guida et al., 2008). The results of the toxicological studies are presented in Fig. 3. No toxicity of the biotic cultures (fungal cultures without xenobiotics) was observed in the tested crustaceans. The biotests indicated that the biodegradation of all used pollutants by the fungus led to the formation of less-toxic derivatives. A comparison of the TU values for the abiotic controls and xenobiotic-treated fungal samples indicated a reduction in toxicity of greater than six- and seven-fold for cultures with 4-CP, seven- and eight-fold for tNP and four and six-fold for samples with 4-*t*-OP, in *A. franciscana* and *D. magna* assays, respectively, after a 24 h incubation. Detoxifying processes were also supported by microscopic observations. Morphological defects of mycelium in the cultures supplemented with tNP, 4-*t*-OP and 4-CP in comparison with the biotic control were observed in the first 6 h of cultivation (Supplementary Fig. 3). The presence of xenobiotics in fungal cultures resulted in morphological changes in hyphae such as protuberances and the formation of intracellular nodules. Such adverse effects may be caused by the effects of tNP, 4-*t*-OP and 4-CP on the uptake of respiratory oxygen (Karley et al., 1997). Moreover, the abnormal shape of the hyphae may be a result of the disruption of the hyphal cytosolic Ca^{2+} gradient or actin cytoskeleton (Krupiński et al., 2013). After 6 h of incubation, microscopic analysis revealed a disappearance of the morphological abnormalities in the mycelium of all tested samples. This was associated with the elimination of xenobiotics by the fungus, which implied that the biodegradation processes lead to a decrease in the toxicity of the growth medium. Removal of 4-CP, 4-*t*-OP and tNP was also accompanied by an increase in fungal biomass (Fig. 1A–C). In all cases, elimination of the tested compound, by approximately 80–90%, contributed to a significant enhancement in *U. isabellina* growth. These observations provided further evidence for the reduction of toxicity during biodegradation processes. A similar relationship between the growth rate and kinetics of xenobiotic removal was reported, e.g., in *G. simplex* cultured with 4-*n*-NP or IT-4 and IT-5 strains cultured with 4-*t*-OP (Różalska et al., 2010; Toyama et al., 2011). Only a few studies have monitored changes in toxicity during microbial biodegradation of several EDCs. In some cases, the results showed the formation of metabolites that were more harmful than the original substance, e.g., during removal of tNP by *P. ostreatus* or 4-*n*-NP by *T. versicolor* (Cajthaml et al., 2009). The experiments carried out in this study demonstrated a relevant decrease in toxicity corresponding with biodegradation of the xenobiotic by the fungus. Therefore, these results may be regarded as evidence of biological treatment leading to a reduction in the ecotoxicological risk of the xenobiotic.

4. Conclusions

Numerous studies have demonstrated that biotransformation processes catalysed by fungi should be considered when addressing the environmental fate and biological effect of pollutants such

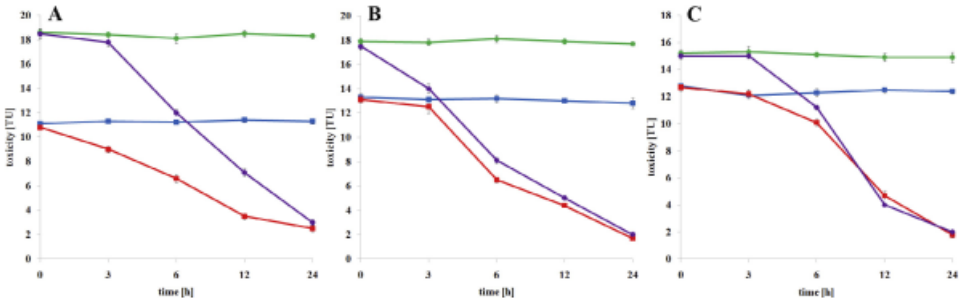

Fig. 3. Toxicity analysis of *U. isabellina* cultures with 4-*t*-OP (A), tNP (B) and 4-CP (C) in *A. franciscana* assay: abiotic controls (■), fungal cultures (■); and *D. magna* assay: abiotic controls (●), fungal cultures (●) during 24 h.

as EDCs. This study provides evidence for the effective biotransformation of 4-*t*-OP, tNP and 4-CP by the non-ligninolytic fungus *U. isabellina*. Moreover, results show that xenobiotic decomposition is accompanied by a decrease in the toxicity of the culture medium. In conclusion, this work indicates a possible role for *U. isabellina* in the degradation of contaminants with endocrine activity, which makes this strain a promising biological tool offering new perspectives for practical applications such as wastewater treatment.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.10.034.

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Supplementary data

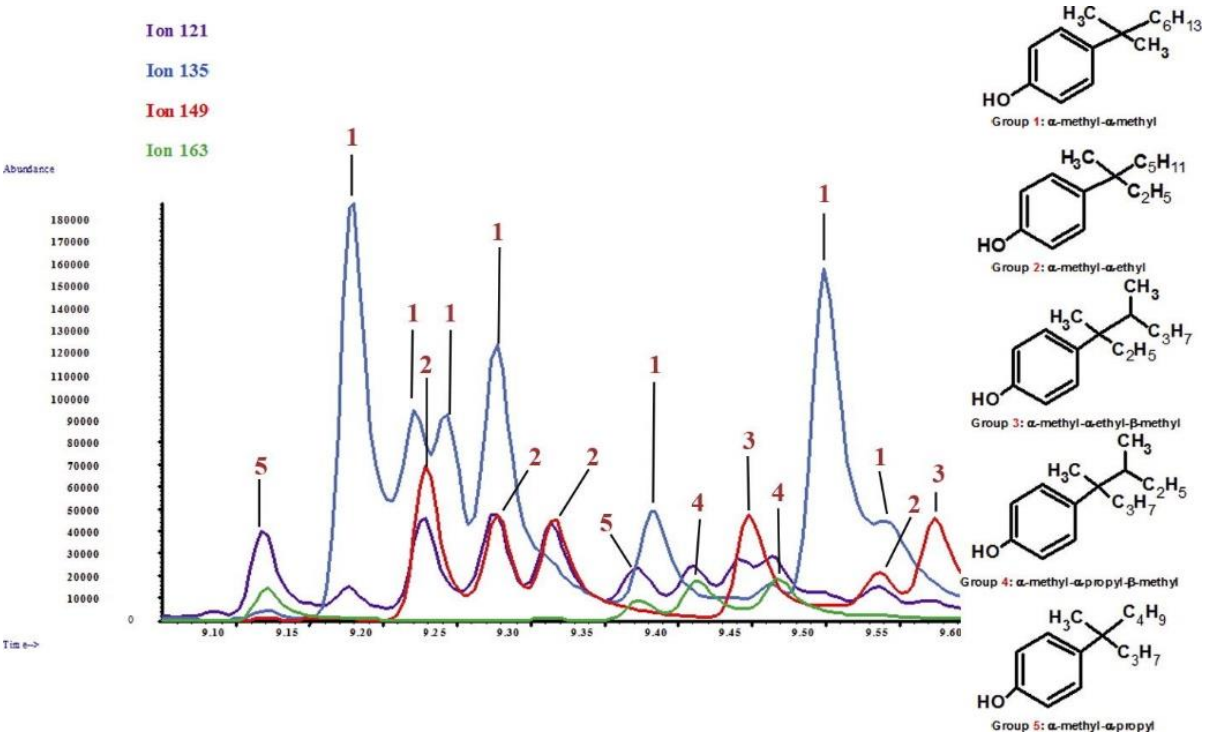

Figure 1. Ion chromatogram of structurally selected ions (*m*/*z* 121, 135, 149 and 163) for quantification of technical NP. Numbers on peaks indicate the assignment of the isomer to one of the five mass spectrometric groups (isomer structures on the right).

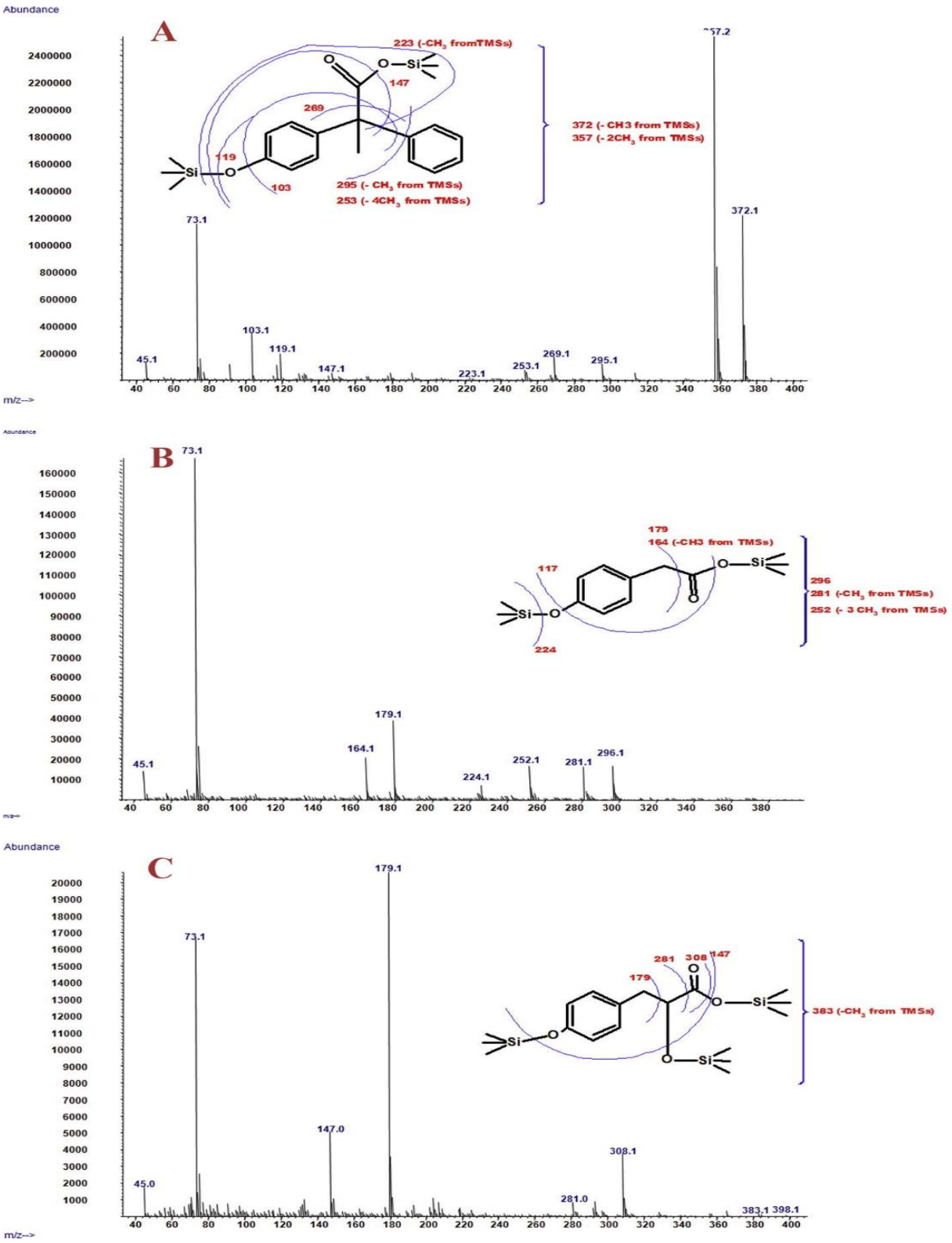

Figure 2. Examples of mass spectra analysis of selected derivatives from *U. isabellina* cultures: (A) 2-(4-hydroxyphenyl)-2-phenylpropanoic acid, (B) 2-(4-hydroxyphenyl) acetic acid, (C) 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid. In all ion chromatograms, *m/z* 45 originates from the carboxyl group, and *m/z* 73 originates from TMS.

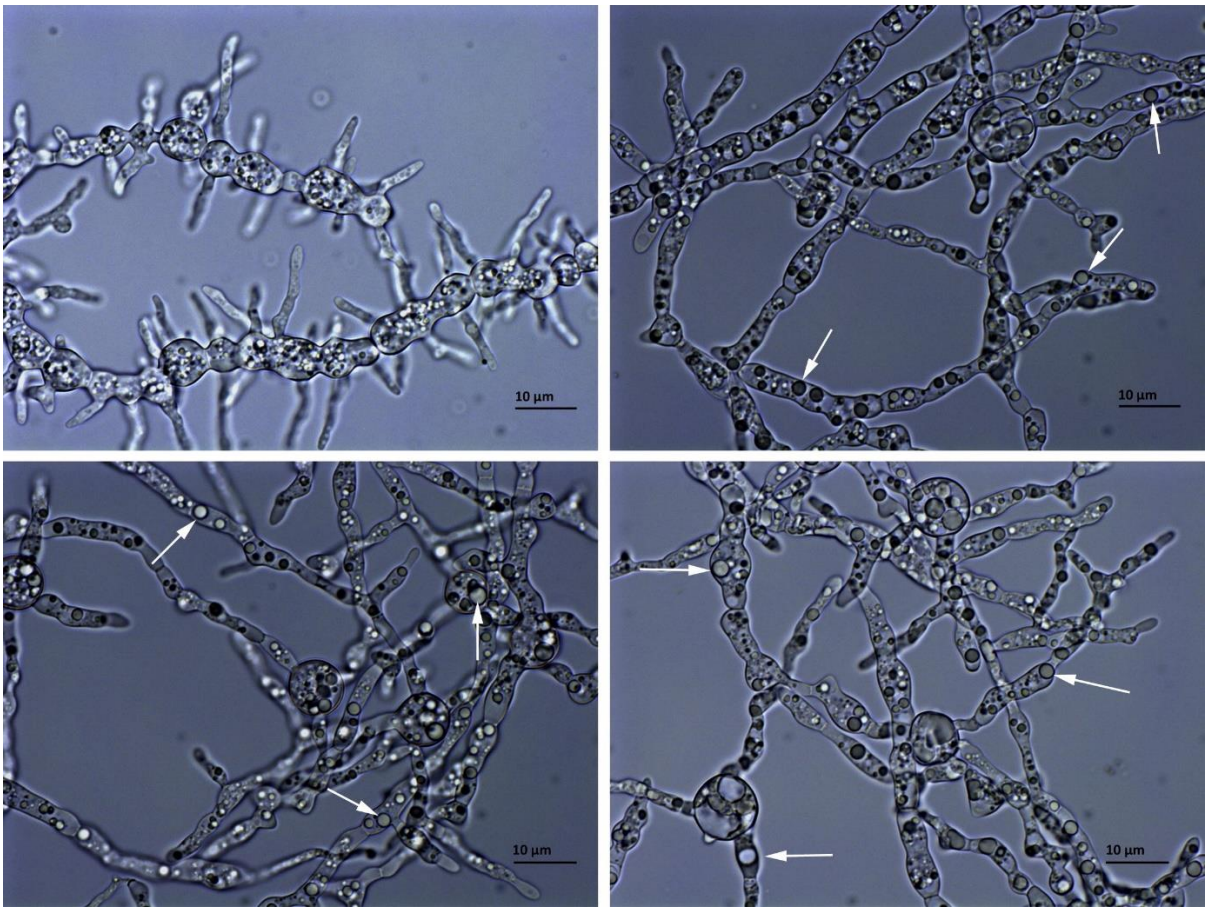

Figure 3. *U. isabellina* photographs after 6 h of incubation on Sabouraud medium without xenobiotics (A) and with tNP (B), 4-CP (C) and 4-*t*-OP (D). Examples of morphological changes are marked by arrows.

Artykuł naukowy P2

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Detoxification and simultaneous removal of phenolic xenobiotics and heavy metals with endocrine-disrupting activity by the non-ligninolytic fungus *Umbelopsis isabellina*

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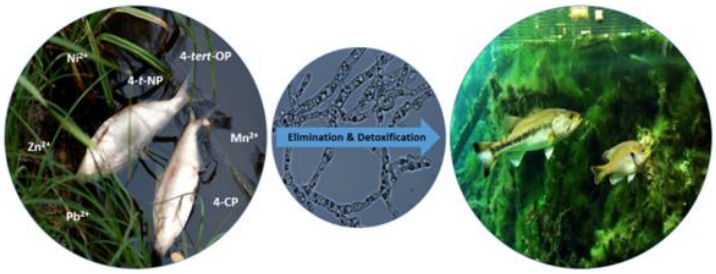
GRAPHICAL ABSTRACT



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ABSTRACT

Organic and inorganic pollutants well known to interfere with the major functions of the endocrine system co-occur widely in contaminated ecosystems. The aim of the study was to evaluate the ability of *Umbelopsis isabellina* fungus to simultaneously remove and detoxify multiple environmentally significant endocrine disruptors: the heavy metals Cd(II), Zn(II), Mn(II), Pb(II) and Ni(II) and the phenolic xenobiotics nonylphenol (*t*-NP), 4-cumylphenol (CP) and 4-*tert*-octylphenol (4-*t*-OP). The effects of the metals on fungal growth and efficiency of single-metal uptake were also investigated. *U. isabellina* exhibited considerable tolerance to Zn(II), Mn(II), Pb(II) and Ni(II), with IC50/24 values ranging from 5.08 for Ni(II) to 13.1 mM for Zn(II). In the presence of CP, the maximum efficiency of Pb(II) removal increased 25% relative to that of the control. Supplementation with Mn (II) or Zn(II) enhanced the 4-*t*-OP degradation by 18 or 9%, respectively, after 6 h of cultivation. Ecotoxicological assays monitoring bioindicators from different aquatic ecosystems revealed detoxification coinciding with the removal of metals and organic xenobiotics from binary mixtures. This work indicates the potential of a single microorganism, *U. isabellina*, to remove both heavy metals and organic xenobiotics from co-contaminated sites, making it a suitable candidate for the development of bioremediation strategies.

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1. Introduction

One of the most serious environmental hazards is posed by chemical factors that affect the endocrine system. Numerous pollutants commonly known as endocrine-disrupting compounds (EDCs) have been recognized to modulate endocrine functions by interfering with the biosynthesis, transport, release or activity of endogenous hormones [1,2].

Technical-grade nonylphenol (*t*-NP), 4-cumylphenol (CP) and 4-*tert*-octylphenol (4-*t*-OP) are typical phenolic xenobiotics possessing endocrine-disrupting activities, mainly known to cause oestrogen-like effects [3,4]. They are widely used as plasticizers, surfactants, detergents, emulsifiers and preservatives in many domestic, agricultural and industrial applications [5–7]. The exposure of wildlife to *t*-NP, CP and 4-*t*-OP was demonstrated to cause multiple harmful effects such as disorders of homeostasis, growth, sexual development and reproductive functions [5,8,9]. Furthermore, some reports suggest that these chemicals can also affect human health by inducing hormone-dependent cancers and by disrupting thyroid function or neurobehavioural development [9–12].

Heavy metals are also known to cause a variety of harmful effects on living species [13]. Manganese (Mn), zinc (Zn), nickel (Ni), lead (Pb) and cadmium (Cd) have attracted much attention as some of the most toxic heavy metals found in polluted environments. All of them have been listed in the ATSDR (Agency for Toxic Substances and Disease Registry) report (2017 ATSDR Substance Priority List) as priority pollutants constituting serious human health hazards. Many studies have demonstrated that exposure to high levels of Mn, Zn, Ni, Pb and Cd can result in disorders of the endocrine system, in particular by modulating the oestrogenic activity of endogenous hormones [1,2,13–15]. For this reason, Zn, Ni, Pb and Cd are often termed metalloestrogens.

The co-occurrence of phenolic compounds such as NP, 4-*t*-OP, and CP with heavy metals possessing endocrine activity is widespread in polluted environments [16–19]. The main sources of the emission of these contaminants into various ecosystems are production, transportation, storage and industrial, agricultural and consumer usage of phenolic compounds and heavy metals containing products. The literature data reveals that these xenobiotics have been detected in the environment of many countries and regions on different continents. As a result of their large-scale usage, they can be found in diverse environmental compartments including: sediments, soil, water (influents and effluents of sewage treatment plants, lakes, rivers, marine areas) and biota samples. All organic and inorganic pollutants used in this work were detected i.a. in the Dianchi Lake (China) and rivers flowing into it in China (in both surface water and sediments) [20–22]. Moreover, phenolic compounds were also identified in fish muscle samples collected from one of these rivers [20]. The coexistence of heavy metals, 4-*tert*-OP and NP was also observed in sediments from the Dokai Bay, Japan [23]. In Europe, Ni(II), Pb(II), Zn(II), Mn(II) and phenolic compounds with endocrine activities were reported to simultaneously occur i.a. in the Elbe River (Germany) and Jarama River (Spain) [24–27]. Most of these xenobiotics have been also detected in municipal wastewater treatment plant effluents in Austria, whereas in the USA the tested contaminants were found e.g. in drinking water samples [28,29]. Therefore, the simultaneous removal of these toxicants in remediation processes is highly important for the protection of both the environment and human health. Moreover, the combined toxicity of such co-contaminated sites is often higher than that of areas contaminated with a single pollutant [30–32]. The treatment of such multi-polluted sites is a complex problem because of the mixed nature of the contaminants, which often necessitates using different purification methods [17,33]. Accordingly, remediation techniques for the effective simultaneous elimination of organic and metallic pollutants are needed [17,33,34].

There are several various physical and chemical techniques used for the treatment of co-contaminated environments such as: chemical precipitation, electrochemical processes, membrane filtration, solvent extraction. Heavy metals and organic pollutants removal methods also include coagulation, flocculation, reverse osmosis, ion exchange or ozone oxidation. However, these conventional physicochemical methods can be inefficient, time-consuming, highly expensive and often less than eco-friendly due to their high reagent requirements and secondary waste generation [34–37]. To overcome these difficulties, many recent studies have focused on the biotechnological potential of microorganisms to eliminate toxicants [31,38–40].

However, to date, limited information is available on the simultaneous removal of heavy metals and phenolic pollutants by fungi in co-contaminated environments [17,38]. Although some studies have addressed the elimination of particular metals and organic xenobiotics from binary mixtures [31,33], the removal of multiple heavy metals and organic toxicants from these environments by a single fungal strain has rarely been investigated and usually refers to the elimination of polycyclic aromatic hydrocarbons (PAHs) coexisting with randomly selected metals [16,17,38]. Moreover, the number of studies reporting toxicity changes during the simultaneous bioelimination of metals and organic xenobiotics is limited.

Our previous work demonstrated the ability of the Zygomycota fungus *Umbelopsis isabellina* to degrade phenolic xenobiotics that disrupt the endocrine system: 4-CP, *t*-NP and 4-*t*-OP [5]. The aim of this study was to evaluate the resistance of this fungal strain to heavy metals with endocrine activities and to investigate its capability of metal removal by biosorption and/or bioaccumulation mechanisms. To the best of our knowledge, the combined effects of particular phenolic xenobiotics and heavy metals with endocrine activities on the removal of both these inorganic pollutants and organic compounds by filamentous microscopic fungi has not been previously investigated. The presented research examines the influence of 4-CP, *t*-NP and 4-*t*-OP on the efficiency of heavy metal removal by *U. isabellina.* An attempt was also made to assess the effect of heavy metals on the biodegradation of the tested xenobiotics when both were present in the culture medium. To estimate the potential toxicity reduction accompanying bioelimination processes in the cultures treated with single metallic and single organic compounds, ecotoxicological assays (Daphtoxkit F and Artoxkit M) were also conducted. As a result, this paper, for the first time, provides evidence for the occurrence of detoxification processes during the simultaneous removal of phenolic organic and inorganic EDCs by a microscopic fungal strain. This work indicates a possible of *U. isabellina* fungus in the simultaneous elimination of heavy metals and phenolic pollutants with endocrine activity from co-contaminated environments which makes this strain a promising biological tool offering perspectives for practical applications in bioremediations processes.

2. Materials and methods

2.1. Microorganisms and growth conditions

The pure culture of *U. isabellina* used in this study originated from the Department of Industrial Microbiology and Biotechnology, University of Lodz, Poland (collection number: IM833). Spores from 10-12-day-old cultures incubated on ZT slants at 28 °C were used to prepare the inoculum. The fungus was inoculated as a conidial suspension (initial concentration of 5×10^7 spores mL^{-1}) in 100 mL Erlenmeyer flasks containing 30 mL Sabouraud medium (Difco). The cultivation of *U. isabellina* was carried out for 24 h at 28 °C on a rotary shaker at 160 rpm.

2.2. Effect of the initial metal concentration on fungal growth

The degree of resistance of *U. isabellina* to heavy metal ions was determined based on the growth of the fungus in the presence of metals and was defined as an IC50 and IC10 values (i.e., the metal ion concentration that inhibits 50 and 10% of the fungal growth based on

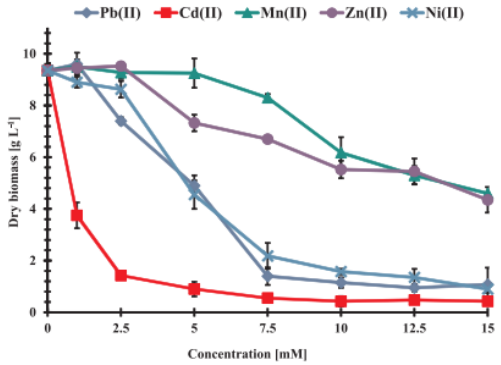

Fig. 1. Effects of different heavy metal ion concentrations on the growth of *U. isabellina* after 24 h of cultivation.

biomass production). The metal concentrations that reduced the fungal growth by 50 and 10% were determined from the linear portion of each curve by regression analysis (Fig. 1). Metal stock solutions were prepared by dissolving their nitrate salts, $Mn(NO_3)_2$ x $4H_2O$, $Zn(NO_3)_2$ x $6H_2O$, $Ni(NO_3)_2$ x $6H_2O$, $Pb(NO_3)_2$, and $Cd(NO_3)_2$ x $4H_2O$, in Milli-Q water and then sterilized by filtration with a syringe filter of 0.22 µm pore size. The stock solutions of individual metals were added separately to Sabouraud medium (100 mL) to obtain 2.5–15 mM concentrations of each heavy metal. The growth experiments were performed in flasks containing fresh medium inoculated with 15% homogenous 24-h-old fungal cultures (obtained as described in Section 2.1). The fungus was cultivated on a rotary shaker (160 rpm) at 28 °C for 24 h. At the end of the experiments, the biomass was harvested by filtration through Whatman No. 1 filter paper and then washed twice with Milli-Q water and dried at 85 °C to obtain a constant weight.

2.3. Metal bioaccumulation and biosorption analysis

The flasks were inoculated and incubated as described in Section 2.2. Based on the data on the growth of *U. isabellina* in the presence of heavy metals, a concentration of each metal corresponding to the IC50 was selected for the biosorption and bioaccumulation studies. Un-inoculated culture medium containing the same concentration of heavy metals was prepared as a control. The metal ion concentrations in dried fungal biomass were measured by atomic absorption spectroscopy (AAS). Dry weight of the biomass was obtained as described in Section 2.2. The biosorption process was estimated by the desorption of metals from previously dried biomass with HCl as the eluent. The HCl-mediated metal desorption was performed by placing 100 mg of metal-loaded biomass in 50 mL of 0.1 M HCl on a rotary shaker at 160 rpm for 30 min at 28 °C. After the desorption step, the biomass was separated by filtration through a Whatman no. 1 filter paper, then washed 3–4 times with Milli-Q water and again dried as described above. Each experiment was repeated three times in three independent test series.

2.4. Sample digestion procedures

A Multiwave 3000 microwave system equipped with a 16HF100 rotor (Anton Paar, Austria) was used for the total digestion of the biomass. The dried fungal biomass (approximately 100 mg) was placed directly in a vessel made of PTFE-TFM, followed by the addition of a HNO_3/$HClO_4$ mixture (4:1). The details of the microwave heating programme are given in Table 1. After digestion, the samples were diluted to 50 mL with Milli-Q water.

Table 1
Operating conditions for microwave digestion.

Step	Power (W)	Time (min)	Fan
1	1200	50	1 (14.4 L s^{-1})
2	0	15	3 (43.8 L s^{-1})

2.5. Metal determination

The metal ion concentrations were quantified by the use of a 240FS AA atomic absorption spectrophotometer equipped with a SIPS 20 sample introduction pump system (Agilent, USA). The metal biosorption/bioaccumulation capacity was calculated as follows: $Q = [(C_i - C_f) / m] V$, where Q is the biosorption/bioaccumulation capacity of metal ion uptake per gram of biomass (mg g^{-1}); C_i is the initial concentration of metal in solution (mg L^{-1}); C_f is the final concentration of metal in solution after the biosorption/bioaccumulation process (mg L^{-1}); m is the amount of dry biomass (g); and V is the volume of the liquid medium (L). The determinations were performed in triplicate.

2.6. Xenobiotic elimination and metal ion removal experiments

The sterilized metal stock solutions were added to the medium to obtain metal concentrations equal to their IC50 (single metal treated cultures and binary mixture) or IC10 values (multi metal and xenobiotic system). Each of the tested xenobiotics (stock solutions 5 mg mL^{-1}, dissolved in DMSO) was individually added to the same shake flask to an initial concentration of 25 mg L^{-1}. The amounts of phenolic compounds used in the experiments were the same as applied in the our previous research concerning separate CP, *t*-NP and 4-*tert*-OP degradation by *U. isabellina* and it corresponded to the concentration, which inhibited fungal growth no more than 50% compared to biomass production in cultures without the xenobiotics [5]. All removal experiments were conducted as described in Sections 2.2 and 2.3. The concentrations of xenobiotics in the extracts from the fungal culture were measured by a 7890 A gas chromatograph equipped with a 5975C mass spectrometer (Agilent, USA). The extraction procedures and quantitative analysis of xenobiotics were performed according to previously described methods [5]. All experiments were conducted in three independent tests and each of them was performed in triplicate.

In order to improve the separation of metals and organic xenobiotics, pH modification (pH ≤ 2) at the mineralization step (metals) and extraction step (phenolic compounds) was performed. In the batch experiments, the tested metals recoveries ranged from 92 to 95% for uninoculated culture media and from 90 to 94% for fungal cultures. Whereas, the *t*-NP, 4-*tert*-OP and CP recoveries were approximately 92–96% for abiotic controls and 91–95% for the *U. isabellina* cultures until the end of experiment, i.e. 24 h of incubation.

2.7. Toxicity assays

The ecotoxicity effects of filtrates from fungal cultures treated with heavy metal ions and xenobiotics were analysed by using acute toxicity tests with bioindicators representative of different aquatic ecosystems: *Artemia franciscana* (marine organism) and *Daphnia magna* (freshwater species). The biotests were performed with the commercial test kits Artoxkit M and Daphtoxkit F (MicroBioTest Inc., Belgium) in accordance with the standard protocols recommended by the producer. Three independent assays were performed by triplicate and the test results were expressed as the LC50. The lethal concentration (in % dilution) after 24 h (*A. franciscana*) and 48 h (*D. magna*) of exposure were then converted to toxic units (TU = $1/LC_{50}$ x 100).

Table 2
Inhibitory concentrations of tested heavy metal ions causing a 10 and 50% reduction in the growth yield of *U. isabellina* [IC10 and IC50] compared with that of the untreated control after 24 h of cultivation.

Metal	$IC50_{/24}$ [mM]	$IC10/_{24}$ [mM]
Zn(II)	13.10	3.11
Pb(II)	5.04	2.70
Mn(II)	11.15	3.02
Ni(II)	5.08	2.55

2.8. Statistical analysis

Results are given as the mean values ± standard deviation (SD). Statistical analyses of the data were performed with the SPSS statistical package for Windows (version 17), and the means were examined by one-way analysis of variance (ANOVA). Duncan's multiple range test was used to compare the differences in all treatments, and P values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Growth and metal uptake

The degree of resistance of *U. isabellina* to heavy metals was estimated based on the measurement of growth inhibition in metal-treated cultures. Moreover, to analyse metal-specific changes in the growth pattern of *U. isabellina*, the IC50 and IC10 values were calculated (Table 2). As shown in Fig. 1, the growth pattern of *U. Isabellina* varied with the addition of different individual heavy metals. Lower biomass production compared with that of the control was observed in cultures supplemented separately with each heavy metal at corresponding concentrations, showing that the toxicity of the tested metals to *U. isabellina* decreased in the order Cd(II) > Pb(II) = Ni(II) > Mn(II) = Zn (II). Considering the metal concentration exposure, Cd(II) had the greatest adverse effect on the biomass yield of *U. isabellina*. When the initial amount of this metal in the medium was increased to 2.5 mM, the growth decreased significantly (approximately 87% in comparison with that of the control). These results corroborate other studies of fungal resistance to Cd(II), which is generally indicated to be one of the metals most toxic to all fungi [34]. A similar substantial inhibition effect of Cd (II) on fungal growth was also previously reported for other Zygomycota strains: *Mucor* sp. CBRF59 and Ascomycota *Paecilomyces marquandii* [34,41]. Based on the presented results, Zn(II) and Mn(II) have been found to be the least toxic to *U, isabellina.* In the cultures supplemented with the highest initial concentration of these metals (15 mM), the biomass amount was reduced to approximately 46 for Zn and 48% for Mn(II) after 24 h of cultivation. A similar relation between the inhibitory effects of Mn(II) and Zn(II) on fungal growth was reported for *Mucor circinelloides*, but this strain exhibited approximately 5-fold lower resistance to Mn(II) and Zn(II) exposure than the newly tested strain [42]. The growth of *U. isabellina* was also significantly influenced by Ni(II) and Pb(II) at their respective initial concentrations. A biomass reduction of approximately 55% compared with that of the control was observed in cultures supplemented with 5 mM Pb(II) or Ni (II). These findings indicate that *U. isabellina* exhibited greater physiological responses and higher tolerance mechanisms to Pb(II) and Ni (II) stress than many filamentous fungi that have been described in other works as Pb(II)- and Ni(II)-resistant. In comparison with this study, higher inhibition of the fungal growth was reported for *P. marquandii* and *Acremonium* sp. (approximately 1.5 and 0.4-fold, respectively) as a result of exposure to 5 mM Ni(II) [41,43]. Lower fungal tolerance to Pb(II) was also observed in *Lasiodiplodia* sp. $MXSF_{31}$ (approximately 1.5-fold), *Paecilomyces fumosoroseus* 4099 (approximately 2-fold) and *Penicillium simplicissimum* (approximately 2.2-fold) that in *U. isabellina* [44–46]. Overall, our research demonstrated that the Zygomycota *U. isabellina* was capable of surviving at high Pb(II), Ni(II), Mn(II) and Zn(II) concentrations and exhibited high resistance to the toxic effects of these metals.

The varied toxicity responses of *U. isabellina* to the different tested metals might be due to one or more types of resistance mechanisms and/or tolerance strategies [31]. Fungi have developed various defence processes to counteract the toxic effects of heavy metals, such as efflux, extracellular precipitation, chemical transformation, cell surface adsorption and intracellular accumulation [34,47,48]. In fungal cells, the main mechanisms of tolerance to heavy metals and simultaneous metal uptake are biosorption – metabolism-independent binding to the cell surface and bioaccumulation – energy-dependent flux into the cell [34,48]. In this study, the ability of *U. isabellina* to remove tested heavy metals by biosorption and/or bioaccumulation mechanisms was investigated. For this purpose, fungal cultures were separately supplemented with each tested heavy metal at concentrations corresponding to the IC50 values (Table 2). Due to the lowest tolerance of the strain to Cd(II) among all the tested metals, biosorption/bioaccumulation studies as well as simultaneous xenobiotic elimination and metal ion removal experiments involving this metal were omitted in further work.

Table 3 shows the differences in the elimination of the tested metals, revealing that in all cases, metal sorption on the fungal cell surface was a predominant removal mechanism. One of the main constituents of the fungal cell wall are lipids containing certain functional groups that participate directly in metal binding [47]. Some reports indicate that *U. Isabellina* is among the oleaginous fungi, which can produce high amounts of lipids under stress [49,50]. These observations suggest that physicochemical interactions between metals and ligands such as ester and carboxyl groups present on the cell surface may be involved in the mechanisms of biosorption by *U. isabellina*.

In comparison to the results in the control medium, Pb(II) was the metal adsorbed by the mycelium in the highest proportion (Table 3). The total Pb(II) uptake yield was determined to be 74.3 mg g^{-1} dry fungal biomass, of which approximately 91% (67.8 mg g^{-1}) was removed by chelation on the cell surface. Our results corroborate findings from several studies describing Pb(II) as the metal most easily removable by filamentous fungi [48]. This strong biosorptive capability can be mostly assigned to the cationic properties Pb(II). In comparison to other heavy metals, the covalent index value of Pb(II) indicates its greater potential to form covalent bonds with biological functional

Table 3
Biosorption and bioaccumulation capacities of *Umbelopsis isabellina* after 24 h of culture without organic treatment (control) and with cumylphenol (CP), 4-*t*-octylphenol (4-*t*-OP) and technical nonylphenol (*t*-NP) separately.

Metal	Xenobiotic	q_s	q_a	q_t	Ct
Mn(II)	–	7.27 ± 0.52	0.13 ± 0.01	8.6 ± 0.53	35.47
	CP	6.79 ± 0.25	0.14 ± 0.01	7.93 ± 0.23	32.71
	4-*t*-OP	4.07 ± 0.51	0.35 ± 0.04	4.42 ± 0.55	18.23
	t-NP	5.11 ± 0.15	0.31 ± 0.01	5.42 ± 0.17	22.35
Ni(II)	–	5.54 ± 0.26	0.22 ± 0.16	5.76 ± 0.44	23.76
	CP	5.16 ± 0.23	0.12 ± 0.01	5.28 ± 0.23	21.78
	4-*t*-OP	1.54 ± 0.02	0 ± 0.01	1.54 ± 0.03	6.32
	t-NP	2.65 ± 0.13	0 ± 0.02	2.65 ± 0.16	10.93
Pb(II)	–	67.84 ± 1.28	6.41 ± 0.50	74.25 ± 1.78	306.28
	CP	88.07 ± 2.29	10.58 ± 0.70	98.65 ± 2.99	406.93
	4-*t*-OP	30.82 ± 1.19	0.32 ± 0.01	31.14 ± 1.20	128.45
	t-NP	35.89 ± 1.83	0.23 ± 0.02	36.12 ± 1.83	148.99
Zn(II)	–	22.86 ± 0.32	0.58 ± 0.11	23.44 ± 0.44	96.69
	CP	21.86 ± 3.66	0.48 ± 0.21	23.34 ± 3.87	96.27
	4-*t*-OP	6.28 ± 1.11	0.11 ± 0.02	6.39 ± 1.13	26.35
	t-NP	6.29 ± 0.74	0.05 ± 0.01	6.34 ± 0.75	26.15

The biosorption (q_s), bioaccumulation (q_a), total metal biosorption and bioaccumulation (q_t) values are estimated for 1 g of dry biomass (mg g^{-1}). The total specific metal ion uptake ($_{Ct}$) was determined as the amount of metal per unit of volume [mg L^{-1}].

groups present on the cell surface [48]. Moreover, the defence mechanisms involved in Pb(II) toxicity reduction might also be linked to the formation of Pb-oxalate crystals. Increased levels of oxalic acid and the occurrence of oxalate crystals on the hyphae surface in the presence of Pb(II) have been suggested as one of the most likely mechanisms of Pb(II) detoxification by filamentous fungi [16]. However, in contrast to several other reports, our results pointed to a higher Pb(II) biosorption capacity by *U. isabellina* than by many other microscopic fungi well recognized for their ability to remove Pb(II) from aqueous solutions [51]. Pb(II) removal efficiency lower than that demonstrated in our study was reported i.e. for *Mucor indicus*, *Aspergillus awamori* and *Trichoderma viride*, with values of 22, 15.6 and 10.3 mg g^{-1} dry biomass, respectively [52,53].

Regarding the removal of other tested metals, the maximum uptake capacities for Zn(II), Mn(II) and Ni(II) were lower than for Pb(II) at 23.4, 7.4 and 6.8 mg g^{-1} dry fungal biomass, respectively, where at least 96% of each metal was deposited extracellularly (Table 3). These results revealed Ni(II) to be the most resistant to microbial action. Similar findings were described by Vargas-Garcia et al. [48], who worked with *Fusarium* and *Penicillium* species, and they suggested that the lower of Ni(II) sorption compared with that of other metals might be significantly lower due to steric hindrances caused by the cationic properties of the metals. However, even though Ni(II) was the least efficiently removed by *U. isabellina*, the maximum uptake of this metal was comparable to those demonstrated in other reports [54,55].

Our results revealed that intracellular accumulation plays a negligible role in the mechanisms of removal of the tested metals. The lower potential of these fungi for intracellular uptake can be attributed to the lack or lesser expression of specific carrier proteins and to the inhibitory effect of metals on cellular processes, resulting in metabolism-dependent uptake [48]. In summary, these investigations have demonstrated that the growing *U. isabellina* strain was capable of the removal of all tested metals, indicating its suitability as a promising biosorbent for the elimination of Pb(II), Zn(II), Mn(II) and Ni(II) ions from aqueous solutions.

3.2. Simultaneous removal of metals and degradation of phenolic xenobiotics

The metal biosorption and bioaccumulation efficiency in cultures with single xenobiotic supplementation are shown in Table 3. As shown, the appearance of an organic co-pollutant in the culture medium affected the total metal uptake differently depending on the type of phenolic compound used. On the other hand, no significant differences were observed in the ratio of the amount of metal accumulated to the amount of the metal absorbed in comparison to the corresponding proportions in the control. In all tested samples, the elimination of phenolic compounds led to the formation of the same several intermediates which have been identified in our previous studies [5]. In general, the total uptake of all tested metals coexisting with NP and 4-*t*-OP separately were considerably reduced compared to those in untreated cultures (Table 3). The highest inhibition of metal removal was observed with 4-*t*-OP and ranged from 48.7% for Mn(II) to 73.3% for Ni(II). In the case of NP supplementation, the lowest metal uptake were found for Zn(II), Ni(II) and Mn(II), and decreased from 23.44, 5.76 and 8.6 in cultures with no xenobiotic to 6.34, 2.65 and 5.42 mg g^{-1} dry biomass in NP-treated cultures, respectively (Table 3). A similar phenomenon was described by Tsekova et al. [33], who found that phenol had a negative influence on Co(II) and Cu(II) biosorption by *Rhizopus archizus*. The inhibitory effect of organic pollutants on heavy metal uptake capacity could be attributed to changes in the composition and chemical-physical properties of the cell wall [35]. Bereketoglu et al. [56] reported the dysregulation of the expression of several genes involved in cell wall biogenesis and cytoskeleton organization in *Saccharomyces cerevisiae* cells exposed to NP. Moreover, some genes encoding cell wall protein were also considerably affected upon exposure to this pollution. Thus, based on our findings as well as the structural similarity between NP and 4-*t*-OP, these xenobiotics could be suggested to influence the amounts and kinds of structural compounds and other chemical activity groups on the fungal call wall, thereby contributing to a decrease in the removal capacities for all tested metals.

In contrast to NP and 4-*t*-OP supplementation, most of the tested metals in cultures with CP showed similar final elimination to those of the controls (Table 3). The only exception was Pb(II). In the presence of CP, the maximum efficiency of Pb(II) removal increased by approximately 25% compared with that in the control and reached 98.65 mg g^{-1} dry biomass (Table 3). Organic contaminants, including phenolic compounds, have been reported to enhance the removal efficiency of different metal ions by fungi in co-polluted aqueous solutions. Chen et al. [32] reported a higher capability of *P. chrysosporium* to eliminate Cd(II) in the presence of 2,4-dichlorophenol in the culture medium, and a similar behaviour was reported by Słaba et al. [57] regarding the ability of *P. marquandii* to remove Zn(II) in alachlor-treated cultures. This improvement in the biosorptive capability could be primarily explained by the influence of selected organic xenobiotics such as CP on the metabolic processes determining the structure and functioning of the fungal cell wall. One of these mechanisms could increase the amounts of specific metal-binding groups embedded in the cell surface, increasing the availability of suitable anionic sites. CP treatment might also cause a loss of cell wall integrity and thereby increase the surface area, leading to the exposure of latent Pb(II) binding ligands [35]. Additionally, our previous work showed that CP elimination by *U. isabellina* was accompanied by the formation of different biodegradation products from those identified in cultures treated with NP or 4-*t*-OP [5]. Moreover, the same metabolites have been detected in present research involving all tested cultures with the addition of CP. Hence, the possibility must be considered that intermediates of CP such as 2-(4-hydroxyphenyl) acetic acid or 2-(4-hydroxyphenyl) 2-phenylpropanoic acid could also impact the fungal cell metabolism and thereby enhance the biosorption of Pb(II) [33,38].

The efficiency of the elimination of *t*-NP, CP and 4-*t*-OP in cultures with single-metal supplementation was also examined. As shown in Fig. 2, in general, when organic xenobiotics were present together with particular metal ions, their removal decreased in the order CP > 4-*t*-OP > *t*-NP. In most cases, metal treatment slowed the elimination of the organic compounds relative to the values for in controls. Compared with cultures without metals the highest inhibition of xenobiotic removal were observed for *t*-NP (from 62% for Pb(II) to 23% for Zn(II) at the end of the experiment) and for 4-*t*-OP, whose elimination yield clearly decreased with Pb(II) supplementation, reaching 44 and 15% after 12 and 24 h of cultivation, respectively. The time necessary to degrade over 95% of 4-*t*-OP in the presence of Ni(II) was found to be within 24 h and was 12 h longer than in cultures without metal. These delays in biodegradation could have been caused primarily by the adverse impact of the tested heavy metals on many key fungal activities such as ATP production, substrate mineralization and cell surface functions that participate in the transport, biotransformation and detoxification of organic xenobiotics [38]. Moreover, many enzymes are regulated by heavy metals at the transcription level and the activity level, and therefore, in most cases, metal ions might inhibit the activities of enzymes involved in the degradation of the tested phenolic compounds [32]. One potential mechanism could reportedly involve the ability of heavy metals to denature proteins and enzymes structures [38]. Furthermore, many heavy metals also negatively affect the removal of organic co-pollutants, inducing oxidative stress associated with the production of reactive oxygen species (ROS), which among other activities, can disrupt ion regulation or directly form DNA and protein adducts [32,38]. Our results showed that Pb(II) supplementation had the greatest adverse influenced the elimination of the tested phenolic compounds. In addition, among all the metals used this work, Pb(II) was characterized by the highest bioaccumulation uptake capability. These findings suggested that one of the possible mechanisms

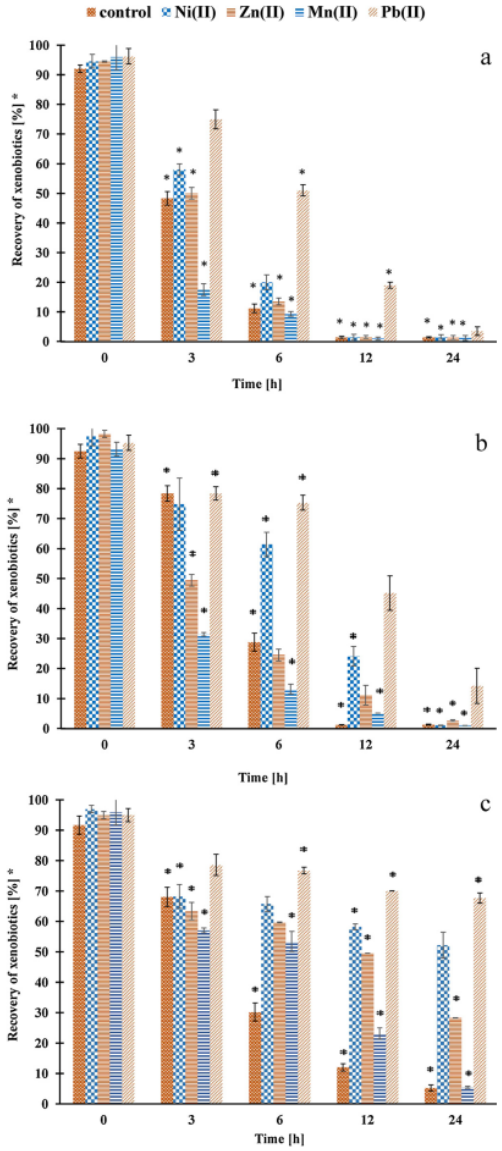

Fig. 2. Elimination rates of CP (**a**); 4-*t*-OP (**b**); and *t*-NP (**c**) by *U. isabellina* in the presence of single heavy metal ions and in the metals untreated cultures. * Data are expressed as the amount of xenobiotic recovery [%] in comparison with appropriate abiotic controls. An asterisk indicates a statistically significant differences from the control ($p < 0.05$).

involved in inhibiting CP, NP and 4-*t*-OP removal in response to Pb(II) stress could be cellular oxidative damage or inactivation of enzymes responsible for xenobiotics metabolism.

The separate addition of Pb(II) to the culture medium considerably inhibited CP removal by *U. isabellina*, resulting in 67 and 19% inhibition after 6 and 12 h of cultivation, respectively, while the opposite behaviour occurred for Mn(II) supplementation. In the presence of this Mn(II), CP removal efficiency was enhanced by approximately 18% in comparison to that of the control after 3 h of cultivation, and almost complete CP elimination (at least 98%) was observed 3 h earlier than in cultures without Mn(II) treatment (Fig. 2). Moreover, our findings indicated that individual Mn(II) or Zn(II) supplementation also increased the 4-*t*-OP degradation by *U. isabellina*, while Mn(II) yielded a higher removal than Zn(II): 9 and 18% of the original levels were observed, respectively, compared with 27% in the control after 6 h of incubation (Fig. 2). This phenomenon was similar to that reported earlier by Wu et al. [30], who found that the amount of phenanthrene elimination by macroscopic fungus *Pleurotus eryngii* was approximately 15% higher in cultures treated with 3 mM of Mn(II) than in the control. A possible explanation of these results could be the enhancement of organic xenobiotics adsorption because of changes in the cell surface properties. Some heavy metals might present stronger electrostatic attraction to the mycelium surface than phenolic co-pollutants, making the cell surface less hydrophilic and thereby promoting the adsorption of hydrophobic compounds such as 4-*t*-OP [38]. Moreover, the enhancement of organic compound degradation could also be attributed to the formulation of an enzyme-metal-substrate complex in which metals act as cofactors. Mn (II) and Zn(II) have been reported to play regulatory roles in the expression and function of enzymes involved in the biotransformation of organic pollutants, and therefore, the action mechanisms of these metals could also contribute to the degradation of the tested organic pollutants. Overall, the biodegradation capacity of some phenolic compounds used in this work was enhanced by supplementation with individual heavy metal ions.

Due to the ubiquitous co-occurrence of tested metals and phenolic compounds in different environmental compartments, the capability of *U. isabellina* to remove these pollutants from the multi metal and xenobiotic mixture was also examined. The mean removal efficiency of metals from fungal cultures co-contaminated with all tested organic xenobiotics is presented in Table 4. Concurrent CP, 4-*t*-OP, *t*-NP and metals supplementation of the culture medium was shown to inhibit the individual metal removal compared to their elimination range from the binary mixture and under single metal exposure (Table 3). In the multi metal and organic xenobiotic system, *U. isabellina* could uptake from 62.01 mg L^{-1} Pb(II) to 3.43 mg L^{-1} Ni(II). These findings suggest that these metals in mixture could interact in a synergistic manner, thereby increasing toxicity [58]. In multiple metal mixture the properties of particular metals such as atomic radius, electronegativity and atomic weight were found to significantly affect their removal efficiency by microorganisms. As indicated in Table 4 and Fig. 2, the coexistence of all organic and inorganic pollutants in culture medium also negatively influenced the ability of the tested fungus to degrade *t*-NP and 4-*tert*-OP. At the end of the experiments (i.e. after 24 h) the elimination of *t*-NP and 4-*tert*-OP by *U. isabellina* decreased by approximately 77 and 33% respectively, compared to the single xenobiotic treated cultures. These observation could result from the fact that the metals uptake occurred

Table 4
Multiple metals and xenobiotics removal capacities of *Umbelopsis isabellina* after 24 h of incubation.

Treatment	q_t	Ct	Removal efficiency (%)
Mn(II)	1.89 ± 0.10	7.20	NA
Ni(II)	0.90 ± 0.04	3.43	NA
Pb(II)	16.31 ± 1.02	62.01	NA
Zn(II)	3.26 ± 0.29	12.45	NA
CP	NA	NA	93.1 ± 2.0
t-NP	NA	NA	15.2 ± 1.3
4-*tert*-OP	NA	NA	65.3 ± 1.9

Total metal uptake (q_t) values were estimated for 1 g of dry biomass (mg g^{-1}). The total specific metal ion uptake ($_{Ct}$) was determined as the amount of metal per unit of volume [mg L^{-1}]; NA – not applicable.

Table 5
Acute toxicity to *D. magna* during the simultaneous elimination of heavy metals and organic xenobiotics by *U. isabellina* from the binary mixture.

Treatments	TU values after time of cultivation (h)					Detoxification
	0	3	6	12	24	0–24 h
Zn(II) + CP	578.0 ± 3.4	521.4 ± 3.0	513.3 ± 2.8	509.0 ± 3.7	507.2 ± 2.3	70.8
Zn(II) + *t*-NP	588.5 ± 2.3	559.7 ± 5.9	532.0 ± 2.4	554.2 ± 3.0	548.1 ± 3.1	40.4
Zn(II) + 4-*t*-OP	590.0 ± 3.7	557.3 ± 2.5	554.2 ± 2.9	550.7 ± 3.3	530.1 ± 2.7	59.9
Pb(II) + CP	132.0 ± 3.0	85.8 ± 1.6	80.3 ± 1.1	72.7 ± 2.0	70.8 ± 2.2	61.2
Pb(II) + *t*-NP	139.7 ± 3.3	104,3 ± 2.6	103.9 ± 3.5	109.3 ± 2.5	102.4 ± 3.3	37.3
Pb(II) + 4-*t*-OP	137.3 ± 2.7	104.4 ± 3.3	101.4 ± 2.4	115.4 ± 2.9	93.6 ± 2,8	43.7
Mn(II) + CP	71.4 ± 1.2	59.7 ± 2.2	55.1 ± 3.0	54.2 ± 2.2	51.8 ± 1.9	19.6
Mn(II) + *t*-NP	76.5 ± 2.0	69.1 ± 2.2	66.8 ± 2.8	63.2 ± 2.4	62.2 ± 1.0	14.3
Mn(II) + 4-*t*-OP	76.1 ± 2.0	66.2 ± 2.4	63.6 ± 1.6	61.1 ± 2.0	61.0 ± 1.9	15.1
Ni(II) + CP	58.2 ± 3.1	43.1 ± 1.1	37.9 ± 2.1	34.7 ± 2.4	32.4 ± 1.2	25.8
Ni(II) + *t*-NP	63.9 ± 2.1	60.3 ± 2.0	59.4 ± 1.8	64.1 ± 2.2	52.0 ± 2.3	10.9
Ni(II) + 4-*t*-OP	62.4 ± 2.0	61.1 ± 1.5	51.2 ± 2.0	62.2 ± 2.6	37.2 ± 2.4	25.2

prior to the phenolic substrates elimination, which led to the saturation of the active sites binding these compounds on the biomass surface [33]. In the case of CP, coexistence of all tested contaminants in culture medium exerted a minimal influence on the removal of this xenobiotic by *U. isabellina*. Comprehensive analysis concerning the simultaneous elimination of these toxicants by tested fungal strain and understanding the mechanisms of these processes require further investigations.

Although the coexistence of metals and organic substrates in culture medium negatively affected their elimination efficiency by *U. isabellina*, this strain was still able to effectively remove these pollutants from co-contaminated aqueous solutions, making it a promising candidate for bioremediation processes. The findings in this work may be crucial for the development an environmental protection technologies to be applied in the elimination of co-pollutants from aquatic ecosystems.

3.3. Ecotoxicity assessment

The metabolic biotransformation and elimination of pollutants by microorganisms can lead to the formation of products more toxic than the original compounds. Therefore, the detoxification efficiency of metals and organic chemicals should be monitored. In this study, toxicity bioassays were performed with the crustacean test species *D. magna* and *A. franciscana*, widely accepted bioindicators for acute toxicity evaluations for both single chemicals and complex environmental contaminant [5,59].

The results of ecotoxicological experiments are summarized in Tables 5 and 6. Detoxification processes occurred for all tested binary mixtures of metal and a phenolic compound, but in various proportions. In the abiotic controls, no significant toxicity changes occurred over time (data not shown), indicating that the toxicity reduction resulted from fungal action. No toxicity was reported in untreated fungal cultures (biotic controls). The bioassay results indicated differential sensitivity of the bioindicators to the tested contaminants. *A. franciscana* exhibited higher resistance to the harmful effects of the tested co-pollutants than *D. magna*. These findings were consistent with the results of our previous research concerning toxicity assessment during the individual degradation of CP, 4-*t*-OP and *t*-NP by *U. Isabellina* [5]. Moreover, compared with results from this earlier report, we also observed synergistic toxicological effects when the phenolic substrate and heavy metal ion were present together in the fungal culture. These findings corroborated the results of other studies emphasizing that combined pollutions may generate unexpected increases in toxicity in many ecosystems [60].

In general, the bioassays used in this study showed that the detoxification pattern was correlated with the observed removal of inorganic and organic compounds in almost all experimental systems. Similar observations have also been reported in other works investigating toxicity changes during the microbial elimination of co-pollutants [59,60]. In this study, the highest toxicity reduction occurred in CP-treated cultures and ranged between 70.8 and 19.6 TU for *D. magna* and between 67.1 and 15.2 TU for *A. franciscana* in the presence of Zn(II) and Mn(II), respectively, after 24 h of cultivation. On the other hand, the slowest detoxification were reported for cultures supplemented with Ni(II) and *t*-NP, whose supernatants became 5.8 and 3.4 times less toxic towards *D. magna* and *A. franciscana,* respectively, at the end of the treatment period. In all cases, the decrease in toxicity was markedly

Table 6
Acute toxicity to *A. franciscana* during the simultaneous elimination of heavy metals and organic xenobiotics by *U. isabellina* from the binary mixture.

Treatments	TU values after time of cultivation (h)					Detoxification
	0	3	6	12	24	0–24 h
Zn(II) + CP	84.3 ± 2.4	27.5 ± 2,1	20.6 ± 2.0	17.7 ± 2.6	17.2 ± 2.7	67.1
Zn(II) + *t*-NP	86.1 ± 2.9	53.4 ± 3.6	43.0 ± 2.2	54.8 ± 2.1	37.0 ± 2.1	49.1
Zn(II) + 4-*t*-OP	86.8 ± 2.7	47.3 ± 2.4	32.8 ± 2.7	33.4 ± 2.5	30.9 ± 1.8	55.9
Pb(II) + CP	43.4 ± 2.6	28.6 ± 3.0	24.1 ± 1.7	17.0 ± 2.0	14.2 ± 2.5	29.2
Pb(II) + *t*-NP	53.3 ± 2.6	39.8 ± 3.1	49.7 ± 3.2	50.7 ± 2.8	32.7 ± 3.3	20.6
Pb(II) + 4-*t*-OP	50.7 ± 2.9	34.5 ± 3.6	30.2 ± 2.8	42.1 ± 3.1	28.3 ± 2.6	22.4
Mn(II) + CP	21.1 ± 1.3	11.6 ± 1.2	7.2 ± 0.9	6.0 ± 0.9	5.9 ± 0.3	15.2
Mn(II) + *t*-NP	28.2 ± 2.2	23.4 ± 1.4	19.1 ± 2.0	17.2 ± 1.8	16.2 ± 1.8	12.0
Mn(II) + 4-*t*-OP	26.7 ± 2.7	14.0 ± 3.1	10.0 ± 1.1	9.1 ± 1.5	8.0 ± 1.2	18.7
Ni(II) + CP	32.8 ± 1.7	15.5 ± 1.8	11.4 ± 1.1	7.7 ± 1.7	6.7 ± 0.5	26.1
Ni(II) + *t*-NP	39.8 ± 1.8	38.3 ± 1.9	35.2 ± 3.1	41.4 ± 2.6	28.2 ± 2.0	11.6
Ni(II) + 4-*t*-OP	40.4 ± 2.3	37.9 ± 2.1	14.0 ± 1.9	29.3 ± 2.9	7.9 ± 1.0	32.5

faster during the first six hours of cultivation, possibly due to the intensive adsorption of metals in the initial stage of cultivation. Our results also indicated that some filtrates from 12 h cultures with *t*-NP and 4-*t*-OP showed more toxic effects towards both tested bioindicators than the supernatants after 6 h and 24 h of incubation. Thus, the increase in toxicity could be primarily explained by the appearance of more harmful *t*-NP and 4-*t*-OP intermediates in the culture medium.

Overall, the ecotoxicological analyses performed in this study clearly showed that the simultaneous removal of tested organic and inorganic pollutants by *U. isabellina* was accompanied by detoxification.

4. Conclusions

This work supplies new information on the potential application of filamentous fungi in the treatment of contaminated environments, thereby leading to a better understanding of the efficiency of bioremediation processes under realistic conditions, i.e., in cases of co-exposure to xenobiotics and heavy metals. Our research demonstrated for the first time the potential of *U. isabellina* to remove selected heavy metals from aqueous solution. Moreover, this fungus had never been previously employed to eliminate pollutants coexisting in the culture medium. Therefore, we propose the *U. isabellina* strain as an efficient candidate for the simultaneous removal of endocrine-disrupting heavy metals and phenolic xenobiotics, especially those inducing significant oestrogen-like effects. Future research should be conducted to improve the elimination capability of the fungus, primarily by the optimization of all biotic and abiotic factors influencing the removal processes. In general, both the chemical and ecotoxicological investigations in this work revealed the high potency of Zygomycota *U. isabellina* strain for the remediation of co-contaminated sites.

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Artykuł naukowy P3

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Ecotoxicological estimation of 4-cumylphenol, 4-t-octylphenol, nonylphenol and volatile leachate phenol degradation by the microscopic fungus Umbelopsis isabellina using a battery of biotests

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Abstract:	The phenolic xenobiotics nonylphenol (NP), 4- tert -octylphenol (4- t -OP) and 4-cumylphenol (4-CP) have the potential to seriously disrupt the endocrine system. Volatile phenols (VPs), especially those present in landfill leachate, also adversely affect the health of numerous organisms. Microbial degradation of xenobiotics can result in the formation of intermediates with a higher toxicity than the precursor substrates. Therefore, the main aim of this study was to assess the changes in environmental ecotoxicity during the biotransformation of NP, 4- t -OP, 4-CP and VPs by Umbelopsis isabellina using a battery of biotests. The application of bioindicators belonging to different taxonomic groups and diverse trophic levels indicated that the biodegradation process was accompanied by detoxification. Removal of 4-CP and 4- t -OP also led to a decrease in the anti-androgenic potential. Moreover, this is the first report demonstrating the anti-androgenic properties of 4-CP. The results showed that U. isabellina is an attractive tool for the bioremediation and detoxification of contaminated environments.
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Highlights

1. The battery of biotests showed lower toxicity of NP, 4-*t*-OP and 4-CP metabolites.
2. *U. isabellina* also detoxifies VPs from post-industrial landfill leachate.
3. 4-*t*-OP and 4-CP exhibit oestrogenic and anti-androgenic properties.
4. *U. isabellina* is an attractive tool for the bioremediation of contaminated areas.

Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol and volatile leachate phenol degradation by the microscopic fungus *Umbelopsis isabellina* using a battery of biotests

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Abstract

The phenolic xenobiotics nonylphenol (NP), 4-*tert*-octylphenol (4-*t*-OP) and 4-cumylphenol (4-CP) have the potential to seriously disrupt the endocrine system. Volatile phenols (VPs), especially those present in landfill leachate, also adversely affect the health of numerous organisms. Microbial degradation of xenobiotics can result in the formation of intermediates with a higher toxicity than the precursor substrates. Therefore, the main aim of this study was to assess the changes in environmental ecotoxicity during the biotransformation of NP, 4-*t*-OP, 4-CP and VPs by *Umbelopsis isabellina* using a battery of biotests. The application of bioindicators belonging to different taxonomic groups and diverse trophic levels indicated that the biodegradation process was accompanied by detoxification. Removal of 4-CP and 4-*t*-OP also led to a decrease in the anti-androgenic potential. Moreover, this is the first report demonstrating the anti-androgenic properties of 4-CP. The results showed that *U. isabellina* is an attractive tool for the bioremediation and detoxification of contaminated environments.

Keywords

Biodegradation

Detoxification

Landfill post-industrial waste

Battery of biotests

Umbelopsis isabellina

1. Introduction

Many phenolic xenobiotics are commonly found in a variety of environmental matrices

due to their wide industrial and household application. Anthropogenic activities result in the continuous release of phenolic xenobiotics into aquatic and terrestrial habitats. Among these compounds, nonylphenol (NP), 4-*tert*-octylphenol (4-*t*-OP) and 4-cumylphenol (4-CP) seriously threaten the biodiversity and function of ecosystems. During the microbial degradation of both natural wastes and organic xenobiotics, toxic volatile phenols (VPs) are formed, which may also have adverse effects on human health and wildlife. These phenols pollute various environmental compartments because they are often a harmful component of leachate from municipal and industrial landfills. Due to their incomplete biodegradation in the treatment plants, together with their toxic derivatives, VPs are frequently detected in different environmental compartments, including surface water (rivers, lakes), groundwater or agricultural lands (Oluwasanu, 2018).

The adverse effects of 4-CP, NP, 4-*t*-OP and VPs on the reproduction, growth, development and homeostasis of different organisms have been reported in several studies (Oluwasanu, 2018; Wang et al., 2016; Yu et al., 2012). Moreover, it has been recognized that these compounds interfere with the normal functioning of the endocrine system; therefore, 4-CP, NP and 4-*t*-OP were included in the categories of endocrine-disrupting chemicals (EDCs) (Wang et al., 2016; Xie et al., 2020; Zhao et al., 2021). Because of the bioaccumulation capacity of 4-CP, NP and 4-*t*-OP, their resistance to degradation and potential negative effects for both humans and wildlife, increasing attention has been given to developing effective methods for their decomposition and detoxification.

Microbial degradation is regarded as an important tool for the elimination and detoxification of xenobiotics. To estimate the biodegradation efficiency, evaluation of the toxicity reduction is a more appropriate and reliable approach than only measuring the residual concentration of the test compound during its biotransformation processes. There are many cases where microbiological degradation of xenobiotics leads to the formation of intermediates

characterized by a higher toxicity than that of the precursor compound (Costa et al., 2016; Oberoi and Philip, 2017). Moreover, antagonistic and synergistic effects due to unknown metabolite interactions may also result in enhanced toxicity. Therefore, from an ecotoxicological perspective, it is very important to use bioassays that allow for a complete evaluation of the toxicity of products resulting from biodegradation processes and prove exact information about their potential environmental impact.

Many biological tests using different organisms as bioindicators have been effectively applied to examine the ecotoxicity of various organic contaminants (Janicki et al., 2018; Kar et al., 2020; Szklarek et al., 2021). Bioindicators allow for relevant information about the biological effects of pollutants on ecosystem structure and function to be obtained.

The use of single bioassays to estimate toxicity changes in xenobiotic biodegradation processes has been reported in several studies (Janicki et al., 2018; Louati et al., 2019; Mtibaà et al., 2020). However, there is little information about the toxicity variation during the microbial biodegradation of VPs and phenolic EDCs, particularly 4-CP, 4-*t*-OP and NP. In our previous paper, we demonstrated the ability of the fungus *Umbelopsis isabellina* to efficiently degrade the phenolic xenobiotics 4-CP, 4-*t*-OP and NP (Janicki et al., 2016). By using assays with two bioindicators belonging to consumers in the trophic chain (*Daphnia magna* and *Artemia franciscana*), we have also shown that the biodegradation processes lead to toxicity reduction. The sensitivity of organisms to contaminants varies widely; therefore, the use of a battery of bioassays involving bioindicators representing different trophic levels is an effective and required tool for predicting comprehensive environmental risk (Costa et al., 2016; Martinez-Haro et al., 2015). Studies concerning a complex assessment of the total hazard posed by all the intermediates formed during microbial bioconversion by using bioindicators from different taxonomic groups remain limited. To our knowledge, the assessment of endocrine activity in the course of microbial degradation of phenolic EDCs has been rarely analysed.

96 Therefore, there is a need to find microbial strains capable of growing and metabolizing in the
97 presence of toxic pollutants and estimate the toxicological effects of intermediates along with
98 precursor contaminants to specify the efficiency of detoxification as a result of biodegradation
99 mechanisms.

100 In this context, the main aim of this study was to assess the toxicity removal efficiency
101 during alkylphenol (4-CP, NP and 4-*t*-OP) degradation by the fungus *U. isabellina* by using
102 selected bioassays covering multiple toxicological endpoints. In the present work, a
103 multispecies toxicological approach was applied by using a battery of biotests containing
104 organisms belonging to different trophic levels and exhibiting different key functions in the
105 ecosystem: decomposers, producers and consumers. Additionally, the endocrine properties,
106 including anti-androgenic activity, of selected phenols were also determined. In the face of
107 increasing environmental pollution with leachates generated from post-industrial landfills and
108 containing various toxic phenols, this work also focused on determining the possibility of using
109 the tested strain to remove and detoxify VPs from leachates loaded with complex organic
110 contaminants. Thus, for the first time, studies were conducted to assess the ability of a single,
111 non-ligninolytic fungal strain to eliminate and detoxify phenolic compounds, including EDCs,
112 both from cultures with single xenobiotic supplementation and from multi-contaminated
113 environmental matrices.

114

115 **2. Materials and methods**

116

117 *2.1. Chemicals*

118

119 NP, 4-CP and 4-*t*-OP were purchased from Sigma-Aldrich (St. Louis. Mo, USA). Other
120 solvents and reagents were of a highly pure grade and obtained from Merck (Darmstadt,

Germany). All constituents of the microbial culture media were provided by Becton Dickinson (Heidelberg, Germany).

2.2. *Landfill leachate preparation*

Landfill leachate was collected from the closed dangerous waste landfill of the former "Boruta" Dye Factory in Zgierz, Poland (Fig. S1), and kindly shared by the Voivodeship Inspectorate of Environmental Protection in Łódź, Poland. Leachate sampling was carried out in accordance with the principles contained in the accredited test procedure based on the ISO 5667-10 standard. The preservation, transport and storage of the tested matrices were performed as specified in the ISO 5667-3:2018 standard. Landfill leachate quality measurements were performed at the Main Research Laboratory in Łódź commissioned by the Voivodeship Inspectorate of Environmental Protection in Łódź, Poland, and are presented in Tables S1 and S2.

2.3. *Microorganism and growth conditions*

The filamentous fungus *U. isabellina* IM 833 was obtained from the Culture Collection of the Department of Industrial Microbiology and Biotechnology, University of Łódź, Poland. Fungal spores from 10-day-old cultures on ZT slants (Janicki et al., 2016) were used to inoculate 20 mL of Sabouraud medium (Difco) in a 100 mL Erlenmeyer flask. Cultivation was conducted at 28 °C for 24 h on a rotary shaker (150 rpm). After 24 h of incubation, precultures were transferred to fresh medium (2 mL of fungal inoculum and 18 mL of Sabouraud medium), and then NP, 4-*t*-OP or 4-CP (dissolved in DMSO, 5 mg/mL stock solution) was aseptically added to the cultures to a final concentration of 25 mg/L. Control samples containing the same volume

of ethanol (abiotic and biotic controls) were also prepared. All cultures were cultivated at 28 °C for 24 h on a rotary shaker (150 rpm). For cultures containing 20 and 40% of the landfill leachate, the incubation process was carried out for 96 h.

2.4. Biomass estimation

The dry fungal biomass was determined by filtering the whole culture through Sartorius filter membranes (0.2 µm pore size) (preweighed and predryed). After filtration, the samples were dried to a constant weight at 100 °C. All analysed samples were triplicated, and the results are expressed as gL^{-1}.

2.5. Ecotoxicological analysis

A series of biotests using bioindicators belonging to different taxonomic groups and diverse trophic levels were carried out to estimate the effectiveness of reducing toxicity by biodegradation processes (Table S3). No toxicity of the untreated fungal cultures (biotic control) was observed for all indicator species used.

2.5.1. Microorganism-based bioassays

Acute toxicity assessment was carried out on the basis of the decrease in *Aliivibrio fischeri* (DSM 7151) luminescence after exposure to the tested post-culture filtrates. The studies were performed according to a standard procedure (ISO 11348-2) using the Microtox M 500 Analyser (Modern Water, USA). Luminescence was measured before introducing the test matrices and after 15 min of incubating bacteria with the samples. After the test, the percentage

171 of bioluminescence inhibition was calculated as the EC50 value by applying MicrotoxOmni
172 software (Modern Water, USA).
173 The antimicrobial activities of the fungal cultures containing xenobiotics and their
174 degradation products were also evaluated using the multi-species microbial assay for risk
175 assessment (MARA) bioassay according to a standard protocol (NCIMB Ltd, Scotland).
176 Biotests were performed in 96-well plates containing lyophilized microorganisms (ten bacterial
177 strains and one yeast) (Table S3). The results were calculated using MARA software (ver. 3.5T)
178 and presented as microbial toxic concentration (MTC) values.
179 Growth inhibition tests using the freshwater green alga *Raphidocelis subcapitata* (SAG
180 61.81) and saltwater algae *Phaeodactylum tricornutum* (SAG 1090-1a) were conducted
181 according to the standard ISO 8692:2012 and ISO 10253:2016 methods, respectively. After 72
182 h of incubation, algal growth reduction was calculated based on the number of cells in the
183 control versus the number of cells in the treated cultures, and the results were expressed as
184 EC50 values.
185
186 *2.5.2. Plant bioassays*
187
188 The phytotoxicity assessment of post-culture filtrates was based on the germination and
189 seedling growth of one monocotyledonous (*Sorghum saccharatum*) and two dicotyledonous
190 (*Lepidium sativum* and *Sinapsis alba*) species using the Phytotoxkit biotest (MicroBioTests
191 Inc., Belgium). The experiments were carried out according to the instructions provided by the
192 manufacturer. The number of germinated seeds and root lengths of the tested plants were
193 examined using GIMP image analysis software. Inhibition of seed germination (IG) and root
194 growth (IR) were calculated using the following formula:
195

196 IG or IR = $[(A - B)/A] \times 100\%$

197

198 where A is the mean seed germination or root length in the control samples and B is the mean
199 seed germination or root length in the test post-culture filtrate samples. To assess the overall
200 germination capacity, the germination index (GI) was calculated by comparing the IG and IR
201 values using the following formula:

202

203 $GI = (Gs \times Ls)/(Gc \times Lc) \times 100\%$

204

205 where Gs and Ls are the seed germination in percent and root elongation in mm for the tested
206 samples, respectively, and Gc and Lc are the corresponding values for the control samples.

207

208 *2.5.3. Invertebrate-based bioassays*

209

210 A commercial test kit Rapidtoxkit (MicroBioTests Inc., Belgium) was used to estimate
211 the acute toxicity of post-culture filtrates with the freshwater crustacean *Thamnocephalus*
212 *platyurus*. The study evaluated the filtration and food intake of crustaceans exposed to the toxic
213 agents present in the test samples in comparison with a reference system. Based on the percent
214 inhibition of food particle uptake (a value ≥30% indicates the presence of harmful substances),
215 EC50 values were determined for the tested cultures. Toxicity levels of filtrates from fungal
216 cultures supplemented with landfill leachate (20 and 40% by volume) were evaluated using
217 Artoxkit M and Daphtoxkit F toxicity bioassays (MicroBioTests, Inc., Belgium). The results of
218 the tests were expressed as LC50 values. Lethal concentrations were determined from the linear
219 portion of each curve using regression analysis.

220 *2.5.4. Endocrine activity assay*

221 The XenoScreen YES/YAS Endocrine Disruptor Assay was used to determine the
222 oestrogenic and androgenic agonistic/antagonistic activity of the tested xenobiotics and post-
223 culture liquids. The tests were performed in accordance with the standard provided by
224 Xenometrix, Switzerland.

225 *2.6. Statistical analyses*

226 Each treatment and control group contained triplicate samples. All experimental data
227 are expressed as the mean ± standard deviation (SD). Statistical comparisons of the results
228 between the control and treated samples were performed using one-way ANOVA followed by
229 Tukey's test. Values of $p \leq 0.05$ were considered to be statistically significant. All statistical
230 analyses were performed with Microsoft Office Excel and STATISTICA 13.3 (Tibco Software
231 Inc., USA) software.

232 *2.7. Spatial analyses*

233 Maps illustrating the location of the landfill and its elements were elaborated based on
234 geographic information systems (GIS) in QuantumGIS (ver. 3.12) software and OSMStandard
235 by OpenStreetMap GIS portal with its spatial database.

236

237 **3. Results and discussion**

238

239 *3.1. Ecotoxicological assessment of biodegradation processes*

240

241 The biodegradation efficiency of the tested phenolic xenobiotics to reduce toxicity was
242 estimated by using a battery of bioassays with different types and durations of exposure, toxicity
243 endpoints and susceptibilities to specific modes of toxicant action. Bioindicator species

244 representative of various ecosystems and comprising a wide range of taxa and exposure routes
245 were applied. Analyzes of the biotests results were carried out in relation to the residual
246 concentrations of the tested xenobiotics, which were measured at different biodegradation times
247 (chemical data presented in our earlier paper (Janicki et al., 2016)).
248
249 *3.1.1. Producers*
250
251 In the present work, the freshwater algae *R. subcapitata* and saltwater diatom *P.*
252 *tricornutum*, species commonly used for ecotoxicological studies, were exposed to *U.*
253 *isabellina* post-culture filtrates supplemented with 4-CP, 4-*t*-OP and NP. As illustrated in Table
254 1, exposure of the microalgae species to the tested filtrates induced their growth inhibition
255 during the assay, with the green alga showing a higher tolerance to the fungal post-culture
256 samples than the diatom.
257
258 **Table 1** Assessment of the acute toxicity effects of *U. isabellina* post-culture filtrates with the
259 addition of 4-CP, NP or 4-*t*-OP by using different bioindicator species.

Xenobiotic	Time (h)	*P. tricornutum*	*R. subcapitata*	*T. platyurus*	*A. fischerii*
		EC50			
4-CP	0	10.1 ± 0.3	22.2 ± 0.5	3.6 ± 0.3	46.2 ± 1.6
	3	9.5 ± 0.5	19.7 ± 0.4	4.0 ± 0.5	59.0 ± 1.8
	6	10.7 ± 0.2	20.1 ± 0.4	5.0 ± 0.4	63.1 ± 2.1
	12	10.9 ± 0.7	19.8 ± 0.3	7.6 ± 0.5	70.4 ± 1.6
	24	16.1 ± 0.7	28.6 ± 0.5	9.8 ± 0.4	74.1 ± 1.5
4-*t*-OP	0	3.3 ± 0.3	4.4 ± 0.2	10.1 ± 0.7	52.6 ± 2.0
	3	4.2 ± 0.5	4.3 ± 0.3	13.2 ± 0.3	62.1 ± 1.1
	6	5.1 ± 0.3	4.5 ± 0.2	14.3 ± 0.6	71.1 ± 1.7
	12	3.8 ± 0.4	5.8 ± 0.3	14.7 ± 0.3	75.7 ± 2.0
	24	7.8 ± 0.5	6.2 ± 0.1	17.9 ± 0.7	74.2 ± 1.4

NP	0	6.5 ± 0.7	4.9 ± 0.2	4.5 ± 0.4	58.3 ± 1.5
	3	6.3 ± 0.3	5.1 ± 0.3	5.9 ± 0.4	70.8 ± 1.3
	6	6.0 ± 0.6	6.4 ± 0.3	5.6 ± 0.2	74.2 ± 1.5
	12	7.4 ± 0.4	9.1 ± 0.2	5.4 ± 0.2	65.2 ± 1.7
	24	19.6 ± 0.7	12 ± 0.4	7.9 ± 0.5	72.8 ± 2.1

Similar differences in toxin sensitivity between marine and freshwater microorganisms were noted in studies on the effect of phenolic compounds on the growth and morphology of selected algae. Additionally, these studies showed that marine algae species were more sensitive to phenolic compounds than freshwater species, which was also shown in the results obtained in the present study. The diatom, compared to the green alga, was characterized by a smaller specific surface due to the small size of the cells. This led to faster absorption of phenolic xenobiotics in its cells and greater toxicity (Duan et al., 2017). The present study showed that the EC_{50} values in the *R. subcapitata* assays performed for 24 h with *U. isabellina* post-culture filtrates treated with 4-CP, NP and 4-*t*-OP increased by 6.4, 1.8 and 7.1%, respectively, compared to the values at the beginning of incubation, thus indicating the detoxification processes occurring during the microbiological degradation of the tested xenobiotics.

A reduction in ecotoxicity was also demonstrated in tests with *P. tricornutum*, where increases in EC_{50} values from 10.1 to 16.1% for 4-CP, from 6.5 to 19.6% for NP and from 3.3 to 7.8% for 4-*t*-OP were observed for filtrates obtained from 0 and 24 h fungal cultures, respectively. Studies on toxicity changes during biological transformation and elimination of pollutants by assessing growth inhibition of various species of algae before and after treatment have also been reported in other works (Inoue et al., 2015; Suda et al., 2012; Yousefi-Ahmadipour et al., 2016).

Plants are important components of ecosystems primarily because they are the main producers of food. Therefore, they are extremely helpful in monitoring pollutants (Długoński, 2018; Wei et al., 2017). Assays based on higher plants are characterized by low costs, quick

282 test activation, and high sensitivity to a broad spectrum of contaminants; do not require
283 qualified personnel; and offer a wide variety of toxicity endpoints (Długoński, 2021; Ghosh et
284 al., 2017; Wieczerzak et al., 2016). In the present work, the phytotoxicity of the post-cultured
285 filtrates was determined on the basis of the number of germinated seeds and root lengths of the
286 tested plants. The results of contact tests with two species of dicotyledons and one species of
287 monocotyledon used in the study showed different reactions of bioindicators to intermediates
288 occurring in post-culture liquids (Table 2).

289 **Table 2** Changes in the phytotoxicity of post-culture filtrates before and after xenobiotic biodegradation processes.

Xenobiotic	Incubation	Germination inhibition (PE%)			Root growth inhibition (PE%)			Germination index (%)		
	time (h)	Sa	Ss	Ls	Sa	Ss	Ls	Sa	Ss	Ls
NP	0	100 ± 0	0 ± 0	0 ± 0	100 ± 0	59.5 ± 4.0	72.5 ± 3.0	0 ± 0	40.5 ± 1.8	27.5 ± 2.2
	3	80 ± 5	0 ± 0	0 ± 0	78.9 ± 3.5	42.8 ± 2.6	67.1 ± 2.9	4.2 ± 1.1	57.2 ± 2.5	32.9 ± 1.9
	6	90 ± 3	10 ± 3	0 ± 0	88.6 ± 2.8	25.3 ± 2.7	60.3 ± 3.3	1.4 ± 0.2	67.0 ± 3.0	39.8 ± 2.5
	12	100 ± 0	0 ± 0	0 ± 0	100 ± 0	25.5 ± 4.5	54.5 ± 2.0	0 ± 0	74.7 ± 3.6	45.6 ± 2.8
	24	90 ± 3	0 ± 0	0 ± 0	90.1 ± 3.1	21.0 ± 3.5	54.1 ± 3.5	1.0 ± 0.2	79.0 ± 2.8	45.9 ± 2.2
4-*t*-OP	0	100 ± 0	0 ± 0	0 ± 0	100 ± 0	65.3 ± 3.1	92.1 ± 3.5	0 ± 0	34.8 ± 1.7	11.7 ± 1.0
	3	90 ± 5	0 ± 0	0 ± 0	99,2 ± 1.0	65.8 ± 2.5	87.1 ± 4.1	0,07 ± 0.1	34.2 ± 1.1	12.9 ± 1.7
	6	100 ± 0	0 ± 0	0 ± 0	100 ± 0	59.9 ± 2.6	87.6 ± 2.6	0 ± 0	40.1 ± 1.0	12.4 ± 1.5
	12	90 ± 5	0 ± 0	0 ± 0	97.7 ± 1.0	56.0 ± 2.6	83.2 ± 3.0	0.23 ± 0.1	44.0 ± 1.5	16.7 ± 1.8
	24	100 ± 0	0 ± 0	0 ± 0	100 ± 0	51.1 ± 2.0	81.4 ± 2.2	0 ± 0	48.9 ± 1.8	18.5 ± 1,5
4-CP	0	90 ± 3	0 ± 0	0 ± 0	98.9 ± 1.2	59.9 ± 4.2	84,4 ± 4.1	0.22 ± 0.1	40.6 ± 3.6	18.6 ± 2.2
	3	90 ± 3	0 ± 0	10 ± 3	95.8 ± 2.7	55.5 ± 3.0	81.1 ± 3.6	0.42 ± 0.1	44.5 ± 2.1	18.9 ± 3.0
	6	90 ± 0	0 ± 0	0 ± 0	95.2 ± 3.8	55.5 ± 3.5	77.3 ± 3.0	0.48 ± 0.1	44.5 ± 2.1	22.8 ± 2.5
	12	100 ± 0	0 ± 0	0 ± 0	100 ± 0	48.5 ± 4.0	76.9 ± 3.3	0 ± 0	51.5 ± 3.8	23.1 ± 2.6
	24	90 ± 3	0 ± 0	0 ± 0	95.3 ± 3.4	36.5 ± 3.2	63.9 ± 3.6	0.45 ± 0.1	63.5 ± 4.2	36.0 ± 3.1

290 Ss – *S. saccharatum*

291 Ls – *L. sativum*

292 Sa – *S. alba*

293 Numerous data indicate that dicotyledons are more sensitive to toxic substances than
294 monocotyledons (Antonkiewicz et al., 2019; Baran and Tarnawski, 2013; Szara et al., 2020).
295 These findings are in agreement with the results obtained in this work for watercress and
296 sorghum, which reveal that *L. sativum* is a more sensitive species for assessing changes in
297 ecotoxicity during biodegradation processes than *S. saccharatum*. IR values ranged from 54.5
298 to 92.1% and from 21.0 to 65.8% for watercress and sorghum, respectively, whereas the GI
299 values varied within the range from 11.7 to 45.9% (*L. sativum*) and from 34.2 to 79.0% (*S.*
300 *saccharatum*) during the whole biodegradation process.

301 Generally, on the basis of the biotests with watercress and sorghum, after 24 h of
302 incubation in all tested xenobiotic-fungus systems, a significant decrease in phytotoxicity was
303 observed compared to the beginning of the experiments (Table 2). These results corresponded
304 with the chemical data presented in our previous work (Janicki et al., 2016), indicating a
305 positive correlation between the amount of the analysed xenobiotics during the *U. isabellina*
306 cultures and the values of the tested endpoints in *L. sativum* and *S. saccharatum*.

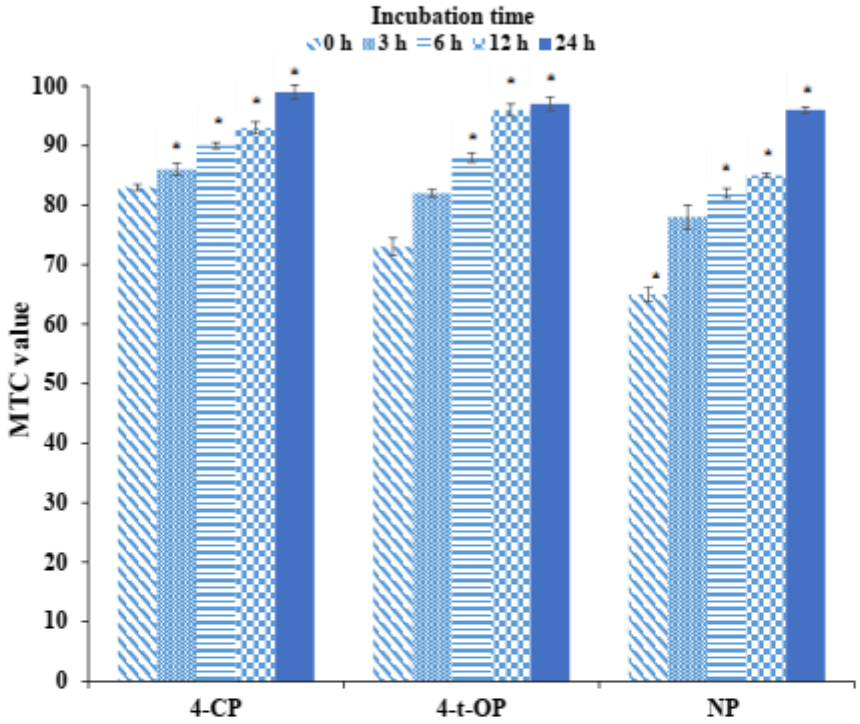
307 Considering the use of mustard in ecotoxicity tests, many studies point out that *S. alba*
308 is a species with higher resistance to the presence of many organic and inorganic pollutants than
309 other bioindicator plants (Baran and Tarnawski, 2013; Szara et al., 2020). However,
310 unexpectedly, the obtained results showed that mustard was the most sensitive test plant to the
311 contaminants present in the post-culture filtrates. Compared to the controls, for all fungal
312 cultures supplemented with xenobiotics, 90 to 100% IG and 80 to 100% IR in *S. alba* tests were
313 observed. In other studies assessing the phytotoxicity of soil from petrochemically
314 contaminated sites, for several tested matrices, *S. alba* was found to be the most resistant species
315 to soil chemicals belonging to the BTEX and PAH groups (Gworek et al., 2018).

316 Our phytotoxicity data confirm that tolerance to stressors such as xenobiotics is a
317 species-specific trait. The contaminants present in the fungal cultures underwent continuous

318 biotransformation processes. These mechanisms significantly determined the bioavailability of
319 the intermediates and thus their toxicity, which turned out to be the highest for mustard. Taking
320 into account the obtained results, it seems interesting to carry out further analyses to determine
321 the chemical profile of the filtrates to better understand the ecotoxicological response of *S. alba*
322 to intermediates formed during 4-CP, NP and 4-*t*-OP biodegradation. As a result, this species
323 may become a key bioindicator of the toxicity of derivatives resulting from the degradation of
324 phenolic xenobiotics.
325
326 *3.1.2. Consumers*
327
328 In our previous studies, we applied two species of crustaceans, *D. magna* and *A.*
329 *franciscana*, to evaluate changes in toxicity during microbial degradation of xenobiotics, and
330 the mortality of the test organisms was used as the toxicity endpoint (Janicki et al., 2016). In
331 the present work, we extended the study to a test that allows for rapid assessment of toxicity
332 with the use of short-term exposure to *T. platyurus* larvae.
333 Our research showed that exposure of *T. platyurus* to post-culture filtrates resulted in
334 inhibition of food ingestion particles by crustaceans in all tested systems, indicating the toxic
335 nature of the contaminants present in the treated samples (Table 1). Nevertheless, based on the
336 Rapidtoxkit test results, we found that after 24 h of incubation of fungal cultures supplemented
337 with the tested xenobiotics, the toxicity of post-culture filtrates decreased 2.7-, 1.8- and 1.7-
338 fold for 4-CP, 4-*t*-OP and NP, respectively. These results clearly show the effectiveness of
339 biodegradation processes in reducing ecotoxicity from *U. isabellina* cultures, which was also
340 observed in our previous studies for other species of bioindicators belonging to consumers
341 (Janicki et al., 2016). Several previously published papers also demonstrated a decrease in
342 sample toxicity for *T. platyurus* during biological and physicochemical methods of pollutant

343 decomposition, e.g., active sludge treatment of wastewater originating from tank truck cleaning
344 or photodegradation of aerucyclamide A (Dries et al., 2014; Sha et al., 2021).
345
346 *3.1.3. Decomposers*
347
348 One of the most sensitive microbiological bioassays is based on the measurement of the
349 bioluminescent activity of the marine bacteria *A. fischeri*. Data on the toxicity of post-culture
350 filtrates for *A. fischeri* during the fungal elimination of 4-CP, 4-*t*-OP and NP are shown in Table
351 1. Bioluminescence readings revealed a decrease in toxicity values during the biodegradation
352 processes for all xenobiotics tested (27.9, 23.6 and 14.5% for 4-CP, 4-*t*-OP and NP,
353 respectively) at the end of the experiment. The rate of toxicity decline was correlated with the
354 degree of xenobiotic biotransformation demonstrated in our earlier paper (Janicki et al., 2016).
355 These results suggested that fungal degradation led to metabolites that were less toxic than the
356 precursor compounds. It is considered that along with the enhancement of the lipophilic
357 character of compounds, their ecotoxicity increases, which strongly inhibits cellular respiration
358 and disturbs bioluminescence (Martínez-Ávila et al., 2021; Mtibaà et al., 2020). Considering
359 the structure of the intermediates we identified earlier, it is suggested that the processes of NP,
360 4-*t*-OP and 4-CP biotransformation by the fungus (aliphatic chain reduction, hydroxylation) led
361 to the formation of more polar derivatives exhibiting reduced toxicity. More hydrophilic and
362 less toxic metabolites than the precursor compounds in the *A. fischeri* test were also noted
363 during the degradation of NP by the fungus *Thielavia* sp HJ22 and polychlorinated biphenyls
364 (PCBs) by *Pleurotus ostreatus* and *Irpex lacteus* strains (Mtibaà et al., 2020; Stella et al., 2017).
365 The toxicity of by-products formed during the biodegradation of the tested xenobiotics
366 by *U. isabellina* was also assessed using the multi-species MARA test. The species used in this
367 assay show different sensitivities to toxicants, and the results obtained give a unique pattern of

368 the microbial growth response (toxic fingerprint) to the tested samples. The data received from
369 the MARA test are summarized in Fig. 1.



370

371 **Fig. 1** Mean MTC values in the MARA test with 4-CP, NP and 4-*t*-OP fungal culture exposure.
372 An asterisk indicates a statistically significant differences from the control ($p \leq 0.05$).

373

374 Additionally, MTC values for each strain are represented in Fig. S2. The dose-response
375 relationship of the different MARA species exposed to the post-culture filtrates was different
376 for each of the xenobiotics tested. Such findings are typical of the MARA bioassay, as they
377 reflect the wide genetic diversity of the species and thus significant differences in susceptibility
378 among microorganisms. Exposure to fungus-treated xenobiotic-polluted media resulted in the
379 growth reduction of almost all tested strains compared to negative controls.

380 The most sensitive microorganism to the cultures supplemented with NP and 4-*t*-OP
381 was *B. diminuta*, while the strongest effect of 4-CP-treated cultures on growth inhibition was
382 observed for *C. testosteroni*. *S. rubidaea* and *C. freundii* were also susceptible to the tested
383 filtrates. The obtained data correspond with previously presented studies showing that these
384 strains were characterized by high sensitivity among MARA species to the presence of other
385 toxicants, e.g., naproxen for *B. diminuta* or doxycycline for *S. rubidaea* and *C. freundii* (Baran
386 et al., 2018; Górny et al., 2019). Relatively low MTC values and, consequently, higher adverse
387 effects of the tested pollutants on the activity of microorganisms commonly found in water and
388 soil ecosystems indicate that these species may be useful markers of treatment efficiency
389 environments contaminated with both phenolic xenobiotics and their derivatives. The results of
390 the MARA assay showed that *P. aurantiaca* was the most tolerant microorganism to the toxic
391 effect of post-culture filtrates. Similar findings were noted during the exposure of the strain to
392 naproxen (Górny et al., 2019).
393 The results indicate that the average MTC values obtained for the post-cultured filtrates
394 increase with increasing incubation time of *U. isabellina* with the tested toxicants. This finding
395 indicates, similar to other biotests, that the intermediates formed during biotransformation are
396 less toxic than the precursor compounds. Our results confirm that the comparative sensitivity
397 of the various bioindicators for the matrices tested cannot be generalized but should be assessed
398 on a case-by-case basis.
399
400 *3.1.4. Yeast screen assay – endocrine activity*
401
402 Because many oestrogenic properties have been reported in the literature, they receive
403 a large amount of attention, and relatively little is known about the influence of various
404 xenobiotics on androgen receptors; in the present work, both the oestrogenic and androgenic

properties as well as the anti-oestrogenic and anti-androgenic properties of 4-CP, 4-*t*-OP and NP were investigated. Moreover, the endocrine activity of post-culture liquids obtained after incubation with *U. isabellina* with the tested alkylphenols was also determined. The endocrine properties of the tested compounds are summarized in Table 3.

409 **Table 3** Activity of test xenobiotics in the XenoScreen YES/YAS Endocrine Disruptor Assay.

Xenobiotic	YES	YAS	Anti-YES	Anti-YAS
4-CP (concentration range: 5000 - 15 µg/L)	Oestrogenic activity (about 10.000 times lower than estradiol)	Lack of androgenic properties	Lack of anti-oestrogenic properties	Weak anti-androgenic activity
4-*t*-OP (concentration range: 5000 - 15 µg/L)	Lack of oestrogenic activity	Lack of androgenic properties	Lack of anti-oestrogenic properties	Weak anti-androgenic activity
NP (concentration range: 200000 - 1500 µg/L)	Oestrogenic activity (about 1.000 times lower than estradiol)	Lack of androgenic properties	Lack of anti-oestrogenic properties	Lack of anti-androgenic properties

410 For each system, the activity of estradiol, DHT, flutamide, 4-hydroxy tamoxifen was determined and also the effect of suppressing the action of

411 individual compounds was checked.

412 Our studies have confirmed that NP is a substance with oestrogenic properties, which was also
413 presented in other publications (Park et al., 2000; Preuss et al., 2006; Uchiyama et al., 2008).
414 In the present study, oestrogenic properties were also reported for cumylphenol (Fig. S3).
415 However, this activity was significantly lower than that of the standard oestradiol (E2). The
416 oestrogenic activity of the tested alkylphenols turned out to be 1000- and 10000-times lower
417 than the activity of the standard for NP and 4-CP, respectively. On the basis of the obtained
418 results, it can also be stated that none of the tested substances show androgenic activity (Table
419 3). No changes in β-galactosidase activity in the presence of NP or bisphenol A (BPA) were
420 noted by Park et al. (2000), which is in agreement with the presented data. Literature data
421 suggest that compounds that exhibit oestrogenic properties may also show anti-androgenic
422 activity (Sohoni and Sumpter, 1998). Examples of such compounds include BPA and 4-*t*-OP
423 (Paris et al., 2002). Therefore, in the next stage, the anti-androgenic properties of selected
424 phenols were also assessed.

425 The conducted analyses showed that 4-*t*-OP and 4-CP exhibit anti-androgenic properties
426 and that the addition of these substances reduces the activity of DHT by approximately 30–
427 40%, depending on the analysed sample. The literature data indicate that alkylphenols such as
428 BPA, NP or OP can interact with androgen receptors. These properties have not yet been
429 demonstrated for 4-CP (Li et al., 2010). To our knowledge, this is the first report to demonstrate
430 the anti-androgenic activity of cumylphenol. On the basis of the obtained results, it can also be
431 concluded that NP does not show anti-androgenic activity. On the other hand, Lee et al. (2003)
432 indicated that NP is an active anti-androgen.

433 In the next stage of the work, the post-culture fluids after incubation with *U. isabellina*
434 with selected phenols were evaluated for endocrine properties. The conducted analyses showed
435 that none of the tested post-culture fluids obtained after incubation of *U. isabellina* with NP or
436 4-CP showed oestrogenic activity, regardless of the incubation time and concentration of the

tested sample. In the test determining the anti-androgenic activity of the tested post-culture liquids, it was shown that properties depend on the time of incubation of *U. isabellina* with the substrates (Table S4). The highest decrease in DHT activity was noted in trials from 0 h. Along with the extension of the incubation time, the anti-androgenic activity of the tested fluids decreased, which indicates that the biodegradation process of the discussed alkylphenols leads to their detoxification.

3.2. Growth ability of U. isabellina in leachate presence

The growth of *U. isabellina* on Sabouraud medium containing 20 and 40% landfill leachate originating from the closed dangerous waste landfill of the former "Boruta" Dye Factory in Zgierz, Poland, is presented in Fig. 2.

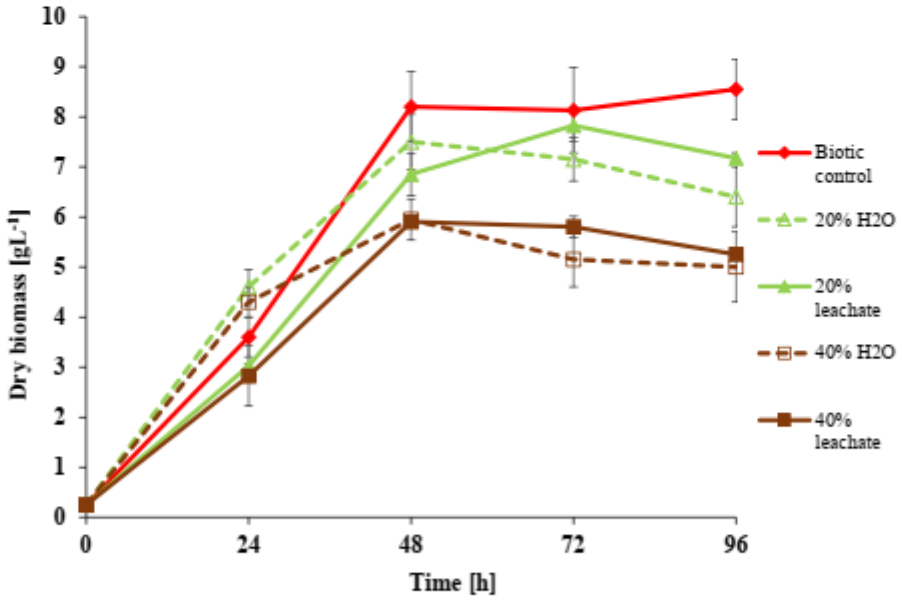


Fig. 2 Effect of supplementation with landfill leachate (20 and 40% of the volume) on the growth of *U. isabellina* during 96 h of incubation.

452 The obtained results show that in 24 h of incubation, the amount of biomass in the culture with
453 20% of the landfill leachate was similar to that in the controls with 20 and 40% water (instead
454 of landfill leachate), as well as in the biotic control without any supplements. In the set with
455 40% landfill leachate, a significantly lower amount of biomass was observed in relation to that
456 in the adequate control supplemented with water. In the following hours of incubation, the
457 difference within both cultures decreased, and at the end of the experiment, the difference was
458 at the same level but much lower than that in the biotic control without any supplements. The
459 obtained results suggest that the fungus is able to adapt to the unfavourable conditions caused
460 by the landfill leachate components even in the case of additional landfill leachate introduction.
461 The basic analyses of the landfill leachate (Table S1) revealed high worth of COD
462 (chemical oxygen demand) as well as total organic carbon (TOC) content and relatively low
463 biochemical oxygen demand (BOD), which suggests the presence of toxic organic and
464 inorganic (high value of conductivity) components for the leachate microbiota. The investigated
465 leachate originates from a dye industry waste landfill located within the boundary of the city
466 Zgierz in the central region of Poland (Fig. 1). The landfill is currently closed but was in use in
467 1995 – 2015 predominantly by the former "Boruta" Dye Industry Plant in Zgierz, Poland. The
468 post-production waste of the former Boruta factory was stored in iron containers, barrels or bins
469 and then covered with a layer of sand, ash, and municipal waste as well as with a layer of ash,
470 gypsum and asbestos (Góralczyk-Bińkowska et al., 2021; Janas and Zawadzka, 2018).
471 The detailed chemical analyses of the leachate (Table S2) showed additionally high
472 amounts of iron (the most likely the result of metal container corrosion) and VPs. VPs are
473 benzene hydroxyl derivatives and other aromatic hydroxyl chemicals that distil water vapour
474 from an acid solution and under specified standard conditions give a colour reaction with 4-
475 aminoantipyrine (PN-EN ISO 14402:2004). VPs are synthesized by microorganisms and are
476 formed during biotransformation and biodegradation processes of some natural compounds and

xenobiotics, e.g., non-volatile phenols, aromatic amino acids, polycyclic aromatic hydrocarbons (PAHs) and polyphenols (Schieber and Wüst, 2020). The leachate from the landfill was sent by the industrial liquid waste system of the former "Boruta" factory to the municipal and industrial wastewater plant in Zgierz, Poland. The possible ability of the tested fungus to degrade and detoxify VPs present in the leachate could be useful for the wastewater plant in Zgierz.

3.3. VP utilization by U. isabellina

Analyses of VPs in cultures of *U. isabellina* (Fig. 3) revealed that in the culture without any supplements (biotic control), the fungus released VPs into the medium over the course of the experiment until the end of incubation at 96 h.

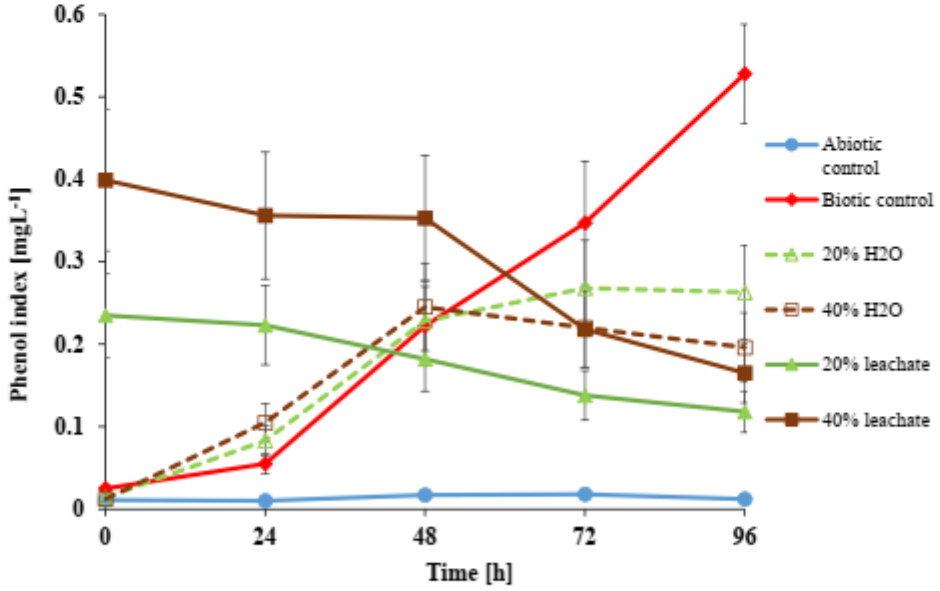


Fig. 3 Volatile phenol content during *U. isabellina* cultures with the addition of 20 and 40% landfill leachate.

492 Fungi are able to produce and utilize numerous volatile organic compounds (VOCs), including
493 VPs (Guo et al., 2021; Kennes et al., 2016). In the investigated control culture with 20% water
494 (instead of landfill leachate) for 48 h, a decrease in VP formation was noticed.

495 In the case of the control set with 40% water (in place of landfill leachate) after 48 h,
496 even a decrease in previously released VPs was observed. These phenomena are correlated with
497 a very rapid breakdown of the VP content after 48 h of culturing in the set with 40% landfill
498 leachate and indicate that VPs are utilized by *U. isabellina*, especially in the stationary phase
499 growth phase when the easily and quickly metabolized components of the growth medium are
500 consumed. This conclusion also seems to be confirmed by a slight decrease in the VPs content
501 in the first 24 h of the experiment in the culture supplemented with 20% leachate and gradual
502 acceleration of this process in the further hours of incubation.
503
504 *3.4. Evaluation of sample toxicity from U. isabellina cultures supplemented with leachate*
505
506 To estimate the detoxification capability of the tested fungus in relation to the post-
507 industrial landfill leachate, toxicity tests using organisms representing different levels of the
508 trophic chain (consumer, decomposer, producer) were carried out.

509 In all of our tests, a significant correlation between the toxicity reduction of filtrates
510 from *U. isabellina* culture in the presence of different leachate concentrations and the decrease
511 in VPs in these samples was observed. Reductions in toxicity: 3.8, 4.9 and 1.9-fold for the 20%
512 leachate volume and 3.7, 4.6 and 2.1-fold for the 40% leachate volume in fungal cultures were
513 achieved during the 96 h of biodegradation experiments for *A. franciscana*, *D. magna* and *A.*
514 *fischeri*, respectively (Table 4).
515

516 **Table 4** Ecotoxicity data (EC50 and LC50 values) for *D. magna*, *A. franciscana* and *A. fischeri*
517 assays in the presence of post-culture filtrates treated with the landfill leachate.
518

Landfill leachate (%)	Time (h)	*A. fischerii*	*A. fransciscana*	*D. magna*
20	0	34.2 ± 0.6	13.4 ± 0.6	9.1 ± 0.6
	24	40.1 ± 0.8	15.6 ± 0.9	14.5 ± 1.1
	48	45.1 ± 1.0	19.7 ± 1.1	26.9 ± 1.4
	72	56.4 ± 0.9	25.6 ± 0.5	30.4 ± 1.0
	96	66.9 ± 1.3	50.4 ± 1.2	44.1 ± 1.5
40	0	15.9 ± 0.4	7.8 ± 1.0	4.4 ± 0.2
	24	24.7 ± 0.5	10.1 ± 0.9	7.2 ± 0.9
	48	28.2 ± 0.9	15.2 ± 1.0	10.0 ± 0.5
	72	31.1 ± 0.5	17.4 ± 1.0	11.5 ± 0.3
	96	34.0 ± 1.0	28.6 ± 1.5	20.2 ± 1.2

519

520 Other studies using *A. franciscana* as a bioindicator showed a 21% decrease in the harmful
521 potential of landfill leachate during treatment processes, which was a similar value to our results
522 (de Almeida et al., 2018). A significant reduction in toxicity for 60% of the landfill leachate as
523 a result of biological treatment over a 35-day period was also observed in the *D. magna* tests
524 (Paskuliakova et al., 2018).

525 The effectiveness of decreasing toxicity after biological treatment of landfill leachate
526 has also been successfully demonstrated in many studies by measuring the bioluminescent
527 activity of bacteria. The application of *A. fischeri* in studies carried out by Kalka (2012) revealed
528 a reduction in toxicity by 67.2% in the treated leachate, while Kalčíková et al. (2014), using the
529 same test, showed a reduction of 33.4% in harmfulness in leachate during the growth of the
530 fungus *Dichomitus squalens* after 3 days of incubation.

Therefore, reports indicate that toxicity assessment with *A. franciscana*, *D. magna* and *A. fischeri* is convenient, effective and useful for monitoring the effectiveness of biological leachate treatment processes (Abbas et al., 2018).

In the present work, toxicity results varied depending on the test organism and selected endpoints, with *D. magna* identified as the most sensitive organism. The lower resistance of daphnids and other freshwater crustaceans to the toxic effect of leachate compared to other bioindicators has already been demonstrated (Kalcikova et al., 2011; Melnyk et al., 2014). The high toxicity of the tested landfill leachate to *D. magna* may be due to the high conductivity and the presence of heavy metals, especially the high TOC values that exceed the standard by more than thirty times (Table S1). The high content of organic matter may reduce the oxygen content in the sample, negatively affecting the survival rate of daphnids (Sackey et al., 2020).

The application of luminescent bacteria in the present study revealed the toxic nature of the landfill leachate; however, among the tested bioindicators, *A. fischerii* showed the lowest sensitivity. A lower frequency of toxic reactions in response to landfill leachate in *A. fischeri* compared to test organisms representing different trophic levels was also reported in another study (Melnyk et al., 2014). It has been suggested that the reason for such findings may be the presence of substances with insecticidal and herbicidal properties in the leachate. The harmful impact of leachate on the luminescent activity of *A. fischerii* could have resulted from the coexistence of several metals, especially Fe^{2+}, the amount of which significantly exceeded the norms. It was noted that the inhibition of bioluminescence by the leachate was positively correlated with the metals present, including Fe^{2+}, that could be adsorbed and trapped in the exo-polysaccharide layer of the bacteria (Schiavo et al., 2020).

Our findings suggest that the toxicity decrease in filtrates in the test with *A. fischeri* may be caused not only by the biodegradation processes of organic substances but also by heavy

556 metal ion biosorption or/and bioaccumulation mechanisms. This is confirmed by the results of
557 our earlier studies, in which we demonstrated the ability of *U. isabellina* to remove selected
558 heavy metals from the growth medium (Janicki et al., 2018).

559 All filtrate samples were evaluated as phytotoxic based on *S. saccharatum* seed
560 germination and root growth, especially at higher tested leachate concentrations. It was found
561 that the use of 20 and 40% of the leachate in fungal cultures resulted in root growth inhibition
562 of 32.5 and 59.1%, respectively, for the filtrates from the start of cultivation. A correlation
563 between a higher leachate content and a greater percent of root growth inhibition of exposed
564 plants was also demonstrated by Šourková et al. (2020), who noticed a similar degree of *S. alba*
565 root growth reduction using 25 and 50% leachate. These findings indicate that the use of large
566 amounts of leachate disrupts the defence system and the metabolism of bioindicator plant
567 species. The phytotoxicity test also showed that toxicity declined in the filtrates obtained during
568 *U. isabellina* incubation with the addition of leachates (Table S5). At 96 h of cultivation, the
569 GI increased by 18.8 and 19.1% for post-culture samples containing 20 and 40% leachate,
570 respectively.

571 Studies on root growth inhibition in the presence of tested supernatants also revealed
572 a reduction in their harmful effects on *S. saccharatum*. The growth inhibition of the roots treated
573 with the 4-day post-culture filtrates was reduced by approximately 19% compared to the starter
574 cultures for both the 20 and 40% leachate volumes. The toxic effect of leachate on *S.*
575 *saccharatum* root elongation may be caused by the high conductivity value. A strong
576 dependence of *S. saccharatum* root growth inhibition on conductivity was also demonstrated in
577 studies on the phytotoxicity assessment of leachate from a controlled municipal landfill in
578 Gdansk (Poland) (Melnyk et al., 2014). The availability value of water-soluble pollutants is
579 high for plants; therefore, they may have the strongest impact on bioindicators, which present
580 the first level of the trophic chain. The results of contact tests with plant seeds also showed an

581 increase in toxicity during the first 48 h of biodegradation processes. This suggests that VPs
582 and other organic contaminants present in the tested leachate could have been degraded into
583 by-products characterized by higher toxicity than the precursor compounds. In conclusion, the
584 obtained data clearly indicate that the microbiological processes of compound metabolism
585 contained in leachate, including VPs, result in their detoxification.
586
587 **4. Conclusions**
588
589 The results obtained in the study provide accurate information on the reduction of
590 cumulative harmful effects of compounds mixtures formed during the degradation of 4-CP, NP
591 and 4-*t*-OP by *U. isabellina* strain and decrease their adverse effect on biota. Moreover, the data
592 presented in this work show that the fungal strain also has the ability to eliminate and detoxify
593 VPs from leachate produced by landfills post-industrial waste. The use of a battery of biotests
594 in our study provided information on the biological activity of leachate treated with biological
595 treatment processes. Thus, it can be concluded that the non-ligninolytic fungus *U. isabellina*
596 shows potential usefulness in bioremediation processes not only in environments contaminated
597 with specific phenolic xenobiotics but also in industrial leachates with similar chemical profiles.
598 The results of the biotests presented in the paper demonstrate sufficient evidence to
599 estimate the effectiveness of hazard reduction by biodegradation processes, indicating the
600 potential of the tested fungal strain to be used as an attractive tool for bioremediation of areas
601 contaminated with phenolic xenobiotics. This creates the prospect of the possible inclusion of
602 this bacterium in wastewater treatment programs as a promising alternative to the often costly
603 and not eco-friendly physico-chemical methods of reducing toxic pollutants.
604
605 **CRediT authorship contribution statement**

606

607 **Krupiński M.** and **Długoński J.** guided the study and drafted the preliminary version
608 of the manuscript, **Janicki T.** and **Krupiński M.** performed the ecotoxicological and statistical
609 analyses, **Długoński A**. elaborated spatial analyses and landfill leachate study, **Felczak A.** made
610 the endocrine activity assays, **Janicki T.** and **Długoński A.** prepared graphical abstract for the
611 paper. All authors participated in the interpretation of the obtained results and preparation of
612 the final version of the manuscript.

613

614 **Declaration of competing interests**

615

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

618

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628

629 **Appendix A. Supporting information**

630

631 Supplementary data associated with this article can be found in the online version at

632

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634

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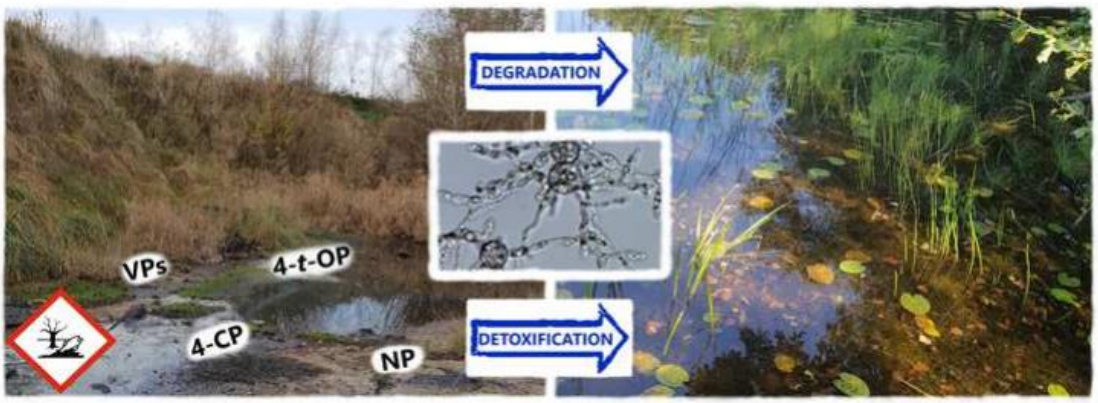
826

Graphical Abstract



Supplementary data

Figure S1. Location of leachate collection from post-industrial landfills. Study area location in Zgierz city (Poland, Central Europe). A. The municipal and industrial wastewater treatment plant, B. The former "Boruta" Dye Industry Plant area, C. The closed landfill for hazardous waste of the former "Boruta" Dye Industry Plant, D. The closed energy ash and gypsum landfill. Note: The figure was elaborated in QuantumGIS Bucuresti (ver. 3.12) based on site observation and mapping analysis according to online software sources (OSM Standard).

Table S1. Basic analysis of the landfill leachate collected from the hazardous waste landfill of the former “Boruta” dye production plants in Zgierz.

Parameter	Method	Unit	Value	Standard
pH	PN-EN ISO 10523:12	pH	7.2 ± 0.4	6.5 – 9.0
Conductivity	PN-EN 27888:1999	µS	11805 ± 673	-
COD_{Mn}	PN-ISO 15705:2005	mg/L O_2	348.8 ± 92.4	125

TOC	PN-EN 1484:1999	mg/L C	1040.5 ± 190.4	30
BOD_5	PN-EN 1899-2:2002	mg/L O_2	300 ± 15.2	-

Table S2. Chemical contaminants of the landfill leachate.

Parameter	Method	Unit	Value	Standard
Nitrites	PN-EN ISO 13395:2001	mg/L NO_2	0.026 ± 006	1
Nitrates	PN-EN ISO 10304-1:2009	mg/L NO_3	< 1.7	30
Sulphates	PN-EN ISO 10304-1:2009	mg/L SO_4	15 ± 3	500
Chlorides	PN-EN ISO 10304-1:2009	mg/L Cl	2.1 ± 0.4	1000
Cyanides (free)	PN-EN ISO 14403-2:2012	mg/L	< 0.008	-
Cyanides (bound)	PN-EN ISO 14403-2:2012	mg/L	0.045 ± 0.008	-
Antimony	PN-EN ISO 11885:2009	mg/L Sb	<0.020	0.3
Arsenic	PN-EN ISO 11885:2009	mg/L As	<0.020	0.1
Barium	PN-EN ISO 11885:2009	mg/L Ba	0.37 ± 0.08	2
Beryllium	PN-EN ISO 11885:2009	mg/L Be	<0.004	1
Boron	PN-EN ISO 11885:2009	mg/L B	20.35 ± 4.68	1
Chromium (total)	PN-EN ISO 11885:2009	mg/L Cr total	0.055 ± 0.011	0.1
Zinc	PN-EN ISO 11885:2009	mg/L Zn	0.067 ± 0.015	2
Aluminum	PN-EN ISO 11885:2009	mg/L Al	0.071 ± 0.016	3
Cadmium	PN-EN ISO 11885:2009	mg/L Cd	0.0015 ± 0.0003	0.4
Cobalt	PN-EN ISO 11885:2009	mg/L Co	<0.002	1
Manganese	PN-EN ISO 11885:2009	mg/L Mn	0.17 ± 0.04	-
Copper	PN-EN ISO 11885:2009	mg/L Cu	0.014 ± 0.003	0.5
Molybdenum	PN-EN ISO 11885:2009	mg/L Mo	0.057 ± 0.009	1
Nickel	PN-EN ISO 11885:2009	mg/L Ni	0.23 ± 0.04	0.5
Lead	PN-EN ISO 11885:2009	mg/L Pb	0.023 ± 0.004	0.5
Selenium	PN-EN ISO 11885:2009	mg/L Se	<0.050	-
Silver	PN-EN ISO 11885:2009	mg/L Ag	<0.010	0.1
Thallium	PN-EN ISO 11885:2009	mg/L Tl	<0.020	1
Titanium	PN-EN ISO 11885:2009	mg/L Ti	0.024 ± 0.005	1
Vanadium	PN-EN ISO 11885:2009	mg/L V	<0.006	2
Iron	PN-EN ISO 11885:2009	mg/L Fe	30.88 ± 5.87	10

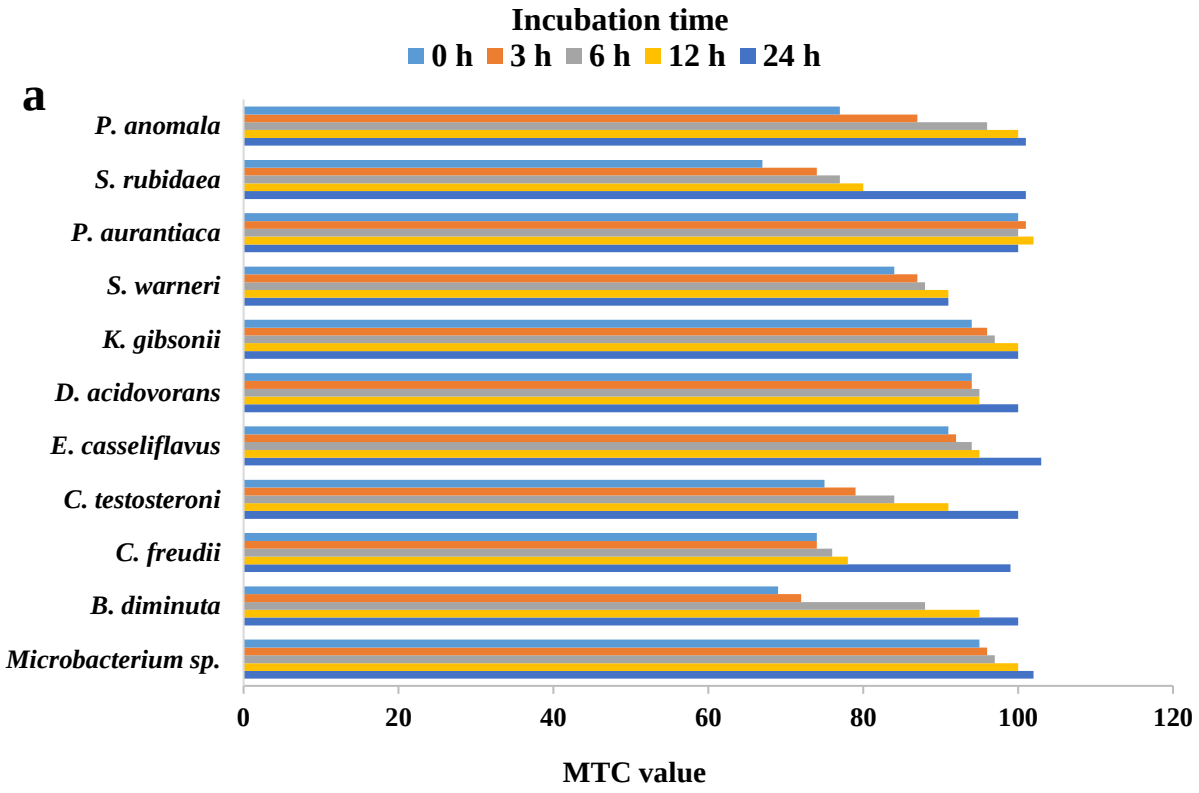
Mercury	EPA 7473 02.2007	mg/L Hg	0.0014 ± 0.0002	0.06
Volatile phenols	PN-EN ISO 14402 : 2004	mg/L	1.68 ± 0.48	0.1
Petroleum hydrocarbons	PN-EN ISO 9377-2:2003	mg/L	7.2 ± 2.3	15

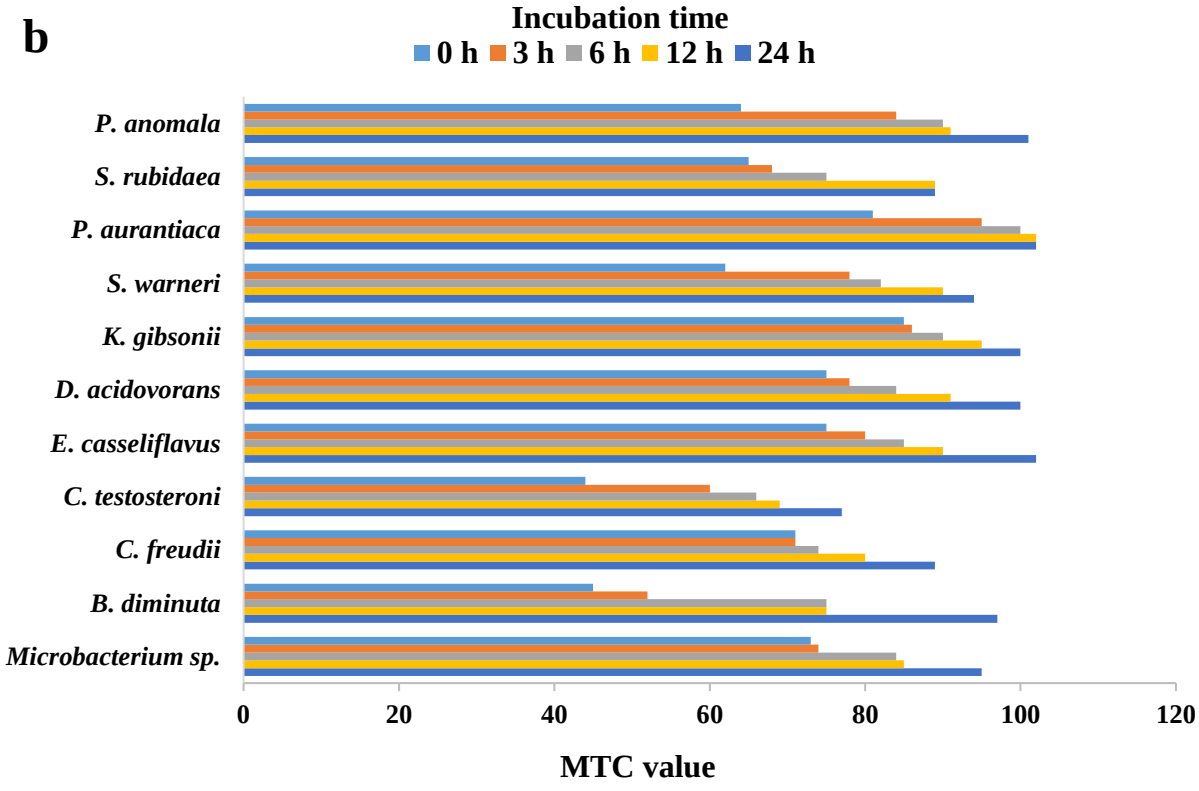
Table S3. Toxicity bioassays selected for the ecotoxicological analysis of filtrates from *U. isabellina* cultures supplemented with xenobiotics and landfill leachate.

Trophic level	Species	Measure of toxic effect (toxicological endpoints)	4NP, 4-CP, 4-*t*-OP samples analyzed	VPs samples analyzed
Decomposer	*Aliivibrio fischeri*	Bioluminescence activity	X	X
	Microbacterium sp.	Growth rate inhibition	X	
	Brevundimonas diminuta	Growth rate inhibition	X	
	Citrobacter freudii	Growth rate inhibition	X	
	Comamonas testosteroni	Growth rate inhibition	X	
	Enterococcus casseliflavus	Growth rate inhibition	X	
	Delftia acidovorans	Growth rate inhibition	X	
	Kurthia gibsonii	Growth rate inhibition	X	
	Staphylococcus warneri	Growth rate inhibition	X	
	Pseudomonas aurantiaca	Growth rate inhibition	X	
	Serratia rubidaea	Growth rate inhibition	X	
	Pichia anomala	Growth rate inhibition	X	

	Saccharomyces cerevisiae	Estrogenic/anti-estrogenic activity Androgenic/anti-androgenic activity	X X	
Producer	*Sorghum saccharatum*	Seed germination rate, root length reduction	X	X
	Lepidium sativum	Seed germination rate, root length reduction	X	
	Sinapis alba	Seed germination rate, root length reduction	X	
	Rhapidocelis subcapitata	Growth rate inhibition	X	
	Phaeodactylum tricornutum	Growth rate inhibition	X	
Consumer	*Thamnocephalus platyurus*	Reduction or complete cessation of food intake	X	
	Daphnia magna	Mortality	X[a]	X
	Artemia franciscana	Mortality	X[a]	X

[a] – tests were carried out in previous studies (Janicki et al., 2016)


a
Incubation time
0 h
3 h
6 h
12 h
24 h
P. anomala
S. rubidaea
P. aurantiaca
S. warneri
K. gibsonii
D. acidovorans
E. casseliflavus
C. testosteroni
C. freudii
B. diminuta
Microbacterium sp.
0
20
40
60
80
100
120
MTC value


b
Incubation time
0 h
3 h
6 h
12 h
24 h
P. anomala
S. rubidaea
P. aurantiaca
S. warneri
K. gibsonii
D. acidovorans
E. casseliflavus
C. testosteroni
C. freudii
B. diminuta
Microbacterium sp.
0
20
40
60
80
100
120
MTC value

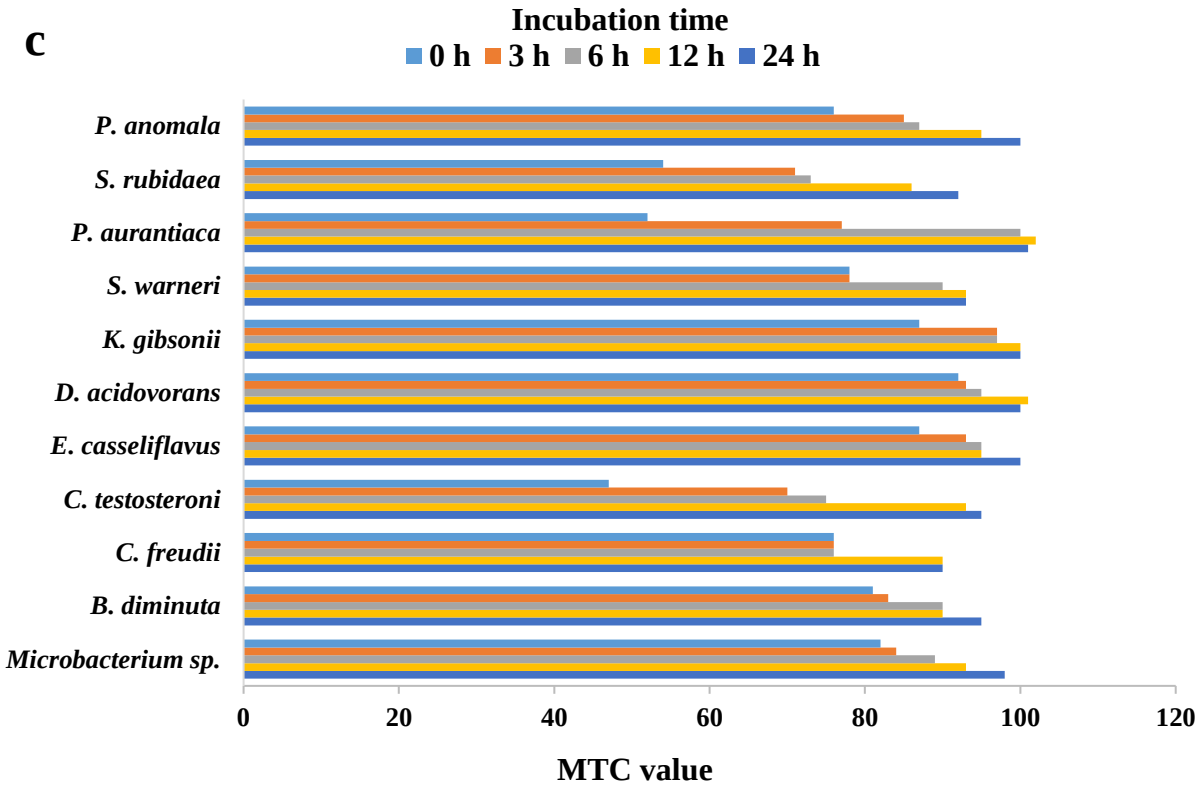


Figure S2. MARA species responses (MTC value) to *U. isabellina* cultures treated with test xenobiotics: a – 4-CP, b – NP, c – 4-*t*-OP.

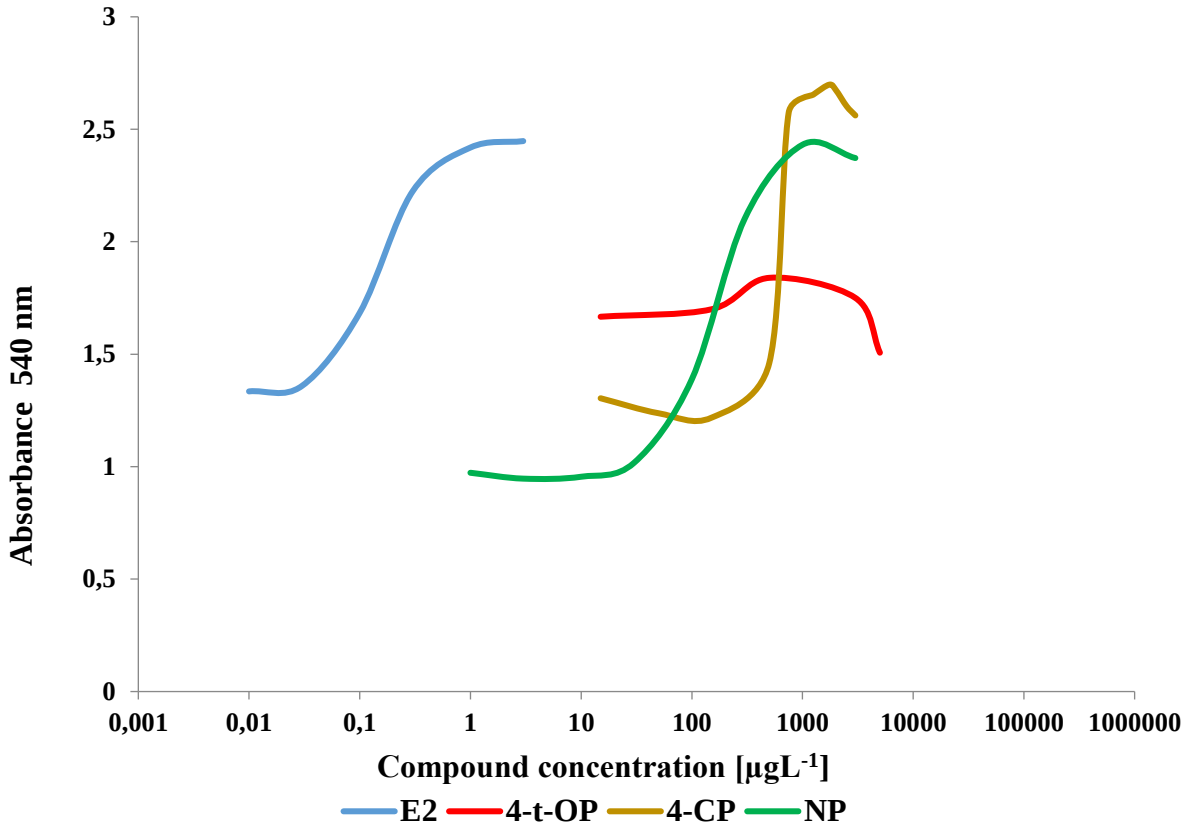


Figure S3. Oestrogenic activity of 4-CP, 4-*t*-OP and NP.

Table S4. Anti-androgenic activity of post-culture filtrates obtained after incubation of *U. isabellina* with NP or 4-CP (post-culture liquid concentration – 4%).

Time (h)	Absorbance of 4-CP (% of control, i.e. DHT)	Absorbance of 4-*t*-OP (% of control, i.e. DHT)
0	65.50 ± 1.52	75.04 ± 1.12
3	75.01 ± 0.90	83.20 ± 1.42
6	82.28 ± 1.23	85.18 ± 0.64
12	81.98 ± 0.56	87.90 ± 0.49
24	86.73 ± 0.70	93.72 ± 0.80

Table S5. Plant biological endpoints for testing the phytotoxicity of fungal post-culture filtrates supplemented with landfill leachate.

Landfill leachate (%)	Time (h)	Germination inhibition (PE%)	Root growth inhibition (PE%)	Germination index (%)
20	0	0 ± 0	32.5 ± 2.8	59.5 ± 2.0
	24	0 ± 0	39.7 ± 2.2	52.4 ± 3.0
	48	0 ± 0	49.6 ± 3.0	43.0 ± 2.9
	72	0 ± 0	29.3 ± 2.7	62.9 ± 3.6
	96	0 ± 0	20.6 ± 3.1	78.3 ± 2.8
40	0	10 ± 2	59.1 ± 3.3	40.6 ± 1.7
	24	0 ± 0	64.7 ± 2.8	35.2 ± 2.6
	48	0 ± 0	69.2 ± 3.4	30.8 ± 2.0
	72	0 ± 0	53.4 ± 3.8	44.6 ± 2.7
	96	0 ± 0	40.1 ± 2.5	59.7 ± 2.1

Oświadczenia współautorów

Tomasz Janicki, Mariusz Krupiński, Jerzy Długoński. 2016. Degradation and toxicity reduction of the endocrine disruptors nonylphenol, 4-*tert*-octylphenol and 4-cumylphenol by the non-ligninolytic fungus *Umbelopsis isabellina*. Bioresource Technology, doi: 10.1016/j.biortech.2015.10.034

Imię i nazwisko	Opis działań	Szacunkowy udział [%]	Podpis
mgr Tomasz Janicki	Opracowanie koncepcji badań nad procesami mikrobiologicznej degradacji. Zaplanowanie i realizacja doświadczeń dotyczących oceny zdolności do wzrostu oraz eliminacji badanych ksenobiotyków przez grzyb strzępkowy *Umbelopsis isabellina*, analiza ilościowa i jakościowa procesów biodegradacji, oszacowanie toksyczności produktów mikrobiologicznego rozkładu badanych związków. Opracowanie i interpretacja otrzymanych wyników. Przygotowanie manuskryptu: opracowanie graficzne wyników oraz abstraktu graficznego, edycja tekstu manuskryptu, przygotowanie danych bibliograficznych, opis materiałów i metod, udział w przygotowaniu wstępu oraz dyskusji.	65	
dr Mariusz Krupiński	Opracowanie koncepcji badań ekotoksykologicznych. Realizacja eksperymentów dotyczących jakościowej oraz ilościowej analizy chromatograficznej. Analiza statystyczna uzyskanych danych. Przygotowanie manuskryptu: opis wyników oraz dyskusji. Udział w przygotowaniu odpowiedzi dla recenzentów.	20	
prof. dr hab. Jerzy Długoński	Opracowanie koncepcji badań . Analiza i interpretacja wyników. Edycja tekstu manuskryptu, udział w przygotowaniu odpowiedzi dla recenzentów.	15	

Dane bibliometryczne z dn. 23.11.2021 r. wg. *Web. Sci.*: IF = 9,642; 33 cytowania (140 punktów MEiN)

Tomasz Janicki, Jerzy Długoński, Mariusz Krupiński. 2018. Detoxification and simultaneous removal of phenolic xenobiotics and heavy metals with endocrine-disrupting activity by the non-ligninolytic fungus *Umbelopsis isabellina*. Journal of Hazardous Materials, doi: 10.1016/j.jhazmat.2018.08.047

Imię i nazwisko	Opis działań	Szacunkowy udział [%]	Podpis
mgr Tomasz Janicki	Opracowanie koncepcji badań obejmujących eliminację zanieczyszczeń organicznych i nieorganicznych przez *Umbelopsis isabellina*. Zaplanowanie i realizacja doświadczeń dotyczących oceny zdolności do wzrostu oraz eliminacji badanych ksenobiotyków przez badany grzyb strzępkowy w obecności wybranych metali ciężkich, analiza zdolności do biosorpcji i bioakumulacji metali oraz ocena ekotoksykologiczna analizowanych procesów mikrobiologicznej eliminacji zanieczyszczeń. Przygotowanie manuskryptu: opracowanie graficzne wyników oraz abstraktu graficznego, edycja tekstu manuskryptu, przygotowanie danych bibliograficznych, opis wstępu oraz materiałów i metod, i wniosków końcowych, udział w przygotowaniu dyskusji oraz wyników.	65	
dr Mariusz Krupiński	Opracowanie koncepcji badań ekotoksykologicznych. Realizacja eksperymentów dotyczących ilościowej analizy chromatograficznej oraz eliminacji metali ciężkich przez badany drobnoustrój. Analiza statystyczna uzyskanych danych. Przygotowanie manuskryptu: opis wyników oraz dyskusji. Udział w przygotowaniu odpowiedzi dla recenzentów.	20	
prof. dr hab. Jerzy Długoński	Opracowanie koncepcji pracy. Analiza i interpretacja wyników. Edycja tekstu manuskryptu, udział w przygotowaniu odpowiedzi dla recenzentów.	15	

Dane bibliometryczne z dn. 23.11.2021 r. wg. *Web. Sci.*: IF = 10,588; 13 cytowań (200 punktów MEiN)

Tomasz Janicki, Andrzej Długoński, Aleksandra Felczak, Jerzy Długoński, Mariusz Krupiński (w trakcie recenzji) „Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol and volatile leachate phenol degradation by the microscopic fungus *Umbelopsis isabellina* using a battery of biotests". Chemosphere, (praca w recenzji).

Imię i nazwisko	Opis działań	Szacunkowy udział [%]	Podpis
mgr Tomasz Janicki	Opracowanie koncepcji badań ekotoksykologicznych. Zaplanowanie i realizacja doświadczeń dotyczących oceny zdolności do wzrostu oraz eliminacji badanych ksenobiotyków przez grzyb strzępkowy *Umbelopsis isabellina* w obecności metali ciężkich, analiza zdolności do biosorpcji i bioakumulacji metali oraz ocena ekotoksykologiczna analizowanych procesów mikrobiologicznej eliminacji zanieczyszczeń - przeprowadzenie biotestów wobec organizmów reprezentujących różne poziomy troficzne. Przygotowanie manuskryptu: opracowanie graficzne wyników oraz abstraktu graficznego, edycja tekstu manuskryptu, przygotowanie danych bibliograficznych, opis wstępu oraz materiałów i metod, udział w przygotowaniu dyskusji oraz wyników.	45	
dr Andrzej Długoński	Opracowanie, analiza oraz interpretacja wyników doświadczeń dotyczących prób środowiskowych zawierających odcieki składowiskowe. Przygotowanie manuskryptu: opis wyników i dyskusji.	15	
dr Aleksandra Felczak	Przeprowadzenie, analiza i interpretacja badań dotyczących zmian potencjału endokrynnego w trakcie procesów mikrobiologicznej eliminacji ksenobiotyków. Przygotowanie manuskryptu: opis wyników i dyskusji.	10	
dr Mariusz Krupiński	Opracowanie koncepcji badań ekotoksykologicznych. Realizacja eksperymentów obejmujących ocenę zmian toksyczności hodowli w trakcie procesów eliminacji testowanych zanieczyszczeń. Analiza statystyczna uzyskanych danych. Przygotowanie manuskryptu: opis wyników, dyskusji i wniosków końcowych.	15	
prof. dr hab. Jerzy Długoński	Opracowanie koncepcji badań obejmujących eliminację zanieczyszczeń z prób środowiskowych zawierających odcieki składowiskowe. Analiza i interpretacja wyników. Edycja tekstu manuskryptu.	15	

Dane bibliometryczne z dn. 20.12.2021 r. wg. *Web.Sci.*: IF = 7,086; (140 punktów MEiN) w recenzji