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Mikrobiologiczna eliminacja oraz toksyczność środowiskowa metyloizotiazolinonu i chloroksylenolu

Microbiological elimination and environmental toxicity of methylisothiazolinone and chloroxylenol

Praca doktorska

wykonana w Katedrze Mikrobiologii Przemysłowej i Biotechnologii

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Składam serdeczne podziękowania
Promotorom ***prof. dr hab. Katarzynie Lisowskiej***
oraz ***dr Katarzynie Niedziałkowskiej***
za okazaną życzliwość, wyrozumiałość, motywację oraz nieocenioną pomoc
w trakcie przygotowywania niniejszej rozprawy doktorskiej.

Dziękuję również pracownikom oraz doktorantom
Instytutu Mikrobiologii, Biotechnologii i Immunologii Uniwersytetu Łódzkiego
za życzliwość, wsparcie, miłą atmosferę pracy oraz
merytoryczną pomoc w trakcie wykonywania badań.

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- **Nowak Marta**, Zawadzka Katarzyna, Lisowska Katarzyna (2020). *Occurrence of methylisothiazolinone in water and soil samples in Poland and its biodegradation by Phanerochaete chrysosporium*, Chemosphere, 254: 126723, doi: 10.1016/j.chemosphere.2020.126723.

  IF_{2020} = 7.086; $IF_{5\text{-letni}}$ = 6.956; pkt. MEiN = 140.

- **Nowak Marta**, Zawadzka Katarzyna, Szemraj Janusz, Góralczyk-Bińkowska Aleksandra, Lisowska Katarzyna (2021). *Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways*, International Journal of Molecular Science, 22(9): 4360, doi: 10.3390/ijms22094360.

  IF_{2021} = 6.208; $IF_{5\text{-letni}}$ = 6.628; pkt. MEiN = 140.

- **Nowak-Lange Marta**, Niedziałkowska Katarzyna, Bernat Przemysław, Lisowska Katarzyna (2022). *In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria*, Scientific Reports, 12: 19068, doi: 10.1038/s41598-022-22981-9.

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- **Nowak-Lange Marta**, Niedziałkowska Katarzyna, Lisowska Katarzyna. *Cosmetic preservatives – hazardous micropollutants in need of greater attention?* International Journal of Molecular Science.

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Na rozprawę doktorską składają się:

- 3 opublikowane prace oryginalne o łącznym IF = 18.291 oraz sumą pkt. MEiN = 420;
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OMÓWIENIE CELU NAUKOWEGO I UZYSKANYCH WYNIKÓW

Wprowadzenie

IV. Omówienie celu naukowego i uzyskanych wyników

IV.1. Wprowadzenie

Wzrost produkcji substancji chemicznych, wytwarzanych w celu zaspokojenia potrzeb ludzkich oraz poprawy jakości życia, stwarza nieustanne zagrożenie skażenia środowiska naturalnego. Rozwój nowoczesnych technologii i wykorzystanie instrumentów analitycznych o wysokiej czułości, umożliwiło znaczne podwyższenie granicy wykrywalności i identyfikację substancji toksycznych nawet przy bardzo niskich stężeniach, takich jak nano – czy mikrogramy. Jednocześnie z wykrywaniem coraz większej ilości różnorodnych zanieczyszczeń środowiska, pojawiły się nowe zagrożenia dla funkcjonowania wielu ekosystemów (Brausch i Rand 2011). W literaturze naukowej szeroka gama nowo identyfikowanych i niebezpiecznych substancji organicznych, o nieokreślonym ryzyku dla zdrowia ludzi i środowiska, określana jest mianem „*contaminants of emerging concern* (CECs)" (García-Fernández 2020). Te potencjalnie niebezpieczne substancje wykrywane są w różnych matrycach środowiskowych, takich jak powietrze, woda, czy gleba. Pomimo, że są to mikrozanieczyszczenia występujące w środowisku w bardzo niskich stężeniach, to charakteryzują się wysoką aktywnością biologiczną, potencjałem do bioakumulacji oraz niską podatnością na degradację.

Do mikrozanieczyszczeń środowiska zaliczane są m. in. farmaceutyki oraz produkty ochrony indywidualnej (ang. *personal care products, PCPs)*. Jednym z aktualnych trendów badawczych jest ocena wpływu PCPs na ekosystemy oraz poszukiwanie efektywnych metod ich eliminacji i detoksykacji (Gavrilescu i wsp. 2015). Obecnie duże obawy budzi zanieczyszczenie różnych ekosystemów dodatkami do produktów higieny osobistej, takimi jak: konserwanty, barwniki, filtry przeciwsłoneczne, surfaktanty czy zapachy, jeszcze do niedawna uważanymi za nieszkodliwe dla środowiska. Wiele z tych produktów okazało się być silnie toksycznymi wobec żywych organizmów (Boxall i wsp. 2012).

Szczególnie niebezpieczną grupę zanieczyszczeń stanowią konserwanty wykorzystywane w produktach higieny osobistej, ponieważ, podobnie jak farmaceutyki, zostały opracowane w celu wywoływania konkretnych efektów biologicznych. Wykorzystanie konserwantów w skali światowej nie jest udokumentowane, a ich rodzaj i zużycie jest różne w zależności od danego kraju. Większość produktów kosmetycznych, ze względu na bogaty skład organiczny oraz wysoką zawartość wody, narażonych jest na zanieczyszczenie mikrobiologiczne. Obecność mikroorganizmów nie tylko obniża jakość wyrobów, ale może także prowadzić do podrażnień lub infekcji u konsumentów, dlatego

konieczne jest uwzględnienie w składzie kosmetyków substancji ograniczających rozwój niepożądanej mikroflory (Alvarez-Rivera i wsp. 2018). Związki konserwujące powinny charakteryzować się trwałością w szerokim zakresie pH oraz odpornością na czynniki fizyko-chemiczne, takie jak światło, podwyższona temperatura, czy dostęp do tlenu, a także być odporne na hydrolizę, co czyni je substancjami trudno degradowalnymi (Andersen i Mose 2016). Stosowanie substancji konserwujących w Unii Europejskiej regulowane jest przez rozporządzenie Parlamentu Europejskiego i Rady Unii Europejskiej, które określa jakie związki chemiczne mogą być dopuszczone do użytku w produktach kosmetycznych (załącznik V do rozporządzenia Parlamentu Europejskiego i Rady Unii Europejskiej nr 1223/2009) (European Comission 2020). Ze względu na swoje właściwości, mogą one działać toksycznie, drażniąco lub indukować reakcje alergiczne, dlatego w ostatnich latach bezpieczeństwo stosowania konserwantów było wielokrotnie kwestionowane przez opinię publiczną. Liczne dowody naukowe świadczące o szkodliwym oddziaływaniu niektórych środków konserwujących na zdrowie konsumentów oraz środowisko naturalne, wpływają na coraz większe ograniczenia stosowania tych związków oraz zastępowanie tradycyjnych konserwantów nowymi substancjami chemicznymi (Alvarez-Rivera i wsp. 2018).

Produkty ochrony indywidualnej przeznaczone są do stosowania zewnętrznego, dlatego też najczęściej trafiają do oczyszczalni ścieków w niezmienionej formie. Wiele z tych związków nie podlega rozkładowi na etapie oczyszczania ścieków, co przyczynia się do zanieczyszczenia środowiska. Oczyszczone ścieki są bowiem zrzucane do wód powierzchniowych, takich jak np. rzeki, jeziora. Mogą być także wykorzystywane do nawadniania pól na terenach zagrożonych suszą. Ponadto, wiele substancji wykazuje tendencję do bioakumulacji w osadach ściekowych, które często stosowane są do nawożenia terenów rolniczych, stanowiąc kolejne źródło zanieczyszczenia środowiska nie tylko substancjami konserwującymi, ale także innymi ksenobiotykami (Chopra i Kumar 2018). Ciągłe przedostawanie się PCPs do środowiska może przyczyniać się do akumulacji tych substancji także w glebie, z której mogą przenikać do wód gruntowych, negatywnie wpływając na organizmy z różnych poziomów troficznych (Gautam i Anbumani 2020). Niebezpieczeństwo, wynikające z przedostawania się biocydów do środowiska naturalnego, stwarza potrzebę analizy stężeń środowiskowych, oceny potencjalnej toksyczności oraz poznania ich losów środowiskowych, a także metod eliminacji tych związków.

Na podstawie badań ostrej toksyczności wobec organizmów wodnych wykazano, że jednymi z najbardziej toksycznych PCPs są konserwanty z grupy związków chloroorganicznych, izotiazolinonów oraz czwartorzędowych związków amonowych.

Konserwanty chloroorganiczne to grupa związków, która na rynku kosmetycznym cieszy się dużą popularnością, lecz są też wśród nich substancje, których użycie jest kontrowersyjne. Chloroksylenol (4-chloro-3,5-dimetylofenol; PCMX) jest jednym z przedstawicieli środków przeciwdrobnoustrojowych, stosowanym w Stanach Zjednoczonych od lat 50. ubiegłego wieku. Wykorzystywany jest w produktach, takich jak płyny do dezynfekcji, mydła w płynie i roztwory stosowane w szpitalach do odkażania narzędzi chirurgicznych (Bruch 1996). PCMX stanowi także składnik aktywny płynu dezynfekcyjnego, znanego pod nazwą handlową Dettol®. Chloroksylenol występuje w postaci białego, krystalicznego proszku, który rozpuszcza się w alkoholu, eterze, benzenie, terpenach, olejach stałych oraz roztworach wodorotlenków alkalicznych. Swoją popularność w ostatnich latach, zyskał na skutek zakazu stosowania niektórych konserwantów w wybranych krajach, na przykład triklosanu. Związek ten wykazuje aktywność przeciwko bakteriom Gram-dodatnim i Gram-ujemnym, grzybom, algom oraz wirusom. Główny mechanizm działania PCMX polega na zaburzeniu funkcjonalności białek błonowych oraz zmianie przepuszczalności osłon komórkowych (Poger i Mark 2019). Pandemia koronawirusa SARS-CoV-2 przyczyniła się do znacznego wzrostu wykorzystania środków dezynfekcyjnych, w odpowiedzi na wzrost standardów higieny, nie tylko w szpitalach, ale również w gospodarstwach domowych. Chloroksylenol został wykorzystany jako składnik aktywny środków skutecznych przeciwko koronawirusowi, których masowe stosowanie przyczynia się do wzrostu jego obecności w ściekach. W konsekwencji, może powodować to zakłócenia prawidłowego funkcjonowania osadu czynnego. Ksenobiotyk ten został zidentyfikowany m. in. w ściekach surowych w Chinach w stężeniu 404 µg/L i pomimo wysokiej wydajności eliminacji w oczyszczalni (>95%), jego stężenie w ściekach oczyszczonych wynosiło od 19 do 3010 ng/L (Kasprzyk-Hordern i wsp. 2008, 2009; Zhong i wsp. 2012). Obecność PCMX w wodach powierzchniowych identyfikowana była w zakresie stężeń od kilkudziesięciu do kilkuset tysięcy ng/L (Dsikowitzky i wsp. 2016, 2017). Jednakże, nadal niewiele jest dostępnych danych literaturowych, opisujących jego występowanie w środowisku naturalnym.

Metyloizotiazolinon (2-metylo-4-izotiazolin-3-on, MIT) jest konserwantem powszechnie wykorzystywanym w produktach ochrony indywidualnej i chemii gospodarczej. Należy do grupy izotiazolinonów, które stosowane są jako konserwanty od lat 70. XX wieku. Izotiazolinony to heterocykliczne pochodne 2H-izotiazolin-3-onu zawierające w swojej strukturze atomy siarki i azotu. Charakteryzują się doskonałymi właściwościami przeciwdrobnoustrojowymi, wobec bakterii Gram-dodatnich, Gram-

ujemnych oraz grzybów strzępkowych. Dzięki obecności atomów siarki, reagują z cząsteczkami nukleofilowymi, wiążą się z grupami tiolowymi białek i w konsekwencji hamują aktywność enzymów, prowadząc do śmierci komórki. W produktach kosmetycznych, głównie w szamponach, odżywkach do włosów oraz żelach pod prysznic, MIT występuje często w postaci mieszaniny z metylochloroizotiazolinonem (Cebulski 2016; Berthet i wsp. 2017; Halla i wsp. 2018; Tomás i wsp. 2020). Stężenia MIT w oczyszczalni ścieków wahają się w zakresie od 39 do 860 ng/L, a wydajność eliminacji wynosi od 55 do 89%. Doniesienia dotyczące występowania MIT w środowisku naturalnym są bardzo ograniczone, co wskazuje na konieczność identyfikacji zanieczyszczeń z tej grupy w próbach środowiskowych.

Zarówno PCMX, jak i MIT charakteryzują się wysoką toksycznością wobec organizmów wodnych. Chloroksylenol wykazuje działanie genotoksyczne, hemotoksyczne, neurotoksyczne, embriotoksyczne, zakłóca także prawidłowe funkcjonowanie układu hormonalnego. Ponadto może wpływać na zmianę struktury zespołów mikroorganizmów w glebie (Capkin et al. 2017; Sreevidya et al. 2018). Metyloizotiazolinon, podobnie jak PCMX, może działać neurotoksycznie, genotoksycznie oraz hemotoksycznie, a także wpływać negatywnie na układ hormonalny (Du i wsp. 2002; He i wsp. 2006; Kresmann i wsp. 2018).

Eliminacja mikrozanieczyszczeń ze środowiska naturalnego jest procesem wieloetapowym, który zależy od licznych czynników, zarówno biologicznych np. potencjału degradacyjnego drobnoustrojów oraz czynników abiotycznych, takich jak temperatura, pH, promieniowanie UV, czy obecność wolnych rodników. Ksenobiotyki najczęściej nie są całkowicie mineralizowane i czasami mogą być przekształcane w bardziej toksyczne pochodne. Dane dotyczące mikrobiologicznej degradacji konserwantów są bardzo ograniczone, dlatego poznanie mechanizmów ich eliminacji ze środowiska stanowi wyzwanie dla biotechnologii środowiskowej. Jedynie nieliczne doniesienia naukowe opisują biodegradację metyloizotiazolinonu i chloroksylenolu.

Poznanie przemian środowiskowych, jakim podlegają MIT i PCMX pod wpływem aktywności metabolicznej mikroorganizmów środowiskowych, pozwoliłoby ocenić realne ryzyko dla ekosystemów w związku z obecnością tych konserwantów i ich metabolitów w wodzie lub glebie. Dlatego, zasadnym było podjęcie badań dotyczących określenia zdolności mikroorganizmów do eliminacji i detoksykacji tych biocydów.

OMÓWIENIE CELU NAUKOWEGO I UZYSKANYCH WYNIKÓW

Cel pracy

IV.2. Cel pracy

Celem badawczym niniejszej pracy doktorskiej było:

- Ocena stężeń środowiskowych metyloizotiazolinonu;
- Określenie zdolności wybranych grzybów strzępkowych do wzrostu w obecności metyloizotiazolinonu i chloroksylenolu;
- Analiza potencjału badanych szczepów do eliminacji metyloizotiazolononu i chloroksylenolu;
- Określenie mechanizmów biologicznej degradacji metyloizotiazolinonu i chloroksylenolu przez testowane drobnoustroje;
- Ocena toksyczności badanych konserwantów oraz ich metabolitów wobec organizmów bioindykacyjnych.

OMÓWIENIE CELU NAUKOWEGO I UZYSKANYCH WYNIKÓW

Metodologia badań

IV.3. Metodologia badań

Tabela 1. Podsumowanie technik badawczych wykorzystanych podczas realizacji pracy doktorskiej.

Technika	Zakres badań
Chromatografia cieczowa sprzężona z tandemową spektrometrią mas, LC-MS/MS, HPLC-MS/MS	• Analiza ilościowa eliminacji metyloizotiazolinonu przez wybrane grzyby strzępkowe • Analiza jakościowa powstających metabolitów w procesie biodegradacji metyloizotiazolinonu • Analiza zmian profilu fosfolipidowego wybranych bakterii glebowych pod wpływem metyloizotiazolinonu i chloroksylenolu
Chromatografia gazowa sprzężona ze spektrometrią mas GC-MS	• Analiza ilościowa eliminacji chloroksylenolu przez wybrane grzyby strzępkowe • Analiza jakościowa powstających metabolitów w procesie biodegradacji metyloizotiazolinonu oraz chloroksylenolu przez wybrane grzyby strzępkowe
Testy toksykologiczne typu Toxkit: ➢ Daphtoxkit F ➢ Phytotoxkit	• Ocena toksyczności metyloizotiazolinonu, chloroksylenolu oraz produktów pośrednich powstających podczas procesu biodegradacji
Spektrofotometria	• Ocena wpływu metyloizotiazolinonu i chloroksylenolu na wzrost bakterii glebowych • Ocena wpływu metyloizotiazolinonu i chloroksylenolu na produkcję biofilmu przez bakterie glebowe • Ocena wpływu metyloizotiazolinonu i chloroksylenolu na produkcję fitohormonu przez bakterie glebowe

Fluorymetria ➢ Test AlamarBlue® ➢ Test SYTOX Green®	• Ocena żywotności komórek bakteryjnych poddanych działaniu metyloizotiazolinonu i chloroksylenolu • Określenie wpływu metyloizotiazolinonu i chloroksylenolu na przepuszczalność osłon komórkowych bakterii glebowych
Real-time PCR	• Określenie udziału cytochromu P450 i reduktazy cytochromu P450 w przebiegu biodegradacji chloroksylenolu
Analiza statystyczna	• Analiza statystyczna otrzymanych wyników z wykorzystaniem oprogramowania Statistica (Statsoft, Polska)

OMÓWIENIE CELU NAUKOWEGO I UZYSKANYCH WYNIKÓW

Syntetyczne omówienie wyników badań przedstawionych w cyklu publikacji wchodzących w skład rozprawy doktorskiej

IV.4. Syntetyczne omówienie wyników badań przedstawionych w cyklu publikacji wchodzących w skład rozprawy doktorskiej

Cykl tematycznie spójnych publikacji otwiera praca pt. „*Cosmetic preservatives – hazardous micropollutants in need of greater attention?*" (Nowak-Lange M., Niedziałkowska K., Lisowska K. (2022). International Journal of Molecular Science. **IF$_{2021}$ = 6.208; MEiN = 140**). Artykuł jest przeglądem publikacji opisujących występowanie, toksyczność oraz mikrobiologiczną degradację wybranych konserwantów kosmetycznych. Szczegółowa analiza dostępnych danych literaturowych wykazała, iż triklokarban, chloroksylenol, metyloizotiazolinon oraz chlorek benzalkoniowy, ze względu na powszechne wykorzystanie w wielu produktach ochrony indywidualnej oraz nieefektywną eliminację w oczyszczalni ścieków, identyfikowane są w różnych matrycach środowiskowych, w tym wodach powierzchniowych, osadach i glebach. Jako główne źródła narażenia ekosystemów na te niebezpieczne ksenobiotyki, wyróżnia się zrzuty z oczyszczalni ścieków i osady ściekowe wykorzystywane w rolnictwie. Najważniejszymi czynnikami wpływającymi na ilość tych konserwantów emitowanych do środowiska są między innymi: wielkość aglomeracji, status ekonomiczny konsumentów oraz technologia wykorzystywana do oczyszczania ścieków. Liczne publikacje naukowe wskazują na potencjał toksyczny omawianych biocydów wobec organizmów wodnych i glebowych. Wśród niekorzystnych efektów wyróżnia się neurotoksyczność, genotoksyczność, embriotoksyczność, zaburzenia wzrostu, ruchliwości, wylęgu oraz wpływ na układ hormonalny. Dane dotyczące mikrobiologicznej degradacji konserwantów są bardzo ograniczone. Wykazano jednakże, że w trakcie procesów rozkładu tych związków mogą powstawać bardziej toksyczne produkty pośrednie. Na podstawie przeglądu literatury naukowej stwierdzono, iż najmniej opisane są metyloizotiazolinon oraz chloroksylenol. W związku z powyższym, to właśnie te dwa konserwanty zostały wykorzystane do badań eksperymentalnych i opisane w niniejszej rozprawie doktorskiej.

Pierwszym etapem badań prezentowanej rozprawy doktorskiej, był skrining grzybów strzępkowych, w kierunku oceny ich zdolności do wydajnej eliminacji metyloizotiazolinonu (wyniki nieopublikowane). Zbadano tolerancję na metyloizotiazolinon oraz skuteczność jego eliminacji przez 19 szczepów grzybów strzępkowych, pochodzących z kolekcji Katedry Mikrobiologii Przemysłowej i Biotechnologii Uniwersytetu Łódzkiego, wyizolowanych z terenów zanieczyszczonych różnymi ksenobiotykami oraz szczepy z niemieckiej kolekcji mikroorganizmów (DSM). Wykazano, że osiem spośród testowanych

szczepów eliminowało ponad 70% metyloizotiazolinonu w stężeniu 30 mg/L po 12 dniach inkubacji, w tym grzyby białej zgnilizny drewna *Phanerochaete chrysosporium* DSM 1556, które charakteryzowały się największą wydajnością w usuwaniu MIT z podłoża wzrostowego. W hodowlach szczepu *P. chrysosporium* inkubowanych z dodatkiem metyloizotiazolinonu, stwierdzono całkowity ubytek substratu po 96 godz. Otrzymane wyniki skłoniły do podjęcia dalszych badań, w celu określenia mechanizmu eliminacji MIT, analizy jakościowej powstających metabolitów oraz ich toksyczności, co było celem pierwszej pracy doświadczalnej składającej się na niniejszą rozprawę doktorską (Nowak M., Zawadzka K., Lisowska K., (2020). *Occurrence of methylisothiazolinone in water and soil samples in Poland and its biodegradation by Phanerochaete chrysosporium*, Chemosphere, 254: 126723, **IF_{2020} = 7.086; MEiN = 140**).

W pracy po raz pierwszy oceniono wpływ metyloizotiazolinonu na wzrost szczepu *P. chrysosporium* DSM 1556 oraz zdolność tego drobnoustroju do eliminacji testowanego ksenobiotyku. Wykazano, iż szczep *P. chrysosporium* charakteryzował się wysoką tolerancją zarówno wobec najniższego (50 µg\L), jak i najwyższego (30 mg/L) stężenia konserwantu. Proces eliminacji metyloizotiazolinonu przez *P. chrysosporium* najefektywniej zachodził w ciągu pierwszych 6 godz. inkubacji, po upływie których odnotowano 90% ubytek substratu. Po 12 godz. hodowli nie stwierdzono obecności biocydu w ekstrahowanych próbach w porównaniu do układów kontrolnych. Analiza jakościowa LC-MS/MS oraz GC-MS pozwoliła po raz pierwszy na identyfikację trzech produktów biodegradacji MIT – hydroksymetyloizotiazolinonu i dihydroksymetyloizotiazolinonu oraz kwasu N-metylomalonamowego.

Grzyby białej zgnilizny drewna, których przedstawicielem jest *P. chrysosporium*, charakteryzują się produkcją zewnątrzkomórkowych enzymów ligninolitycznych, o potencjale degradacyjnym wobec różnorodnych ksenobiotyków. W związku z tym oceniono wydajność eliminacji substratu inkubowanego z płynami pohodowlanymi *P. chrysosporium*. Stwierdzono wysoki stopień eliminacji MIT, dlatego też, ocenie poddano aktywność enzymów ligninolitycznych, takich jak lakaza, peroksydaza manganowa oraz peroksydaza ligninowa. Odnotowano aktywność lakazy, której maksymalna wartość wynosiła 41,85 nkat/L w 6 godz. inkubacji. Natomiast nie zaobserwowano aktywności peroksydazy manganowej oraz peroksydazy ligninowej. W wielu procesach mikrobiologicznej degradacji ksenobiotyków uczestniczą kompleksy cytochromu P450, dlatego też, w prezentowanej pracy postanowiono ocenić udział tych kompleksów enzymatycznych w usuwaniu MIT przez *P. chrysosporium*, inkubując hodowle grzybowe

z jednoczesnym dodatkiem MIT i inhibitorów cytochromu P450. Stwierdzono, że zastosowanie inhibitorów cytochromu P450 nie spowodowało zahamowania procesu eliminacji tego związku.

Liczne dane literaturowe opisują toksyczność MIT wobec organizmów wodnych, dlatego kolejnym etapem badań była ocena potencjału *P. chrysosporium* do detoksykacji MIT. Ocenę toksyczności MIT oraz powstających metabolitów przeprowadzono z wykorzystaniem skorupiaków *Daphnia magna* jako organizmów wskaźnikowych. Stwierdzono wysoką toksyczność MIT w stężeniach 5 i 50 mg/L. Natomiast analiza płynów pohodowlanych grzyba *P. chrysosporium* z dodatkiem metyloizotiazolinonu, wykazała spadek toksyczności o około 90% w porównaniu do kontroli abiotycznej, potwierdzając zdolność badanego drobnoustroju do detoksykacji MIT.

Powszechne wykorzystanie PCPs przyczynia się do przedostawania się ksenobiotyków do środowiska naturalnego. W ostatnim czasie obserwuje się wzrost zainteresowania badaczy identyfikacją zanieczyszczeń antropogenicznych w różnych matrycach środowiskowych. Substancje chemiczne identyfikowane w niskim zakresie stężeń, określane są jako mikrozanieczyszczenia, które ze względu na ciągłe przedostawanie się do środowiska naturalnego, mogą negatywnie wpływać na organizmy żywe. Dane literaturowe dotyczące identyfikacji MIT w próbach wody oraz gleby są bardzo ograniczone. W niniejszej pracy po raz pierwszy zidentyfikowano metyloizotiazolinon w próbkach ścieków oraz gleb pobranych z różnych regionów w Polsce. Najwyższe stężenie konserwantu zidentyfikowano w glebie pobranej z okolic myjni samochodowych w Poddębicach (10,8 µg/kg) oraz Tomaszowie Mazowieckim (5,92 µg/kg), a także w próbkach piasku pobranych z plaży w okolicach polskiego wybrzeża (2,19 – 4,48 µg/kg). MIT zidentyfikowano także w surowych ściekach w oczyszczalni ścieków w Łodzi, w stężeniu 1,21 µg/L.

Kolejnym etapem realizacji pracy doktorskiej był skrining grzybów strzępkowych, w kierunku oceny ich zdolności do degradacji chloroksylenolu. Analizie poddano 19 szczepów grzybów strzępkowych testowanych we wcześniejszej pracy (wyniki nieopublikowane). Na podstawie uzyskanych wyników wybrano dwa szczepy, które charakteryzowały się największym potencjałem degradacyjnym. Szczepy *Trametes versicolor* IM 373 oraz *Cunninghamella elegans* IM 1785/21GP zdolne były do eliminacji około 75% chloroksylenolu z podłoża wzrostu w ciągu 120 godz. inkubacji (30 mg/L). W związku z tym, podjęto badania zmierzające do określenia mechanizmów biodegradacji chloroksylenolu przez te szczepy.

Otrzymane wyniki opublikowano w pracy pt. „*Biodegradation of chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into ecotoxicity and metabolic pathways*" (Nowak M., Zawadzka K., Szemraj J., Góralczyk-Bińkowska A., Lisowska K. 2021. International Journal of Molecular Sciences, 22, 4360, **IF$_{2021}$ = 6.208; MEiN = 140**). Wykazano, iż badane drobnoustroje cechowały się wysoką tolerancją wobec chloroksylenolu. Jedynie najwyższe stężenie ksenobiotyku (50 mg/L) znacząco hamowało wzrost *T. versicolor* i *C. elegans* w porównaniu z hodowlami kontrolnymi (odpowiednio, 77% i 86%). Analiza ilościowa techniką GC-MS wykazała, iż eliminacja chloroksylenolu jest ściśle skorelowana ze wzrostem grzybni. Oba szczepy prowadziły wydajną eliminację substratu w zakresie stężeń, które nie wpływały hamująco na wzrost grzybni (5-25 mg/L). Po 24 godz. i 120 godz. inkubacji testowanych grzybów z dodatkiem PCMX w stężeniu 25 mg/L, wykazano odpowiednio 67 i 79% eliminacji konserwantu w hodowlach *T. versicolor* oraz 61 i 70% ubytku substratu dla *C. elegans*. W ekstraktach pohodowlanych *C. elegans*, po raz pierwszy zidentyfikowano dwa metabolity chloroksylenolu, 2,6-dimetylobenzeno-1,4-diol oraz 2,5-dihydroksy-3-metylobenzaldehyd, powstające na drodze dehalogenacji, hydroksylacji pierścienia aromatycznego oraz utlenienia grupy metylowej. Proces biodegradacji chloroksylenolu przez szczep *T. versicolor* zachodził na drodze dehalogenacji, hydroksylacji oraz utleniania, prowadzących do rozszczepienia pierścienia. Po raz pierwszy zidentyfikowano trzy metabolity rozkładu PCMX, kwas 4,6-dioksoheks-2-enowy, kwas 5-metylo-6-oksoheksa-2,4-dienowy oraz kwas 3-chloro-2,4-dimetyloheksa-2,4-dienodiowy.

W kolejnej części pracy analizowano mechanizm biodegradacji chloroksylenolu przez testowane mikroorganizmy. W pierwszym etapie określono aktywność cytochromu P450, inkubując hodowle grzybowe z jednoczesnym dodatkiem PCMX oraz inhibitorów badanego kompleksu enzymatycznego. Stwierdzono, że w obecności inhibitorów cytochromu P450 proces biotransformacji konserwantu przez *C. elegans* został zahamowany. Zaobserwowano ok. 82-96% odzysk ksenobiotyku po 120 godz. inkubacji. W celu potwierdzenia udziału enzymów cytochromu P450 w tym procesie, przy użyciu techniki RealTime-PCR, przeprowadzono ilościową analizę ekspresji genów cytochromu P450 oraz reduktazy cytochromu P450 w komórkach *C. elegans*. Wzrost ekspresji genów, kodujących wyżej wymienione enzymy, zaobserwowano już po 6 godz. inkubacji grzybów z chloroksylenolem. Natomiast szczyt indukcji ekspresji odnotowano w 24 godz. Otrzymane wyniki były skorelowane z dynamiką eliminacji chloroksylenolu, sugerując, że proces ten katalizowany jest przez enzymy cytochromu P450. W przypadku *T. versicolor* nie

zaobserwowano zmian w wydajności eliminacji chloroksylenolu, w obecności inhibitorów cytochromu P450, co może sugerować brak udziału tego kompleksu enzymatycznego w procesie biodegradacji PCMX u testowanego drobnoustroju. Dlatego też, kolejnym etapem badań było określenie udziału enzymów ligninolitycznych w eliminacji PCMX. Stwierdzono, że *T. versicolor* wykazywał zdolność do produkcji lakazy w podłożu suplementowanym chloroksylenolem. Najwyższy poziom tego enzymu zaobserwowano w 72 godz. inkubacji z substratem (156,4 U/L). Podczas dalszej inkubacji *T. versicolor* z PCMX zanotowano zarówno spadek produkcji lakazy, jak i zmniejszenie wydajności eliminacji biocydu. Na podstawie przeglądu danych literaturowych i otrzymanych wyników stwierdzono, iż metabolizm chloroksylenolu w komórkach grzybów białej zgnilizny drewna, takich jak *T. versicolor*, może zachodzić z udziałem enzymów ligninolitycznych, takich jak lakazy.

W ostatnim etapie badań ocenie poddano toksyczność zarówno chloroksylenolu, jak i jego metabolitów wobec bezkręgowców *D. magna* oraz roślin *Lepidium sativum* i *Sorghum saccharatum*. Wartość 48 LC_{50} chloroksylenolu wobec larw *D. magna* wynosiła 8,78 mg/L, natomiast 72 EC_{50} dla *L. sativum* i *S. saccharatum* wynosiły odpowiednio 30,79 mg/L oraz 15,4 mg/L. Stwierdzono znacząco niższą toksyczność płynów pohodowlanych (powyżej 50%) *C. elegans* oraz *T. versicolor* inkubowanych z dodatkiem chloroksylenolu, w porównaniu do kontroli abiotycznej, wykazując iż proces eliminacji PCMX ma charakter detoksykacji.

Jednymi z kluczowych źródeł zanieczyszczeń środowiska naturalnego PCPs są osady ściekowe oraz oczyszczone wody, które są wykorzystywane w rolnictwie do nawożenia gleb. Pozostałości substancji konserwujących, obecne w osadach ściekowych i oczyszczonych wodach, stanowią poważne zagrożenie dla ekosystemów glebowych, zaburzając procesy w nich zachodzące. Zarówno MIT, jak i PCMX, identyfikowane są w próbkach gleby, jednakże ich wpływ na organizmy bytujące w tych środowiskach nie został dokładnie zbadany. Dlatego też, ostatnia praca wchodząca w skład niniejszej rozprawy doktorskiej, obejmowała analizę toksyczności środowiskowej metyloizotiazolinonu oraz chloroksylenolu wobec bakterii glebowych *Pseudomonas putida* DSM 291, *Pseudomonas moorei* DSM 12647, *Sphingomonas mali* DSM 10565 i *Bacillus subtilis* DSM 3657 (Nowak-Lange M., Niedziałkowska K., Bernat P., Lisowska K., (2022). *In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria*. Scientific Reports, 12: 19068, **IF_{2021} = 4.997 oraz pkt. MEiN = 140**). Drobnoustroje te zaliczane są do bakterii poprawiających żyzność gleby oraz biorących

udział w obiegu pierwiastków. Ponadto, mikroorganizmy te zdolne są do produkcji fitohormonów, eliminacji zanieczyszczeń oraz ograniczania działania czynników stresogennych, takich jak zasolenie, czy obecność metali ciężkich, co prowadzi do poprawy warunków wzrostu roślin. Obecność biocydów, takich jak MIT oraz PCMX, może wpływać negatywnie na liczebność mikroorganizmów glebowych oraz zaburzać prawidłowe funkcjonowanie ekosystemu glebowego.

Stwierdzono, że inhibicja wzrostu wszystkich badanych mikroorganizmów skorelowana jest ze wzrostem stężenia ksenobiotyku. Wykazano, iż MIT jest związkiem bardziej toksycznym niż PCMX. Spośród badanych drobnoustrojów, większą opornością na zastosowane stężenia konserwantów charakteryzowały się szczepy z rodzaju *Sphingomonas* i *Bacillus*. Stwierdzono także, iż niższe stężenia badanych związków powodowały stymulację wzrostu szczepów *Pseudomonas* oraz *Bacillus*, sugerując, iż drobnoustroje mogą wykorzystywać te substraty jako źródło węgla i energii. Zbadano również wpływ chloroksylenolu i metyloizotiazolinonu na żywotność bakterii, która była powiązana z inhibicją wzrostu mikroorganizmów. W kolejnym etapie badań oceniono wpływ metyloizotiazolinonu i chloroksylenolu na tworzenie biofilmu przez bakterie glebowe. U badanych drobnoustrojów, narażonych na działanie MIT i PCMX, zaobserwowano zahamowanie produkcji biofilmu. Toksyczność testowanych ksenobiotyków wobec drobnoustrojów glebowych oceniono również na podstawie pomiaru ilości reaktywnych form tlenu (ROS). W hodowlach badanych bakterii, suplementowanych PCMX i MIT, zaobserwowano indukcję ROS wraz ze wzrostem stężenia konserwantów.

Bakterie glebowe charakteryzują się zdolnością do syntetyzowania hormonów roślinnych, stymulujących wzrost oraz wspomagających mechanizmy obronne roślin. Jednym z przykładów fitohormonów są auksyny, których produkcja może być hamowana w odpowiedzi na obecność substancji toksycznych w środowisku wzrostu bakterii glebowych. Dlatego też, oceniono wpływ metyloizotiazolinonu i chloroksylenolu na produkcję kwasu indolo-3-octowego (IAA), będącego przedstawicielem hormonów z grupy auksyn. Spośród badanych bakterii, tylko szczepy *Pseudomonas* wykazywały zdolność do wydajnej produkcji fitohormonu w układach kontrolnych bez dodatku ksenobiotyków. Zarówno metyloizotiazolinon, jak i chloroksylenol, powodowały inhibicję produkcji IAA przez testowane szczepy, przy czym metyloizotiazolinon wykazywał silniejszą toksyczność. Ostatni etap badań obejmował analizę wpływu metyloizotiazolinonu i chloroksylenolu na modyfikację struktury i integralności błony komórkowej badanych mikroorganizmów. Ocenę zmiany przepuszczalności błon bakteryjnych przeprowadzono z wykorzystaniem

testu SYTOX Green. Stwierdzono, iż metyloizotiazolinon i chloroksylenol w stężeniach powyżej 0,489 mg/L oraz 15,625 mg/L, powodowały zwiększenie przepuszczalności błony komórkowej. Fosfolipidy są głównym składnikiem błon komórkowych, a ich zawartość może się zmieniać w odpowiedzi na niekorzystne warunki środowiskowe, dlatego też zbadano profil fosfolipidowy testowanych drobnoustrojów. Analiza profilu wykazała, że dominującymi fosfolipidami w komórkach traktowanych ksenobiotykami oraz w kontrolach biotycznych były fosfatydyloetanoloamina (PE) i fosfatydyloglicerol (PG). U wszystkich badanych bakterii zaobserwowano zmianę stosunku PE/PG, co może sugerować modyfikację płynności błony komórkowej w odpowiedzi na toksyczne działanie tych biocydów. Uzyskane wyniki wskazują, że MIT i PCMX mogą stanowić zagrożenie dla ekosystemu glebowego, co stwarza konieczność prowadzenia monitoringu środowiskowego w kierunku obecności tych konserwantów.

WNIOSKI I STWIERDZENIA KOŃCOWE

V. Wnioski i stwierdzenia końcowe

Przegląd danych literaturowych pozwolił stwierdzić, że konserwanty kosmetyczne, takie jak triklokarban, chloroksylenol, metyloizotiazolinon oraz chlorek benzalkoniowy, są identyfikowane w ściekach, wodach powierzchniowych, osadach oraz glebach. Spośród opisywanych biocydów, najmniej poznanymi są metyloizotiazolinon oraz chloroksylenol. Doniesienia naukowe wskazują na toksyczność wymienionych konserwantów. Istnieją jedynie nieliczne prace dotyczące zdolności wybranych mikroorganizmów do degradacji i detoksykacji tych ksenobiotyków, brakuje natomiast danych opisujących przemiany środowiskowe konserwantów kosmetycznych.

W niniejszej pracy po raz pierwszy wykazano, że metyloizotiazolinon jest identyfikowany w Polsce, w próbach gleby i ścieków. Stwierdzono, że wśród badanych drobnoustrojów najwyższą tolerancją wobec metyloizotiazolinonu charakteryzowały się grzyby białej zgnilizny drewna *Phanerochaete chrysosporium* DSM 1556. Wykazano, że szczep *P. chrysosporium* całkowicie eliminuje MIT z podłoża wzrostowego. Analiza jakościowa LC-MS/MS i GC-MS/MS umożliwiła identyfikację trzech nowych metabolitów degradacji MIT przez testowany szczep: hydroksymetyloizotiazolinonu, dihydroksymetyloizotiazolinonu i kwasu N-metylomalonamowego. Wykazano także udział lakazy w tym procesie. Stwierdzono, że proces eliminacji ksenobiotyku przez *P. chrysosporium* DSM 1556 ma charakter detoksykacji.

Wykazano, że szczepy *Trametes versicolor* IM 373 oraz *Cunninghamella elegans* IM 1785/21Gp charakteryzują się najwyższą wydajnością eliminacji chloroksylenolu. Stwierdzono po raz pierwszy, że metabolizm tego konserwantu przez *C. elegans* przebiega na drodze dehalogenacji, hydroksylacji pierścienia aromatycznego oraz utlenienia grupy metylowej z wytworzeniem dwóch metabolitów chloroksylenolu: 2,6-dimetylobenzeno-1,4-diolu oraz 2,5-dihydroksy-3-metylobenzaldehydu. W przypadku *T. versicolor* zidentyfikowano po raz pierwszy trzy metabolity: kwas 4,6-dioksoheks-2-enowy, kwas 5-metylo-6-oksoheksa-2,4-dienowy oraz kwas 3-chloro-2,4-dimetyloheksa-2,4-dienodiowy, wykazując, że proces biodegradacji PCMX przez ten szczep przebiega na drodze dehalogenacji, hydroksylacji i utlenienia, prowadzących do rozszczepienia pierścienia. Stwierdzono, że degradacja chloroksylenolu przebiega z udziałem enzymów cytochromu P450 u *C. elegans* oraz lakazy u *T. versicolor*, a proces ma charakter detoksykacji.

Badania, pozwoliły także stwierdzić, że zarówno metyloizotiazolinon, jak i chloroksylenol, wykazują działanie toksyczne wobec bakterii glebowych. Stwierdzono, że dodatek ksenobiotyków do hodowli bakterii *Pseudomonas putida* DSM 291, *Pseudomonas moorei* DSM 12647, *Sphingomonas mali* DSM 10565 i *Bacillus subtilis* DSM 3657, powoduje obniżenie żywotności, zdolności do produkcji biofilmu oraz zahamowanie syntezy fitohormonu — kwasu indolo-3-octowego oraz wzrost ilości reaktywnych form tlenu. Zaobserwowano także modyfikacj struktury błony komórkowej bakterii w odpowiedzi na toksyczne działanie konserwantów.

Przeprowadzone badania wykazały możliwość wykorzystania w procesach biotechnologicznych drobnoustrojów zdolnych do wydajnej eliminacji i detoksykacji metyloizotiazolinonu i chloroksylenolu. Ponadto, uzyskane wyniki pozwoliły na poznanie potencjalnych przemian środowiskowych badanych ksenobiotyków oraz ocenę ich ryzyka toksykologicznego wobec organizmów wodnych i glebowych.

STRESZCZENIE

VI. Streszczenie

VI.1. Streszczenie w języku polskim

Produkty ochrony indywidualnej (ang. Personal Care Products, PCPs) należą do mikrozanieczyszczeń coraz częściej identyfikowanych w różnych matrycach środowiskowych. Szczególne obawy budzi zanieczyszczenie ekosystemów dodatkami do PCPs, takimi jak konserwanty, jeszcze do niedawna uważanymi za nieszkodliwe dla środowiska. Pomimo, iż związki te występują w środowisku w niskich stężeniach to charakteryzują się wysoką aktywnością biologiczną, potencjałem do bioakumulacji oraz niską biodegradowalnością. Obecność substancji konserwujących w środowisku naturalnym stanowi poważne zagrożenie dla funkcjonowania ekosystemów wodnych i glebowych, co stwarza potrzebę poszukiwania efektywnych metod ich eliminacji i detoksykacji.

W pracy przeglądowej wykazano, iż konserwanty, takie jak triklokarban, chloroksylenol (PCMX), metyloizotiazolinon (MIT) oraz chlorek benzalkoniowy, ze względu na powszechne wykorzystanie w wielu produktach ochrony indywidualnej oraz nieefektywną eliminację w oczyszczalni ścieków, identyfikowane są w wodach powierzchniowych, osadach dennych oraz glebach. Dane dotyczące ich mikrobiologicznej degradacji są bardzo ograniczone. Spośród opisywanych związków, najmniej poznanymi są MIT i PCMX, pomimo znacznego wzrostu ich wykorzystania w produkcji środków dezynfekcyjnych w ostatnim czasie. Dlatego też ksenobiotyki te zostały wybrane jako przedmiot badań prezentowanej pracy.

Rozprawa doktorska miała na celu poznanie przemian środowiskowych, jakim podlegają te konserwanty, ocenę ich ryzyka toksykologicznego wobec organizmów wodnych i glebowych, a także wyselekcjonowanie drobnoustrojów zdolnych do eliminacji tych związków.

W pracy wykazano po raz pierwszy, iż MIT jest identyfikowany w Polsce w próbach gleby i ścieków. Stwierdzono, że wśród badanych drobnoustrojów najwyższą tolerancją wobec MIT charakteryzował się szczep *Phanerochaete chrysosporium* DSM 1556. Dlatego też oceniono zdolność tego drobnoustroju do biodegradacji testowanego ksenobiotyku. Wykazano, że szczep *P. chrysosporium* całkowicie eliminuje MIT z podłoża wzrostowego. Analizy LC-MS/MS i GC-MS/MS ekstraktów pohodowlanych umożliwiły identyfikację trzech nowych produktów biodegradacji – hydroksymetyloizotiazolinonu, dihydroksymetyloizotiazolinonu i kwasu N-metylomalanowego. Wykazano także udział lakazy w tym procesie. Analiza toksyczności testowanego związku oraz powstających

metabolitów z wykorzystaniem skorupiaków *Daphnia magna* wykazała, że proces eliminacji MIT ma charakter detoksykacji.

W kolejnym etapie badań analizowano mechanizm rozkładu chloroksylenolu przez szczepy *Cunninghamella elegans* IM 1785/21GP oraz *Trametes versicolor* IM 373, które charakteryzowały się największym potencjałem degradacyjnym. Stwierdzono po raz pierwszy, iż metabolizm tego konserwantu przez *C. elegans* przebiega na drodze dehalogenacji, hydroksylacji pierścienia aromatycznego oraz utlenienia grupy metylowej z wytworzeniem dwóch metabolitów: 2,6-dimetylobenzeno-1,4-diolu oraz 2,5-dihydroksy-3-metylobenzaldehydu. W przypadku *T. versicolor* zidentyfikowano po raz pierwszy trzy metabolity: kwas 4,6-dioksoheks-2-enowy, kwas 5-metylo-6-oksoheksa-2,4-dienowy oraz kwas 3-chloro-2,4-dimetyloheksa-2,4-dienodiowy, wykazując, że proces biodegradacji PCMX przez ten szczep przebiega na drodze: dehalogenacji, hydroksylacji i utlenienia, które prowadzą do rozszczepienia pierścienia. Stwierdzono, że degradacja chloroksylenolu przebiega z udziałem enzymów cytochromu P450 u *C. elegans* oraz lakazy u *T. versicolor*, a proces ma charakter detoksykacji.

Ostatnim etapem badań była ocena toksyczności środowiskowej metyloizotiazolinonu i chloroksylenolu wobec bakterii glebowych *Pseudomonas putida* DSM 291, *Pseudomonas moorei* DSM 12647, *Sphingomonas mali* DSM 10565 i *Bacillus subtilis* DSM 3657. Stwierdzono, iż zarówno MIT, jak i PCMX, powodują obniżenie żywotności, zdolności do produkcji biofilmu, zahamowanie syntezy fitohormonu – kwasu indolo-3-octowego oraz wzrost ilości reaktywnych form tlenu u badanych bakterii. Odnotowano także zmiany przepuszczalności osłon komórkowych oraz zmiany profilu fosfolipidowego badanych bakterii, w odpowiedzi na toksyczne działanie MIT i PCMX.

Przeprowadzone badania wykazały możliwość wykorzystania w procesach biotechnologicznych drobnoustrojów zdolnych do wydajnej eliminacji i detoksykacji metyloizotiazolinonu i chloroksylenolu. Ponadto, uzyskane wyniki pozwoliły na poznanie potencjalnych przemian środowiskowych badanych ksenobiotyków oraz ocenę ich ryzyka toksykologicznego wobec organizmów wodnych i glebowych.

VI.2. Streszczenie w języku angielskim

Personal Care Products (PCPs) are micropollutants that are increasingly identified in various environmental matrices. Of particular concern is the contamination of ecosystems with additives to PCPs, such as preservatives, until recently considered harmless to the environment. Despite the fact that these compounds occur in the environment in low concentrations, they are characterized by high biological activity, the potential for bioaccumulation and low biodegradability. The presence of preservatives in the natural environment poses a serious threat to the functioning of water and soil ecosystems, which creates the need to search for effective methods of elimination and detoxification.

A review of the literature data showed that, due to their widespread use in many personal protection products and ineffective elimination in sewage treatment plants, preservatives such as triclocarban, chloroxylenol (PCMX), methylisothiazolinone (MIT) and benzalkonium chloride, are identified in surface waters, bottom sediments, and soils. Scientific reports also indicate the toxicity of these preservatives. However, data on their microbial degradation are minimal. Of the described compounds, the least known are MIT and PCMX, therefore these preservatives were selected for the study.

The aim of the doctoral dissertation was to investigate the environmental changes that both xenobiotics are subject to, assess their toxicological risk to aquatic and soil organisms, as well as find microorganisms capable of eliminating these compounds.

It was demonstrated for the first time that MIT is found in soil and sewage samples in Poland. It was revealed that the strain *Phanerochaete chrysosporium* DSM 1556 was characterized by the highest tolerance to MIT among the tested microorganisms. Therefore, the ability of this microorganism to eliminate the tested xenobiotic was assessed. The *P. chrysosporium* strain was shown to completely eliminate MIT from the growth medium. LC-MS/MS and GC-MS/MS analyses of post-culture extracts enabled the identification of three new biodegradation products: hydroxymethylisothiazolinone, dihydroxymethylisothiazolinone and N-methylmalamic acid. The participation of laccase in this process was also demonstrated. The analysis of the toxicity of the tested compound and the formed metabolites with the use of *Daphnia magna* crustaceans showed that the process of MIT elimination is based on detoxification.

In the next stage of the research, the mechanism of chloroxylenol biodegradation by the strains *Cunninghamella elegans* IM 1785 / 21GP and *Trametes versicolor* IM 373, which were characterized by the highest degradation potential, was analyzed. It was found for the

first time that the metabolism of this preservative by *C. elegans* proceeds by dehalogenation, hydroxylation of the aromatic ring and oxidation of the methyl group with the formation of two chloroxylenol metabolites. In the case of *T. versicolor*, three metabolites were identified for the first time, showing that the biodegradation of PCMX by this strain proceeds by dehalogenation, hydroxylation and oxidation leading to ring cleavage. It was revealed that the degradation of chloroxylenol is mediated by cytochrome P450 enzymes in *C. elegans* and laccase in *T. versicolor*, and the process is based on detoxification.

The last stage of the research was the assessment of the environmental toxicity of methylisothiazolinone and chloroxylenol against soil bacteria *Pseudomonas putida* DSM 291, *Pseudomonas moorei* DSM 12647, *Sphingomonas mali* DSM 10565 and *Bacillus subtilis* DSM 3657. It was found that both MIT and PCMX caused a reduction of vitality and the ability to form biofilm, as well as inhibited phytohormone synthesis - indole-3-acetic acid, and increased the number of reactive oxygen species in the tested bacteria. Changes in the permeability of the cell membranes and in the phospholipid profile of the tested bacteria were also demonstrated in response to the toxic effects of MIT and PCMX.

The conducted research showed the possibility of using microorganisms capable of efficient elimination and detoxification of methylisothiazolinone and chloroxylenol in biotechnological processes. Moreover, the obtained results made it possible to learn about the potential environmental changes of the xenobiotics studied and to assess their toxicological risk to aquatic and soil organisms.

DOROBEK NAUKOWY

VII. Dorobek naukowy

VII.1. Sumaryczny dorobek naukowy

- Publikacje – 7, IF_{Σ} = 46,917, h = 4, MEiN = 1040, liczba cytowań[1]: 37;
- Doniesienia konferencyjne – 18;
- Szkolenia i kursy – 7;
- Organizacja konferencji – 3.

VII.2. Spis publikacji niewchodzących w skład rozprawy doktorskiej

- *The synergy of ciprofloxacin and carvedilol against staphylococcus aureus – prospects of a new treatment strategy?* Katarzyna Zawadzka, **Marta Nowak**, Ireneusz Piwoński, Katarzyna Lisowska. Molecules, 2019; 24(22), 24224104. IF_{2019} = 3.267, $IF_{5\text{-letni}}$ = 3.589, MEiN = 140
- *Antimicrobial effect of chitosan films on food spoilage bacteria.* Natalia Wrońska, Nadia Katir, Katarzyna Miłowska, Nisrine Hammi, **Marta Nowak**, Marta Kędzierska, Aicha Anouar, Katarzyna Zawadzka, Maria Bryszewska, Abdelkrim El Kadib, Katarzyna Lisowska. International Journal of Molecular Sciences, 2021; 22(11), 5839. IF_{2021} = 6.208, $IF_{5\text{-letni}}$ = 6.628, MEiN = 140
- *Antimicrobial activity and toxicological risk assessment of silver nanoparticles synthesized using an eco-friendly method with Gloeophyllum striatum.* Katarzyna Zawadzka, Aleksandra Felczak, **Marta Nowak**, Aleksandra Kowalczyk, Ireneusz Piwoński, Katarzyna Lisowska. Journal of Hazardous Materials, 2021; 418, 126316. IF_{2021} = 14.224, $IF_{5\text{-letni}}$ = 12.984, MEiN = 200
- *Effect of Quinoline on the Phospholipid Profile of Curvularia lunata and Its Microbial Detoxification.* Aleksandra Felczak, Katarzyna Zawadzka, Przemysław Bernat, **Marta Nowak-Lange**, Katarzyna Lisowska. Molecules, 2022; 27(7), 2081. IF_{2021} = 4.927, $IF_{5\text{-letni}}$ = 5.110, MEiN = 140

[1] Dane z dnia: 9.11.2022 r. wg bazy Scopus

VII.3. Doniesienia konferencyjne

- *Screening for microscopic filamentous fungi applicable to the elimination of benzalkonium chloride*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – International Conference for Young Researchers 2018, Kraków;
- *Evaluation of the antimicrobial activity of carbazole derivatives*; Katarzyna Zawadzka, **Marta Nowak**, Katarzyna Lisowska – European Biotechnology Congress 2018, Ateny;
- *Modification of the cell membrane structure of the filamentous fungi in response to pharmaceuticals and personal care products*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – 4th International Conference of Cell Biology, Kraków;
- *Microbial elimination of personal care product- methylisothiazolinone*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – Biotechnologia- Nauka i Aplikacje 2018, Wrocław;
- *The use of liquid chromatography and mass spectrometry in the analysis of environmental fate of the compounds belonging to personal care products (PCPs)*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – XII Copernican International Young Scientists Conference 2018, Toruń;
- *Microbial degradation of selected compounds belonging to the group of personal care products (PCPs)*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – XII Copernican International Young Scientists Conference 2018, Toruń;
- *Determination of biocides in environmental samples using LC-MS/MS*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – 8th European Young Engineers Conference 2019, Warszawa;
- *The use of LC-MS/MS in the analysis the role of cytochrome P-450 in the PCPs elimination by fungi of the genus Basidiomycetes*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – 8th European Young Engineers Conference 2019, Warszawa;
- *Eliminacja związków z grupy PCPs przez grzyby strzępkowe z rodzaju Trichoderma*; Aleksandra Tończyk, Magdalena Ulanowska, **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – V Ogólnopolska Konferencja Doktorantów Nauk o Życiu BIOOPEN 2019, Łódź;
- *Eliminacja chloroxylenolu (PCMX) przez szczep Trichoderma citrinoviridae*; Magdalena Ulanowska, Aleksandra Tończyk, **Marta Nowak**, Katarzyna Zawadzka,

Katarzyna Lisowska – V Ogólnopolska Konferencja Doktorantów Nauk o Życiu BIOOPEN 2019, Łódź;

- *Mikrobiologiczna degradacja chloroksylenolu*; Aleksandra Tończyk, Magdalena Ulanowska, **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – III Sesja Młodych Mikrobiologów Środowiska Łódzkiego 2019, Łódź;
- *Microbial degradation of selected compounds belonging to the group of isothiazolinones*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – Pomiędzy naukami VIII Edycja Konferencji dla Młodych Naukowców 2019, Chorzów;
- *Enhancement of antimicrobial activity by co-adminitraion of titanium oxide nanoparticles and chosen antibiotics*; Katarzyna Zawadzka, **Marta Nowak**, Aleksandra Felczak, Mariusz Krupiński, Katarzyna Lisowska – 7th Central European Congress of Life Science 2019, Kraków;
- *Detoxification of selected N-heterocyclic compounds by the soil fungus Trichoderma citrinoviride*; Aleksandra Felczak, **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – 7th Central European Congress of Life Science 2019, Kraków;
- *Antibacterial activity of chitosan-metal oxide films*, Natalia Wrońska, **Marta Nowak**, Abdelkrim El Kadib, Katarzyna Lisowska – 7th Central European Congress of Life Science 2019, Kraków;
- *Occurrence and ecological risk of preservatives present in personal care products*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – 7th Central European Congress of Life Science 2019, Kraków;
- *The elimination of preservatives present in personal care products by filamentous fungi*; **Marta Nowak**, Katarzyna Zawadzka, Katarzyna Lisowska – 7th Central European Congress of Life Science 2019, Kraków;
- *Biodegradacja metyloizotiazolinonu przez Phanerochaete chrysosporium*, **Marta Nowak**, Katarzyna Zawadzka, Aleksandra Tończyk, Katarzyna Lisowska – MycoRise Up! Młodzi w Mykologii 2021, online (wyróżnienie).

VII.4. Działalność naukowa

VII.4.1. Projekty

- *Mikrobiologiczna degradacja związków z grupy Personal Care Products (PCPs)*. Finansowanie: Ministerstwo Nauki i Szkolnictwa Wyższego. 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. Okres realizacji projektu: 1.10.2019-31.12.2019; kwota finansowania: 5 000 zł; stanowisko: kierownik.

- *Badanie aktywności biologicznej nowosyntetyzowanych nanokompozytów chitozanowych.* Finasowanie: Narodowe Centrum Nauki, OPUS 13. Okres realizacji projektu: 24.01.2018-23.01.2023; kwota finansowania: 652 320 zł; stanowisko: stypendysta.

VII.4.2. Kursy i szkolenia

- *Wysokorozdzielcza Spektrometria Mas… dla każdego*; Warsztaty firmy SCIEX 14.03.2019, Łódź;
- *Dni Thunder Leica Imager*; Warsztaty firmy Leica Microsystems 8.05.2019, Łódź;
- *Jak dbać o spektrometr mas. Część 2: Czyszczenie źródła jonów i elementów drogi optycznej*; Webinar firmy Bioanalytic 22.06.2021, online;
- *Co może łączyć super sportowy samochód i Spektrometr Mas?*; Webinar firmy Bioanalytic 23.06.2021, online;
- *Wystąpienia publiczne – tego można się nauczyć!*; Webinar Fundacji Science Watch Polska 2.03.2022, online;
- *Spektrometria mas w diagnostyce laboratoryjnej*; Webinar firmy Bioanalytic 30.03.2022, online;
- *Chromatografia cieczowa – najczęstsze problemy i ich rozwiązywanie*; Webinar firmy Bioanalytic 7.09.2022, online.

VII.4.3. Pobyty naukowe

- Rezerwat Dzikiej Przyrody i Centrum Edukacji „Our Land” Tajlandia, Prowincja Kanchanaburi; poszukiwanie nowych szczepów mikroorganizmów zdolnych do biodegradacji ksenobiotyków; 02.2020.

VII.5. Działalność organizacyjna

VII.5.1. Organizacja konferencji

- IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu BIOOPEN, Łódź, 24-25.05.2018;
- II Sesja Młodych Mikrobiologów Środowiska Łódzkiego, 8.06.2018;
- V Ogólnopolska Konferencja Doktorantów Nauk o Życiu BIOOPEN, Łódź, 30-31.05.2019.

VII.5.2. Komisje UŁ

- Członek Uczelnianej Komisji Stypendialno-Socjalnej dla Doktorantów UŁ – lata 2017-2021;
- Członek Wydziałowej Komisji Doktoranckiej ds. stypendium doktoranckiego i jego zwiększenia z dotacji projakościowej – od 2018 do chwili obecnej;
- Członek Komisji Dydaktycznej ds. kierunku Biotechnologia – od 2020 do chwili obecnej.

VII.5.3. Popularyzacja nauki

- Współorganizator warsztatów w ramach projektu „Uniwersytet Łódzki dla Dzieci” – lata 2017, 2018, 2019;
- Współorganizator warsztatów w ramach projektu „Instytut Kreatywnej Biologii” – lata 2017, 2019, 2020;
- Członek i opiekun Studenckiego Koła Naukowego Biotechnologiczno – Mikrobiologicznego „Bio-Mik” – od 2017 do chwili obecnej.

LITERATURA UZUPEŁNIAJĄCA

VIII. Literatura uzupełniająca

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IX. Załączniki

IX.1. Kopie publikacji wchodzących w skład rozprawy doktorskiej

Cosmetic preservatives – hazardous micropollutants in need of greater attention?

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Review

Cosmetic preservatives- hazardous micropollutants in need of greater attention?

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Abstract: In recent years, Personal Care Products (PCPs) have surfaced as a novel class of pollutants due to their release to wastewater treatment plants (WWTPs) and receiving environments by sewage effluent and biosolid-augmentation soil, which poses potential risks to non-target organisms. Among PCPs, there are preservatives which are added to cosmetics, for protection against microbial spoilage. This paper reviews the occurrence in different environmental matrices, toxicological effects and mechanisms of microbial degradation of four selected preservatives (triclocarban, chloroxylenol, methylisothiazolinone and benzalkonium chloride). Due to the insufficient removal in WWTP, cosmetic preservatives have been widely detected in aquatic environments and sewage sludge at concentrations mainly below tens of μg L-1. These compounds are toxic to aquatic organisms such as fish, algae, daphnids and rotifer, as well as toward terrestrial organisms. Summary of the mechanisms of preservatives biodegradation by microorganisms and analysis of emerging intermediates was also provided. Formed metabolites are often characterized by lower toxicity compared to the parent compounds. Further studies are needed for an evaluation of environmental concentrations of preservatives in diverse matrices, toxicity to more species of aquatic and terrestrial organisms, and understanding of the mechanisms of microbial degradation. The research should focus on chloroxylenol and methylisothiazolinone because these compounds are the least understood.

Keywords: preservatives;; ecotoxicity; microbial degradation; personal care products; triclocarban; chloroxylenol; methylisothiazolinone; benzalkonium chloride

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1. Introduction

Control and monitoring of environmental pollution have so far focused on priority pollutants that are regulated and considered hazardous, toxic, persistent or accumulative. However, in the last decade, there has been a significant increase in interest in the occurrence of new pollutants, and their fate in the environment and potential toxicity. Many of these substances are not newly discovered chemicals, but compounds that have been used for decades and are only now questionable. Many of them are considered potentially significant environmental pollutants despite the lack of regulations and restrictions [1–3].

Among these pollutants, there are additives used in daily products named personal care products (PCPs), which are used to improve the quality of everyday life. The Global Beauty Market (GMB) is usually divided into five main business sectors: hair care, skin care, color (makeup), fragrances and toiletries. Statistical studies conducted in the United States have shown that one woman and one man use 12 and 6 cosmetics products per day, respectively [4]. The widespread use of PCPs, the cult of beauty and strong competition on the cosmetics market make this industry one of the fastest growing industries in the world. The majority of global premium cosmetics sales is concentrated within the

developed markets (mostly the USA, Japan, and France). For the production of cosmetics, slightly more than 12 thousand chemicals are used, of which less than 20% have been recognized as completely safe for human health and the environment [5,6]. In addition, not all countries are required to control products entering the market. Therefore, in the last few years there have been concerns about the widespread use of personal care products and their impact on the environment [4]. These compounds are released to the environment mainly from anthropogenic sources and are defined by the Environmental Protection Agency of US (US-EPA) as new compounds without regulatory status whose impact on the environment and human health is poorly understood [7]. PCPs are most often intended for external use, thus they get directly to the sewage treatment plants, not subject to previous metabolic changes. Their extensive use and improper disposal contribute to the contamination of soils and aquatic ecosystems by PCPs. The largest sources of PCPs are sewage effluents from wastewater treatment plants (WWTPs) [8,9], where the removal of PCPs in many is still unsatisfactory and requires continuous optimization of elimination processes. The treated effluents are discharged into receiving waters, including small streams, rivers and lakes and there are even places where the wastewater is released into the environment without previous treatment, being directly discharged into riverine habitats or water bodies. Reclaimed water and sewage sludge can be used in agricultural production and become a source of soil pollution. Excessive penetration of PCPs into the environment may contribute to the accumulation of these xenobiotics in the soil, they can then enter groundwater or be absorbed by plants and crops and enter food chains. As they occur in the natural environment in low concentrations (ng L^{-1}, μg L^{-1}), they are known as micropollutants. Furthermore, due to their continuous entry into the environment and synergic effects resulting from the coupled parallel activity, even low concentrations of chemicals may have undesirable consequences in the environment [8,10,19–22,11–18].

Owing to the global use of PCPs and their potential for negative effects in human and wildlife, a rising number of studies assess the presence of additives for PCPs in environmental matrices. This review summarizes recent publications regarding the occurrence, toxicity and transformation of most ecotoxic cosmetic preservatives found in the natural environment.

2. Characteristics of preservatives in cosmetics

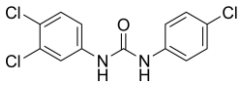
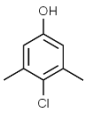
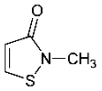
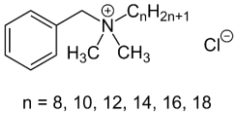
The presence of water, a large amount of nutrients and the way the consumer uses the cosmetics promote the proliferation of microorganisms in selected products. Such impurities can pose a threat to the health of the users and also adversely affect the organoleptic properties of the products [23,24]. In order to prevent microbial growth in cosmetics while extending their shelf life and the period of use of the products, most manufacturers use synthetic preservatives. Because of their biological activity, preservatives present a wide spectrum of undesirable effects for consumers, such as toxicity, irritation or sensitization. Therefore, the safe use of these compounds is always being called into question. Nowadays, more and more producers decide not to use traditional preservatives in view of the negative public opinion about them. Manufactures who use new and little known chemicals, claim that their products are “free” from potentially toxic compounds. Literature data of the possible harmful effects of some preservatives has led to increasing various international regulations and some of chemicals have been banned in cosmetic products. In the European Union, European Chemical Agency (ECHA) created a list of compounds for Personal Care Products preservation from microbial spoilage, according to Annex V, Regulation 1223/2009/EC on Cosmetic Products, as amended by Regulation (EU) 2021/1902, 3 November 2021 [25]. In the United States, the Cosmetic Ingredient Review (CIR), led by a panel of medical experts collaborates with the US Food and Drug Administration (FDA) to provide a review and assessment of the safety of ingredients used in cosmetics. Regulations regard the type and amount of preservatives added to cosmetics, nevertheless, the regulatory issues concerning preservatives in cosmetics are different in other countries [23,26,27]. It is important to point out that the regulatory status of

preservatives is very dynamic and varies from region to region, even from country to country [27,28].

The microbial stability of cosmetic preparations without preservatives is very short, therefore it is impossible to completely exclude them from cosmetic products manufactured on a large scale. There are some characteristics to take into consideration in preservative selection. The agents should have a broad spectrum and be active against all possible bacteria and fungi. When choosing preservatives, their stability is also very important in a wide range of pH and temperatures, no interaction with other cosmetic ingredients and resistance to light and oxygen. Compounds serving as preservatives should be colorless, tasteless and palpable and should not undergo hydrolysis. All these features mean that these substances can be considered potentially harmful to the environment and to humans. Furthermore, given their widespread use in daily life, treatment of environmental contamination is challenging, as preservative avoidance may be very difficult to achieve.

Nowadays, most ecotoxicity preservatives, based on the acute toxicity tests on aquatic organisms include organochloride compounds, isothiazolinones and quatenary ammonium compounds (QACs). This section will characterize most controversial examples of preservatives used in cosmetics (Table 1).

Table 1. Basic information on the target preservatives.

INCI Name	**Triclocarban**	**Chloroxylenol**	**Methylisothiazolinone**	**Benzalkonium chloride**
Acronym	**TCC**	**PCMX**	**MIT**	**BAC**
CAS Number	101-20-2	88-04-0 / 1321-23-9	2682-20-4	63449-41-2 / 68391-01-5 / 68424-85-1 / 85409-22-9
Formula	$C_{13}H_9Cl_3N_2O$	C_8H_9OCl	C_4H_5NOS	$C_9H_{13}ClNR$ (R = C_8H_{17} to $C_{18}H_{37}$)
Molecular weight	315.58 g mol^{-1}	156.61 g mol^{-1}	115.1 g mol^{-1}	-
Structure				 n = 8, 10, 12, 14, 16, 18

2.1. Organochloride compounds

Organochloride preservatives have rather varying success in the marketplace, ranging from highly controversial and almost banned molecules, to very popular and successful. Preservatives from this group include chemicals, such as triclocarban or chloroxylenol. Triclocarban [1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl)urea; TCC] is a trichlorinated, binuclear phenylurea pesticide that has been used globally as an ingredient in disinfectants, deodorants, soaps, toothpastes and mouthwashes. Its concentration in the products has been approved by the European Union (EU) in 0.2% [29]. TCC has been made and marketed on a massive scale since 1957, and its consumption was achieved at 227-454 tons in the USA annually [30–32]. Most commercially obtainable TCC is available in the solid form as a white to off white crystalline powder with a slight aromatic odor. TCC's mechanism of action is unknown, however, it is thought to be comparable to that of triclosan (TCS, another antiseptic active component commonly found in PCPs), which has a similar structure [33]. TCS exhibits a biostatic and biocidal efficacy against Gram-positive and Gram-negative bacteria, fungi, as well as against viruses. It permeates the bacterial cell wall and targets multiple cytoplasmic and membrane sites, including RNA synthesis and the production of macromolecules [34]. TCS also blocks synthesis of fatty acids through inhibition of enoyl reductase, but has no effect on bacterial spores [34–36].

Chloroxylenol [4-Chloro-3,5-dimethylphenol; PCMX] is an antibacterial agent applied in disinfectant products in the United States since the 1950s, such as liquid soaps, hand washing liquid, and solutions used in hospitals to clean surgical instruments etc. [37]. PCMX is a white to off-white crystalline powder soluble in alcohol, ether, benzene,

terpenes, fixed oils, and solutions of alkali hydroxides and it is sparingly soluble in water. Some antibacterial ingredients, such as triclosan in PCPs, have been banned in some countries, leading to an increase in the use of antibacterial alternatives such as PCMX. The use of PCMX as an antibacterial ingredient in PCPs has been on the rise [38]. PCMX is the active ingredient in Dettol disinfectant solution, and has unique in vitro and in vivo antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, algae, and viruses. The main mechanisms of its action are altering the integrity of membrane proteins, changing the permeability of the cell wall and disrupting its biological processes. The maximum concentration of PCMX in ready for use preparation is 0.5% [39]. The worldwide SARS-CoV-2 pandemic has forced a significant increase in the amount of used disinfectants. Increased hygiene standards became applicable not only in hospitals but also in households. The Singapore's National Environment Agency (NEA) has prepared a list of active substances that are effective against the virus. One of the active components is chloroxylenol, which has a concentration of 0.12%. [40].

2.2. Isothiazolinones

Isothiazolinones are a group of chemicals with antimicrobial effect, which since the 1970s have been used as preservatives in cosmetics, in other consumer products, and in chemical products for occupational use. These compounds are heterocyclic derivatives of 2H-isothiazolin-3-one chemicals containing vicinal sulfur and nitrogen atoms. Isothiazolinones exhibit excellent broad-spectrum antimicrobial activity against gram positive, gram negative bacteria and fungi at low concentrations and over a wide range of pH. Due to the sulfur heterocycle, they react with nucleophilic molecules, bind to the thiol groups of proteins and consequently inhibit the activity of enzymes that are essential for growth and metabolism, which leads to microbial cell death after a few hours of contact. A number of isothiazolinones exist which all may be applied in products for occupational use, while only two have been permitted in cosmetic products. These preservatives are often masked under the chemical names of their mixtures: the mixture of methylchloroisothiazolinone with methylisothiazolinone (MCI/MI) in 3:1, also often named by its tradename Kathon™. The cosmetics industry commonly includes Kathon in a wide range of both rinse-off and leave-on formulations such as shampoos, gels and hair and skin care products. Methylisothiazolinone (MI, MIT) is one of the most used preservatives in shampoos and one of the most effective. In March 2013 MIT has been called an "Allergen of the Year" and its usage has been self-restricted to rinse-off applications. In consumer products other than cosmetics, different isothiazolinones are used, including benzisothiazolinone (BIT) and octylisothiazolinone (OIT) [27,41–46].

2.3. Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) mainly represent cationic surfactants. In terms of chemical structure, quaternary ammonium compounds belong to ionic compounds that contain 4 organic groups in the molecule and are associated with nitrogen atoms (including 3 covalent and 1 coordination bonds).The antimicrobial activity of QACs depends on the length of the N-alkyl chain, which confers lipophilicity. Benzalkonium chloride (BAC) is one of the most important quaternary ammonium compounds and has been used since 1935 as an antimicrobial additive in various cosmetic preparations in concentration 0.1% as well as in pharmaceutical preparations. BAC is a mixture of alkylbenzyl dimethylammonium chlorides with several analogues varying in the length of the aliphatic alkyl chain. In commercial preparations the aliphatic alkyl chains possess lengths of 12, 14 and 16 carbon atoms. The optimum activity against Gram-positive bacteria and yeast is obtained with chain lengths of 12 to 14 alkyls, while the optimum activity against Gram-negative bacteria is obtained with chain lengths of 14–16 alkyls. Compounds with N-alkyl chain lengths <4 or >18 are virtually inactive. The sensitivity of microorganisms to the action of QACs depends also on the concentration. At low concentrations (0.5–5 mg

L^{-1}), these compounds act biostatically on most bacteria, mycobacteria, spores, fungi and algae. At medium concentrations (10–50 mg L^{-1}) they show a biocidal effect on bacteria and fungi, while even in very high concentrations they do not have a biocidal effect on spores, mycobacteria and prions. Quaternary ammonium salts also act on lipid enveloped viruses, including HIV (human immunodeficiency virus) and HBV (hepatitis B virus). Thanks to their high antiviral activity, products containing QAC as the active ingredient, have been included in the *List N: Disinfectants for Use Against SARS-CoV-2* where there are over of 500 products meeting the US EPA criteria for the control of SARS-CoV-2 [47,48]. QACs are frequently used at high levels in hair washing and conditioning products because of their anti-static and softening properties. These compounds are widely used not only in cosmetics, but also in agriculture (fungicides, pesticides, insecticides), in health care (medicines) and in industry (anti-corrosive and anti-electrostatic agents) [25,47,49–52].

3. Occurrence and ecotoxicity

3.1. Occurrence in sewage and sludge

The extensive application of PCPs in industrial and consumer products has led to widespread contamination of the environment. There are several direct and indirect pathways through which preservatives can be introduced into the aqueous environment (Figure 1). Municipal wastewater with residues of xenobiotics is identified as the major route responsible for water contamination with micropollutants like preservatives and different PCPs. All four preservatives were reported in WWTPs influents, effluents and sludge samples between 2011 and 2021. According to the conducted research, described preservatives were found in different geographical regions of the world mainly in some Asia, North America, and European countries. The concentrations of four preservatives ranged significantly, from below the limit of quantification (LOQ) to a few tens of micrograms per liter or kilogram dry weight and usually showed high detection rates (Table 2). The analytical results from WWTPs in the world, revealed that the highest influent concentration level was obtained for chloroxylenol with the detection frequency 80% and maximum of 404.09 μg L^{-1} in the sewage treatment plants (STPs) in the Tianjin region in China. In this case PCMX was identified only in influents, which indicates the high efficiency of the traditional activated sludge and anaerobic-anoxic-oxic (A^2O) techniques which are two commonly used treatment technologies in this STPs [53]. The removal rates of PCMX in some European (United Kingdom) WWTPs were approximately 98% and effluent concentrations of PCMX were lower than 140 ng L^{-1} [54]. There is a lack of information concerning chloroxylenol concentrations in WWTP sludge.

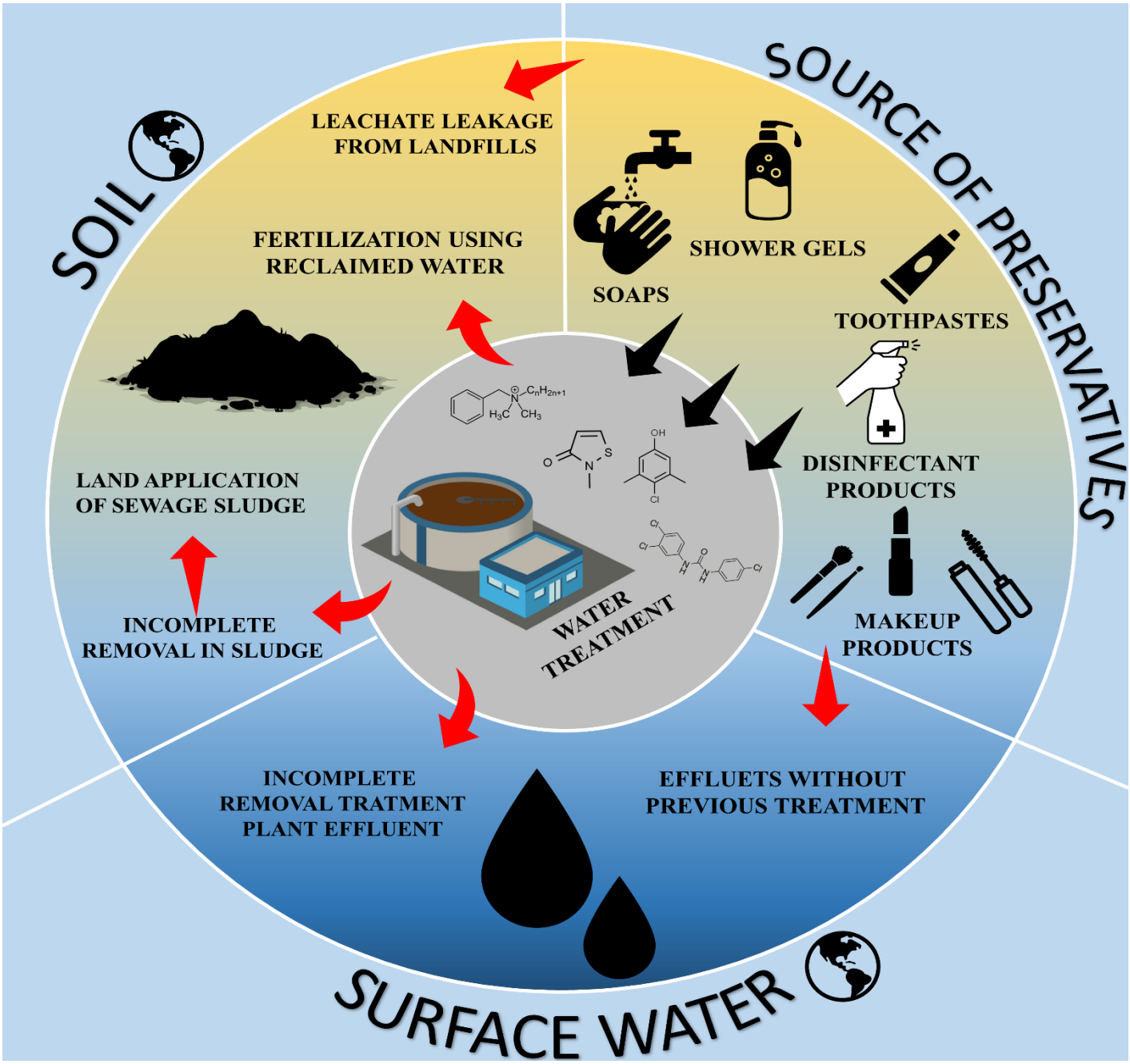


Figure 1. Environmental pathways for cosmetic preservatives.

BAC was found in lower influent concentration levels in comparison to PCMX with the highest concentration of 43.5 μg L^{-1} detected for BAC-C12 in Korea [55]. Also high levels was detected in European countries like Sweden, the Netherlands or France where BAC-C12 ranged from 200 to 29655 ng L^{-1} with detection frequency 100% [56–58]. Among the BAC homologues, BAC-C14 was also highly abundant, with maximum concentration of 8903 ng L^{-1} in WWTP in Sweden [56]. BAC was not only found in the liquid phase, but it was also detected in the suspended particulate phase, where the concentrations were higher than in the liquid phase due to their high adsorption on suspended particulate matter. The sludges in WWTP in Sweden were mainly contaminated by BAC-C12 and BAC-C14 with detection frequency 100% and maximum concentrations 89000 and 60000 ng g^{-1} dw, respectively [56,59]. In effluents, the levels of BACs were much lower than in influents, ranging from below detection limit to 500 ng L^{-1} with the removal rate 98% in China WWTPs [56,59,60].

Among the discussed preservatives, the most research was focused on TCC. For example, the highest concentrations of 515-10000 ng L^{-1} with detection frequency 100% were reported for TCC in influents from India [61–63]. In China, Chen et al. reported the mean concentration of 267 ng L^{-1} of TTC, while in another study from China, Sun et al. reported influents in the concentrations range 4.7-76.2 ng L^{-1} [64,65]. High concentrations in influent were also observed in North America, where the mean concentrations reported by Hedgespeth et al. were 4566 and 4644 ng L^{-1} with detection frequency 100% [66]. Similarly, Lozano et al. reported the mean concentration of 4920 ng L^{-1} in the USA [67]. On the other hand, in the study of Oliveira et al. the mean concentrations of TCC in different WWTPs in the USA ranged from 210 to 390 ng L^{-1} [68]. In European countries, influent concentrations of TCC achieved a maximum of 140 ng L^{-1} [69]. TCC elimination effectiveness varied between 11.4 and 97 % in WWTPs. However, due to its high octanol-water distribution coefficients, TCC was more often detected at a high concentration in the primary sludge from WWTPs and the high removal rates are accounted for by its attachment to solids [67,70,71]. The sludges were highly contaminated by TCC in India WWTPs. Subedi et al. reported high concentrations of TCC in sewage sludge samples collected in 2012, where preservative concentrations ranged from 5570 to 6740 ng g^{-1} [62]. In the next study of Subedi et al. in samples collected in 2013 the concentration of TCC was significantly higher with maximum of 10000-28000 ng L^{-1} and detection frequency 100% [63]. High concentrations of TCC in sewage sludge were reported in Canada and China, in the range 1200-8900 ng g^{-1} dw and 887-8450 ng g^{-1}, respectively [64,72–74]. The effluent concentrations of triclocarban were generally lower than μg L^{-1}, except for a sample from India WWTP, where the mean concentration achieved 5860 ng L^{-1} [62]. In most WWTPs in the world detection frequency of TCC in effluents was approximately 100%.

There is a lack of information concerning MIT concentrations in WWTPs in the world. Limited data showed the presence of MIT in plant wastewater in Poland at concentration 1210 ng L^{-1} [20]. Paijens et al. detected MIT in French WWTPs influent and effluent samples in the range 350-860 and 39-110 ng L^{-1}, respectively [59]. In another sampling campaign in French WWTPs, Paijens et al. revealed MIT at median concentrations 620 ng L^{-1} and 150 ng L^{-1} in influent and effluent, respectively [58]. The removal of this preservative was ranged from 55 to 89%. There are no data about the occurrence of MIT in other countries and sewage sludge samples.

Table 2. Occurrence of preservatives in the wastewater treatment plants.

Compound	Region	Location	Date/ n[a]	Influent	Effluent	Sludge	Analytical method	Reference
TCC	**Asia**	China	2008/ 5		390 ng L^{-1}[b]		LC-MS/MS	[75]
					183 ng L^{-1}			
		China	Unknown/ 3	267 ng L^{-1}	36.6 ng L^{-1}	887 ng g^{-1}	UHPLC-MS/MS	[64]
		South Korea	2011/ 40			<LOQ[c]-1260 ng g^{-1}[d] (100%)[e] 1630-5090 ng g^{-1} (100%) 362-6930 ng g^{-1} (100%)	HPLC-MS/MS	[61]
		India	2012/ unknown	515 ng L^{-1}	22.4 ng L^{-1}	5620 ng g^{-1}	HPLC-ESI-MS/MS	[62]
				933 ng L^{-1}	457 ng L^{-1}	6740 ng g^{-1}		
				8880 ng L^{-1}	5860 ng L^{-1}	8460 ng g^{-1}		
				1150 ng L^{-1}	48.4 ng L^{-1}	5570 ng g^{-1}		
				2100 ng L^{-1}	375 ng L^{-1}			
		China	2014/ 12	4.7-76.2 ng L^{-1} (100%)	27.6-109 ng L^{-1} (100%)	1130-2180 ng L^{-1} (100%)	LC-QqQ MS	[73]

		Singapore	2015/ 4	423.9-933.9 ng L^{-1} (100%)	143.1-214.5 ng L^{-1} (100%)		UHPLC-MS/MS	[71]
					49.1-263.9 ng L^{-1} (100%)			
		India	2013/ 7	1300-4300 ng L^{-1} (100%)	300-860 ng L^{-1} (100%)	13000-28000 ng L^{-1} (100%)	HPLC-MS/MS	[63]
				1200-10000 ng L^{-1} (100%)	215-358 ng L^{-1} (100%)	10000-23000 ng L^{-1} (100%)		
		China	2009-2014/ 100			8450 ng g^{-1}[c]	LC-MS/MS	[74]
	North America	USA	2009-2010/ unknown	4566 ng L^{-1} (100%)	617 ng L^{-1} (100%)		HPLC-MS/MS	[66]
				4644 ng L^{-1} (100%)	311 ng L^{-1} (100%)			
		USA	2009/ 5	4920 ng L^{-1}	20 ng L^{-1}		LC-MS	[67]
		Canada	Unknown/ 36	14-270 ng L^{-1} (86%)	3.1-33 ng L^{-1} (92%)	1200-8900 ng g^{-1} dw[h]	LC-MS/MS	[72]
		USA	2013/ 6		0.19 μg L^{-1}		LC-MS/MS	[68]
			2013/ 5	0.21 μg L^{-1}	0.05 μg L^{-1}			
			2013/ 8	0.37 μg L^{-1}				
			2013/ 8	0.39 μg L^{-1}	0.07 μg L^{-1}			
			2013/ 8	0.21 μg L^{-1}				
	Europe	France	2010/ 2	97-140 ng L^{-1}			UPLC-MS/MS	[69]
		Ireland	2015/ 16			0.08 μg g^{-1}	LC-MS/MS	[32]
PCMX	**Asia**	China	unknown	404.09 μg L^{-1} (80%)	n.d.[f]		GC-MS	[76]
				16.22 μg L^{-1}	n.d.			
				3.68 μg L^{-1}	n.d.			
				7.29 μg L^{-1}				
	Europe	United Kingdom	Unknown/ 9		19-140 ng L^{-1}		GC-MS	[54]
MIT	**Europe**	Poland	2018/ 3	1.21 μg L^{-1}			LC-MS/MS	[20]
		France	unknown	860 ng L^{-1}	110 ng L^{-1}		HPLC-MS/MS	[59]
				430 ng L^{-1}	65 ng L^{-1}			
				39 ng L^{-1}	<LOQ			
		France	2018-2019/ 6	35-860 ng L^{-1} (100%)	39-350 ng L^{-1} (100%)		HPLC-MS/MS	[58]
BAC	**Asia**	Korea	2016/ unknown				HPLC-MS/MS	[55]
		BAC12		18 μg L^{-1}	0.479 μg L^{-1}			
				43.5 μg L^{-1}	0.014 μg L^{-1}			
		China	2016/ unknown				UPLC-MS/MS	[60]

	BAC12	Autumn	0.308 μg L^{-1}	<MDL[g]			
		Winter	0.480 μg L^{-1}	<MDL			
		Autumn	0.622 μg L^{-1}	<MDL			
		Winter	0.650 μg L^{-1}	0.010 μg L^{-1}			
	BAC14	Autumn	0.121 μg L^{-1}	<MDL			
		Winter	0.161 μg L^{-1}	<MDL			
		Autumn	0.141 μg L^{-1}	<MDL			
		Winter	0.220 μg L^{-1}	<MDL			
	China	unknown				HPLC-MS/MS	[77]
	BAC12		1800 ng L^{-1}	3.7 ng L^{-1}			
			1400 ng L^{-1}	6.8 ng L^{-1}			
			1300 ng L^{-1}	4.8 ng L^{-1}			
	BAC14		670 ng L^{-1}	1.9 ng L^{-1}			
			610 ng L^{-1}	3.3 ng L^{-1}			
			480 ng L^{-1}	2.3 ng L^{-1}			
North America	USA	2018/ 13				LC-HRMS/MS	[78]
	BAC12			23 ng L^{-1}			
	BAC14			216 ng L^{-1}			
Europe	Sweden	unknown				LC-MS/MS	[56]
	BAC10		2-64 ng L^{-1} (100%)	<LOQ-3 ng L^{-1} (12%)	24-210 ng g^{-1} dw (100%)		
	BAC12		1725-29655 ng L^{-1} (100%)	<LOQ-310 ng L^{-1} (67%)	8800-89000 ng g^{-1} dw (100%)		
	BAC14		454-8903 ng L^{-1} (100%)	<LOQ-84 ng L^{-1} (58%)	3200-60000 ng g^{-1} dw (100%)		
	BAC16		<LOQ-1485 ng L^{-1} (88%)	<LOQ-13 ng L^{-1} (6%)	990-4900 ng g^{-1} dw (100%)		
	Netherlands	2014/ 15	15.5 μg L^{-1}	<LOQ		LC-MS/MS	[57]
			10 μg L^{-1}	0.5 μg L^{-1}			
	France	unknown				UPLC-MS/MS	[58]
	BAC12		200 ng L^{-1}	100 ng L^{-1}			
			500 ng L^{-1}	400 ng L^{-1}			
			2000 ng L^{-1}	300 ng L^{-1}			
	BAC14		<LOQ	<LOQ			
			400 ng L^{-1}	200 ng L^{-1}			
			300 ng L^{-1}	80 ng L^{-1}			
	BAC16		200 ng L^{-1}	<LOQ			

[a]Date/ n: sampling date and number. [b]Mean or median value. [c]limit of quantification. [d]Concentration range. [e]Detection frequency. [f]not detected. [g]method detection limits. [h]dry weight.

3.2. Occurrence in surface waters

Deficient removal of preservatives in WWTPs and following discharge of effluents lead to contamination of the receiving environments. Concentrations of selected preservatives in surface waters range from ng L^{-1} to tens of μg L^{-1}, thus demonstrating the ubiquity of these pollutants. BAC shows much higher concentrations and detection frequency than other analyzed preservatives. Analyses conducted in water samples, obtained from eight different located ponds in Changsha city in Hunan Province in China, showed the maximum concentration of tetradecyl benzyl ammonium chloride (BAC-C14) up to 8.1 mg L^{-1}

[79]. Li et al. examined the occurrence of cationic surfactants and other pollutants from the Songhua River, the Second Songhua River and the Nen River, BAC-C12 was detected in 87% of total 196 samples, with maximum concentration 41 ng L^{-1} and average concentration of 3.5±5.3 ng L^{-1} [80]. Also, BAC-C14 was detected in samples with detection frequency 98% and with maximum concentration 13 ng L^{-1}. The authors suggested that the low concentrations of cationic surfactants in surface waters are caused by the limited usage of these chemicals in North China. Meanwhile, in Korea the maximum concentrations of BAC-C12 and BAC-C14 in surface waters reached 35.8 μg L^{-1} and 21.6 μg L^{-1}, respectively. In the case of European countries, the analyses of samples from five different sites in the city of Gdańsk (Poland) showed maximum concentration of hexadecyl benzyl dimethyl ammonium chloride (BAC-C16) up to 243 μg L^{-1}. Also, high contents of BAC-C12 and BAC-C14 were observed, with maximum concentrations 99.6 μg L^{-1} and 157 μg L^{-1}, respectively [81]. Ruman et al. examined the occurrence of five cationic surfactants from the Kłodnica River in Poland in four seasons [82]. BAC-C12, BAC-C14 and BAC-C16 were detected in water samples, with maximum concentrations of 99.1 μg L^{-1}, 76.1 μg L^{-1} and 89.4 μg L^{-1}, respectively. All of the maximum concentrations were detected in cold season.

Studies about the occurrence of chloroxylenol in receiving aquatic environments, in the last ten years, were conducted only in Asian countries. Recent research of the occurrence of PCMX in surface water in China was conducted by Tan et al. [83]. Nine surface water samples were collected from two urban streams, the Pearl River located in Guangzhou and an outlet of the STP. The PCMX concentrations in the urban streams achieved 8.89 μg L^{-1}, in the Pearl River they ranged from 1.62 to 3.60 μg L^{-1} and in the outlet of the sewage treatment plant the average was 2.45 μg L^{-1}. Dsikowitzky et al. estimated the concentrations of lipophilic organic contaminants of surface water in Jakarta (Indonesia), in seven locations, in two of which PCMX was detected in the range 20-30 ng L^{-1} [84]. A year before, the same team examined 18 spots in the rivers in Jakarta city which go into Jakarta Bay. PCMX occurred in 13 in the concentrations range from 60 to 1200 ng L^{-1} [85]. The occurrence of PCMX in another Asian country was detected by Kimura et al. in Tokushima city [86]. Out of four river samples PCMX was detected only in one from the autumn sampling campaign at a concentration of 17.8 ng L^{-1}.

Triclocarban is one of the best studied preservatives in the world due to its environmental impact. Many studies describe its occurrence in receiving environments. One of them was conducted by Vimalkumar et al. where concentrations of triclocarban in three major rivers in India were analysed [29]. Sampling was done during wet and dry seasons from 29 locations. The highest concentration of TCC was detected in the Karei River in the range from 8 to 1119 ng L^{-1}. In the Thamiraparani River and Vellar River the average concentrations were 55.6 and 25.3 ng L^{-1}, respectively. TCC was detected in all tested samples. TCC was also found in another Indian river (Torsa River) at the maximum concentration of 77 ng L^{-1} [87]. On the other hand, TCC was not detected in another Indian river- the Arkavathi River [88]. Slightly lower concentrations of TCC were noticed in samples collected from Sri Lanka aquatic environments. Triclocarban was detected in 100% of them, with a maximum concentration of 31 ng L^{-1} [89]. High concentrations of preservatives in surface water were detected in samples closer to the outfall of effluents from WWTPs pointing to the major source of these pollutants. Juksu et al. examined the total emission of TCC from WWTPs based on the estimated consumption in Thailand [90]. TCC was one of the biggest pollutants, with >30 tons/year for the entire country. Moreover, TCC had the highest concentration in receiving riverine environments (4030 ng L^{-1}) and in sea water in coastal environments of Pattaya city (248 ng L^{-1}). The occurrence of TCC in China rivers environments was examined in the Songhua River, the Second Songhua River and the Nen River [80]. The total concentrations of TCC in river water ranged from below detection limit to 27 ng L^{-1}, with detection frequency of 96%. Comparatively, the levels of TCC in sea water were in the range of <LOD-13.2 ng L^{-1} in coastal areas of Zhejiang in the East China Sea and Xiamen Bay [91,92]. TCC was also detected in North America surface waters in the concentrations range of 2.5 - 102 ng L^{-1} [93,94]. TCS was reported to occur in

low concentrations in European countries, e.g. in the Italian, Spanish, Romanian and Polish surface waters (>0.8 ng L^{-1}; 0-15 ng L^{-1}; 3 ng L^{-1}; 0.6-54 ng L^{-1} and 5 μg L^{-1}) [95–99]. TCC was also found in the samples from the western basins of the Mediterranean Sea and the North Sea in the concentrations range from 0.0036 to 0,07 ng L^{-1} [100,101].

There is little literature data concerning the occurrence of MIT in surface water. Paijens et al. described contamination of Paris waste water by MIT at concentration 14 ng L^{-1} [59]. MIT was also a subject of the research by Nowak et al. who examined the contamination of the Vistula River (Poland), where the preservative was not detected [20].

3.3. Occurrence in surface water sediments

There are only a few papers on the presence of preservatives in sediments. Accessible research concerns merely two compounds discussed in this work: triclocarban and benzalkonium chloride. Due to high adsorption and resistance to microbial removal, BAC may be detected in surface water sediments, though at significantly lower levels than in sewage sludge samples [102]. The most contaminated samples were from the Hudson River Estuary, New York, USA, with maximum concentrations 8900, 4000, 3800 and 1000 ng g^{-1} detected for BAC-C14, C18, C16 and C12, respectively [103]. Comparing the median concentrations of different BAC homologs, the highest median value was determined for BAC-C18. The authors suggested that BAC-C18 is most used in PCPs that may reach WWTPs. Relatively lower concentrations of BAC homologous were determined in the rivers of China (the Songhua River, the Second Songhua River, the Nen River, the Pearl River, and the Zhujiang River). Concentrations for total BAC in the Chinese rivers were measured by Li et al. and ranged from 49.3 to 1530 ng g^{-1} [104]. The average concentrations of BAC-C12 and BAC-C14 were determined by Li et al. and were 1 ±1.6 ng g^{-1} dw and 0.44 ±0.69 ng g^{-1} dw, respectively [80]. Domestic wastewater is the main source of biocides in rivers, and its concentrations depend on the economic level of the area, the population of cities along the river, everyday activities of the populace, different physical and chemical properties of various sites, and the type of surface water system receiving treated wastewater. However, the area of influence of WWTP and the location of the sampling site seems to dictate the contamination levels. The levels of TCC in surface waters sediments in the USA were measured by Venkatesan et al. and Maruya et al. [105,106]. Samples collected from Minnesota freshwater sediments were contaminated by TCC from 5 to 822 ng g^{-1} dw. TCC concentrations in sediment collected from the near area of influence of WWTP discharge were higher than those observed in any river or creek sediments downstream of WWTP discharges [105]. In southern California, the highest detectable concentration of TCC in river sediments was 183 ng g^{-1} dw. It was higher than in estuarine sediments, which again may indicate the influence of physicochemical properties, i.e. pH or salinity, on the fate of biocides in the natural environment [106]. Other researchers reported the occurrence of TCC in sediments from European surface waters sediments. The concentrations ranged from 0.7 to 6.91 ng g^{-1}, in the upstream and downstream of the River Lambro in Northern Italy, and in the Lake Lugano and Greifensee in Switzerland they reached 7 ng g^{-1} dw [107,108]. In another study, an analysis of 19 grab samples of sediments from the Albufera Natural Park in Spain showed the occurrence of TCC at a concentration below 10 ng g-1 with a detection frequency of 42% [99]. Elevated levels of TCC were also found in sediments from Asia surface waters. The mean concentration of TCC detected in the Thao Praya River in Bangkok, Thailand was 3370 ng g^{-1} [90]. Widespread occurrence of TCC was reported in the Pearl River Delta, at concentrations range 0.53-103 ng g^{-1}. The concentrations of TCC were lower in the adjacent tributaries than in the mainstream, suggesting that municipal sewage is the main source of contaminants [109]. The lowest concentrations were observed in coastal areas of Zhejiang in the East China Sea. Detection frequency was 100%, and concentrations range from 0.12 to 6.6 ng g^{-1} [92].

3.4. Occurrence in soil

The available literature data describing the concentrations of preservatives in soil concern mainly agricultural soils. The widespread use of sewage sludge, solid waste, or reclaimed water for soil fertilization and irrigation poses a serious risk of soil contamination with residual chemicals that are not completely removed in the wastewater treatment process. In addition, the use of pesticides, which may contain preservatives, is also a source of soil contamination. Despite the concerns, little data is available. As with surface water sediments, the majority of papers describe BAC and TCC concentrations. Both described compounds due to the high log organic carbon-water partitioning coefficient (K_{OC}), indicate strong trends to accumulate in the soil [75,110].

A study revealed that the BAC homologs are able to persist in soils for half a year after application. With the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS), Kang and Shin for the first time, showed BAC concentrations in soil [111]. The total BAC was detected in Korean soil samples from sentry posts, cattle farms and migratory bird habitats in the concentration range from 0.001 to 28.5 mg kg^{-1}. BAC-C12 occurred in all tested soil samples. Scarce water resources in arid and semi-arid regions of Earth caused reclaimed wastewater to be used for irrigation of soil. Chen et al. reported a range of concentrations from 8.5±11.9 to 105±38.9 μg kg^{-1} dw for TCC in Hebei (China) at different soil depths irrigated with reclaimed wastewater, and evaluated TCC half-life, which was 108 days [112]. TCC was also detected in biosolid-amended soils in China provinces (Zhejiang, Hunan, Shandong) at concentrations ranging from 111 to 1584 μg kg^{-1} dw. The authors observed that the concentrations of TCC in soils several times fertilized with biosolids were significantly higher than those of a single application. Moreover, the occurrence of TCC in soil samples after three years since the first application of biosolids implies a tendency of TCC to persist in the soil. TCC concentrations in the control plots, free from biosolids applications, were below the detection limit or very low, suggesting that the occurrence of contaminants, such as TCC, is caused by the use of biosolids [113]. In another study, TCC was also detected in biosolid-amended soil in North America in the state of Idaho. Tested soil samples were treated with biosolids for seven years, however, the TCC concentration range was lower than in China (14.8-27.3 ng g^{-1} dw). Similar results were received by Viglino et al. and Negahban-Azar et al. in Canada (13±2-53±9 ng g^{-1}) and the USA (2.5-9.1 μg kg^{-1}) [114,115]. The lower levels of contaminations in these studies could result from different physio-chemical properties of soil, an alternative method of wastewater treatment, and different amounts of application biosolid [116]. The data from another state (Virginia) collected from commercial farms by Lozano et al. showed that the highest measured concentration of TCC (131.9±76.1 ng g^{-1} dw) was observed in fields that received biosolids multiple times [117]. A lower concentration (107.1±43.7 ng g^{-1} dw) was observed in soil from the field which received a single application of biosolids. Additionally, a trace quantity of TCC (<19.7±3.7 ng g^{-1} dw) was observed in soil never fertilized with biosolids. Moreover, the authors evaluated TCC concentrations seven and eight years after biosolids application, which were on the level 45.8±6.09 and 72.4±15.3 ng g^{-1} dw, respectively. The last analysis proves that TCC has high persistence in soil.

Soil can be contaminated not only through fertilization or irrigation but also through general human activity. One of the reasons for soil contamination with preservatives may be inadequate solid waste disposal. The research carried out by Nowak et al. team showed the presence of MIT in sand samples collected in the summer season from the Baltic Sea coast [20]. In the publication, authors suggested that sand contamination may be due to the excessive use of skin-protecting agents against UV radiation. MIT concentrations in sand samples ranged from 2.19±0.47 to 4.48±1.04 μg kg^{-1}).

There is a lack of literature data describing the occurrence of chloroxylenol in soil samples.

3.5. Aquatic toxicity

The toxic effects of preservatives on non-target organisms were studied on several model species from different trophic levels. Some of the acute and chronic toxicity effects

for triclocarban, chloroxylenol, benzalkonium chloride and methylisothiazolinone are listed in Table 3. Histopathological alterations, modification of proteins, neurotoxicity and genotoxicity, reproduction and structural abnormalities, embryotoxicity and endocrine disturbance have been observed as a result of preservatives toxicity in aquatic organisms (Figure 2).

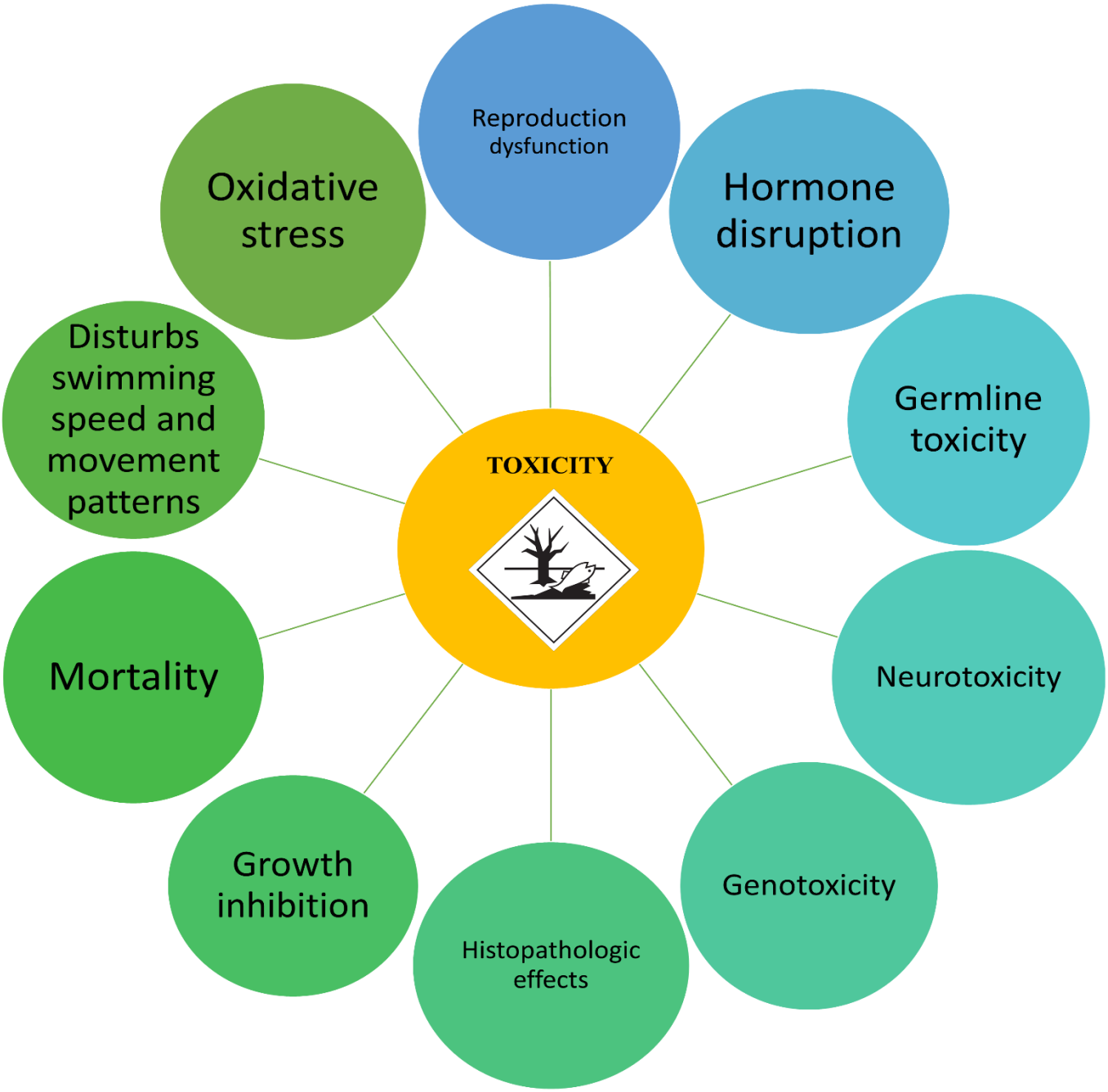


Figure 2. Toxicity of cosmetic preservatives.

3.5.1. Triclocarban

The acute toxicity of triclocarban for water flea (*Daphnia magna*) immobility was described with the EC_{50} (Effective concentration, 50%) of 5.9 μg L^{-1} at 24 h [118]. Sreevidya et al. investigated the ecotoxicity of triclocarban on two aquatic organisms, nematode *Caenorhabditis elegans*, and zebrafish *Danio rerio* [119]. TCC below 1 mg L^{-1} could result in

disorder in reproduction and abridgment lifespan of *C. elegans*. Moreover, TCC induced germline toxicity in the exposed worm, manifested by the increased occurrence of the "green eggs" phenotype. The measured median lethal concentration (LC_{50}) was 0.91 mg L^{-1} for *C. elegans*. TCC at 0.1 mg L^{-1} and 0.5 mg L^{-1} induced larval mortality of zebrafish after 72 hour fertilization (hpf). Among the many different toxic effects of TCC, an interesting observation is that this xenobiotic caused developmental neurotoxicity in the *Danio rerio* embryos. Zebrafish embryos exposed to 0.1-0.5 mg L^{-1} showed abnormalities in secondary motor neurons. The TCC developmental toxicity in *D. rerio* was also confirmed in a study by Dong et al. [120]. The calculated LC_{50} value for *D. rerio* was 215.8 μg L^{-1}. Furthermore, obtained results showed that exposure to 133.3 μg L^{-1} of TCC influenced thyroid hormone activity and disturbed the expression of genes. The toxicity potential of TCC, at low concentrations, was evaluated for silver catfish *Rhamadia quelen*. Environmental concentrations of TCC induced sublethal effects such as deformities of embryos, oxidative damage, and neurotoxic effects [121]. Jimoh and Sogbanmu observed the dose-dependent gill histopathological alterations in *Clavias gariepinus* as well as embryotoxic effects [122]. Three typical freshwater algae *Chlorella vulgaris*, *Scenedesmus obliquus*, *Chlorella pyrenoidosa* were less vulnerable to triclocarban than fish and nematodes, with the 96 h EC50 of 8.474, 9.11, 8.76 mg L^{-1} respectively, based on the growth inhibition. Triclocarban significantly altered the content of chlorophyll α and disturbed the activity of peroxidase and superoxide dismutase enzyme, destroying the antioxidant functions of cells. Moreover, the growth of malondialdehyde content indicated elevated stress levels in algae [123].

3.5.2. Chloroxylenol

Won et al. reported that the LC_{50} value of PCMX for the rotifer *Brachionus koreanus* was 24.264 mg L^{-1} [124]. The population growth and reproduction ability of rotifers were significantly inhibited in response to PCMX in a dose-depend manner. Swimming speed as well as movement track of the tested organisms were disturbed after PCMX exposure. The occurrence of this disinfectant in *B. koreanus* environment caused an increase in ROS (reactive oxygen species) generation. PCMX showed mutagenic activity in the DNA of the fish (Rainbow trout) erythrocytes [125]. Similarly to TCC, PCMX disturbed the reproduction and lifespan of *C. elegans* and showed germline toxicity and neurotoxicity toward *D. rerio*. Moreover, PCMX in *D. rerio* induced embryonic malformations, for example, body curvature, and caused an increase in mortality. The reported LC_{50} value of PCMX to *C. elegans* after 24 h of exposure was 31.8 mg L^{-1} [119]. The acute values of chloroxylenol towards whirligig beetle (*Orectogyrus alluaudi*) for mortality were reported at the LC_{50} of 21.587, 16.744, 11.638, 7.819 mg L^{-1} at 24, 48, 72, 96 h, respectively. The observed increase in mortality of *O. alluaudi* was dependent on the concentration and exposure duration. These results suggest that *O. alluaudi* has a higher vulnerability toward PCMX than other invertebrate [126]. However, the 48 h LC_{50} for *Daphnia magna* was lower and achieved 8.78 mg/L [127]. These differences in the sensitivity of aquatic organisms (Table 3) can be associated with differences in biochemical responses, exposure routes and psychological responses.

3.5.3. Methylisothiazolinone

Different species of invertebrates have different sensitivities towards methylisothiazolinone (Table 3). The short-term median lethal concentration values (LC_{50}) of MIT on three freshwater invertebrates *Daphnia similis*, *Dugesia japonica*, and *Neocaridina denticulata* varied from 1.83 to 198.34 mg L^{-1} at 24 h [128]. In contrast, EC_{50} values for MIT for *D. magna* were reported at a concentration of 510 μg L^{-1} [129]. Moreover, MIT at the level of 15 μM caused alterations in regeneration and wound healing in planaria (*D. japonica*) as well as defects in neuromuscular and epithelial integrity [130]. Wang et al. evaluated the tolerance of microalgae (*Scenedesmus sp*. LX1) to MIT by testing their growth inhibition [131]. The results indicated that MIT caused growth inhibition of microalgae by photosynthesis

disturbance. The EC_{50} value for these aquatic organisms was 1 mg L^{-1}. Capkin et al. confirmed the genotoxic and histopathologic effects of MIT on rainbow trout, causing DNA damage in red blood cells and upregulation of all studied genes [132]. In addition, MIT at a concentration of 300 μg L^{-1} disturbed the ability to hatch and survive of zebrafish larvae and caused deregulation of thyroid hormone genes expression, resulting in a reduction of the content of triiodothyronine and thyroxine in the whole body of *D. rerio* larvae [133].

3.5.4. Benzalkonium chloride

Studies regarding the toxicity of benzalkonium chloride have been focused on fish, algae, and invertebrates such as daphnids and rotifer. The 48 h acute toxicity of BAC to *D. magna* (EC_{50}) is 41.1 μg L^{-1}, which is significantly lower than its toxicity toward microalgae *Phaeodactylum tricornutum* (EC_{50}: 131.9 μg L^{-1}), *Tisochysis lutea* (EC_{50}: 86 μg L^{-1}) and *Pseudokirchneviella subcapitata* (EC_{50}: 255 μg L^{-1}) [55,134,135]. The hazardous potential of BAC toward aquatic environments was evaluated by Qian et al., who analyzed the toxicity of BAC on freshwater cyanobacteria *Microcystis aeruginosa* [136]. All of the BAC-C12 concentrations examined strongly inhibited cyanobacteria growth, with the 96 h EC_{50} value identified as 3.61 mg L^{-1}. Moreover, the exposition of *M. aeruginosa* to BAC-C12 resulted in the inhibition of photosynthetic efficiency by disturbing the structure of chlorophyll-protein-lipid and photosynthetic organelle. The toxicity effects of BAC were also manifested via an increase in oxidative stress and greater permeability of cell membranes. Therefore, it can be suggested that BAC-C12 might enhance the release of microcystins by *M. aeruginosa* and increase its level in the aquatic environment making a higher risk for aquatic ecosystems. Similarly to chloroxylenol, BAC inflects antioxidant enzymatic activities and the level of ROS, and disturbs swimming speed and movement patterns in *B. koreanus* [124]. Some in vitro and in vivo studies have examined the endocrine-disrupting effects of benzalkonium chloride. BAC at concentration 3 μg L^{-1} was reported to possess endocrine disrupting properties by Kim et al. in an vivo assay using the measurement of vitellogenin gene transcription, which is a biomarker of estrogenic activity in male fish *Oryzias latipes* [55]. In another study, molecular response to long-term exposure to BAC was analyzed with the use of a proteomic approach. BAC showed interactions with proteins responsible for the endocrine and nervous systems, oxidative stress, signaling pathways, cellular proteolysis, and cytoskeleton in *O. latipes* [137].

Table 3. Aquatic toxicity values for preservatives.

Compound	Species	Effect	Duration	Endpoint	Value	References
TCC	*Daphnia magna*	Immobility	48 h	EC_{50}	5.9 μg L^{-1}	[118]
	Daphnia magna	Mortality	96 h	LC_{50}	0.087 μM	[138]
	Daphnia similis	Immobility	48 h	EC_{50}	0.044 μM	[139]
	Pseudokirchneriella subcapitata	Growth inhibition	72 h	IC_{50}[a]	1.01 μM	[139]
	Chlorella vulgaris	Growth inhibition	96 h	EC_{50}	8.474 mg L^{-1}	[123]
	Scenedesmus obliquus	Growth inhibition	96 h	EC_{50}	9.11 mg L^{-1}	[123]
	Chlorella pyrenoidosa	Growth inhibition	96 h	EC_{50}	8.76 mg L^{-1}	[123]
	Clarias gariepinus	Fingerlings mortality	96 h	LC_{50}	41.57 mg L^{-1}	[122]
	Clarias gariepinus	Embryos mortality	24 h	LC_{50}	46.08 mg L^{-1}	[122]
	Clarias gariepinus	Hatching	26 h	EC_{50}	41.93 mg L^{-1}	[122]
	Caenorhabditis elegans	Reproduction	96 h	EC_{50}	0.38 μmol L^{-1}	[140]
	Caenorhabditis elegans	Growth	96 h	EC_{50}	0.66 μmol L^{-1}	[140]
	Caenorhabditis elegans	Mortality	24 h	LC_{50}	0.91 mg L^{-1}	[119]
	Caenorhabditis elegans	Reproduction	4-6 days	LOEC[b]	0.01 mg L^{-1}	[119]
	Caenorhabditis elegans	Lifespan	4-6 days	LOEC	0.05 mg L^{-1}	[119]
	Caenorhabditis elegans	Germline toxicity	24 h	LOEC	0.01 mg L^{-1}	[119]
PCMX	*Brachionus koreanus*	Mortality	24 h	LC_{50}	24.264 mg L^{-1}	[124]
	Brachionus koreanus	Mortality	24 h	NOEC[c]	15 mg L^{-1}	[124]
	Daphnia magna	Mortality	48 h	LC_{50}	8.78 mg L^{-1}	[127]
	Caenorhabditis elegans	Mortality	24 h	LC_{50}	31.8 mg L^{-1}	[119]
	Caenorhabditis elegans	Reproduction	4-6 days	LOEC[b]	1 mg L^{-1}	[119]

	Caenorhabditis elegans	Lifespan	4-6 days	LOEC	10 mg L^{-1}	[119]
	Caenorhabditis elegans	Germline toxicity	24 h	LOEC	5 mg L^{-1}	[119]
	Orectogyrus alluaudi	Mortality	24 h	LC_{50}	21.587 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	48 h	LC_{50}	16.744 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	72 h	LC_{50}	11.638 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	96 h	LC_{50}	7.819 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	24 h	NOEC	6.754 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	48 h	NOEC	2.789 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	72 h	NOEC	1.1535 mg L^{-1}	[126]
	Orectogyrus alluaudi	Mortality	96 h	NOEC	0.5485 mg L^{-1}	[126]
MIT	Daphnid	Mortality	48 h	LC_{50}	4.7 mg L^{-1}	[141]
	Algae	-	96 h	EC_{50}	0.4 mg L^{-1}	[141]
	Fish	Mortality	96 h	LC_{50}	3.8 mg L^{-1}	[141]
	Daphnia magna	Immobility	48 h	EC_{50}	510 μg L^{-1}	[129]
	Cell line RTL-W1 from *Oncorhynchus mykiss*	Vitality	48 h	EC_{50}	10400 μg L^{-1}	[129]
	Daphnia similis	Mortality	24 h	LC_{50}	1.83 mg L^{-1}	[128]
	Daphnia similis	Mortality	48 h	LC_{50}	0.81 mg L^{-1}	[128]
	Dugesia japonica	Mortality	24 h	LC_{50}	2.36 mg L^{-1}	[128]
	Dugesia japonica	Mortality	48 h	LC_{50}	2.06 mg L^{-1}	[128]
	Dugesia japonica	Mortality	72 h	LC_{50}	1.58 mg L^{-1}	[128]
	Dugesia japonica	Mortality	96 h	LC_{50}	1.54 mg L^{-1}	[128]
	Neocaridina denticulata	Mortality	24 h	LC_{50}	198.34 mg L^{-1}	[128]
	Neocaridina denticulata	Mortality	48 h	LC_{50}	84.48 mg L^{-1}	[128]
	Neocaridina denticulata	Mortality	24 h	LC_{50}	43.82 mg L^{-1}	[128]
	Neocaridina denticulata	Mortality	48 h	LC_{50}	35.36 mg L^{-1}	[128]
	Scenedesmus sp. LX1	Growth inhibition	72 h	EC_{50}	1 mg L^{-1}	[131]
BAC	*Daphnia magna*	Immobility	48 h	EC_{50}	41.1 μg L^{-1}	[55]
	Oryzias latipes	Mortality	96 h	LC_{50}	246 μg L^{-1}	[55]
	Oryzias latipes	Mortality	96 h	LC_{50}	2.12 mg L^{-1}	[137]
	Phaeodactylum tricornutum	Growth inhibition	72 h	EC_{10}	69 μg L^{-1}	[134]
	Phaeodactylum tricornutum	Growth inhibition	72 h	EC_{50}	131.9 μg L^{-1}	[134]
	Tisochrysis lutea	Growth inhibition	72 h	EC_{10}	57.1 μg L^{-1}	[134]
	Tisochrysis lutea	Growth inhibition	72 h	EC_{50}	86 μg L^{-1}	[134]
	Pseudokirchneriella subcapitata	Growth inhibition	72 h	EC_{10}	0.092 mg L^{-1}	[135]
	Pseudokirchneriella subcapitata	Growth inhibition	72 h	EC_{50}	0.255 mg L^{-1}	[135]
	Pseudokirchneriella subcapitata	Growth inhibition	72 h	NOEC	0.023 mg L^{-1}	[135]
	Microcystis aeruginosa	Growth inhibition	96 h	EC_{50}	3.61 mg L^{-}	[136]
	Brachionus koreanus	Mortality	24 h	LC_{50}	0.483 mg L^{-1}	[124]
	Brachionus koreanus	Mortality	24 h	NOEC[c]	0.3 mg L^{-1}	[124]
	Cell line RTgill-W1 from *Oncorhynchus mykiss*	Metabolic activity	24 h	EC_{50}	1098 μg L^{-1}	[142]
	Cell line RTgill-W1 from *Oncorhynchus mykiss*	Membrane integrity	24 h	EC_{50}	1628 μg L^{-1}	[142]
	Cell line RTgill-W1 from *Oncorhynchus mykiss*	Rybosomal integrity	24 h	EC_{50}	690 μg L^{-1}	[142]

3.6. Soil toxicity

The aquatic toxicity of preservatives has been studied extensively, however their toxicity toward terrestrial organisms is also in need of attention, as sewage sludge land application has become one of the major soil fertilization methods. Biosolids contaminated by preservatives together with many other xenobiotics may be a potential risk to soil

organisms. In the study by Yang et al. BAC up-regulated the N fixation gene (nifH) and nitrification genes (AOA and AOB) in the soil and down-regulated the denitrification gene (narG). Moreover, it reduced the variety of soil microbial communities and caused an increased quantity of *Crenarchaeota* and *Proteobacteria* [143]. TCC was demonstrated to decrease the abundance of soil bacteria and reduce the degradation level of pesticides in soil resulting in their persistence in the environment [144]. Ali et al. also proved activity inhibition of soil microflora by TCC at a concentration of 450 $\mu g\ g^{-1}$ [145]. Bioaccumulation of TCC was reported in earthworm (*Eisenia fetida*) tissues at bioaccumulation factor values ranging from 5.2 to 18 $g_{soil}\ g_{tissue}^{-1}$. The calculated LC_{50} value for *E. fetida* was 40 mg kg^{-1} fine sand [146]. The database on the toxicity of preservatives to soil organisms is still fragmentary.

4. Microbial degradation of preservatives

Human economic activity and progressive urbanization pose a constant threat of environmental contamination with xenobiotics. The presence of cosmetic preservatives in water and soil samples, confirmed by numerous studies, is the cause of many undesirable processes that contribute to the disturbance of the biological balance, as well as the emergence of unfavorable changes at the level of ecosystems. Due to the pollution of the natural environment with xenobiotics and their toxic properties, research on their removal has been developing on a large scale and enjoys great interest. However, little literature data is available describing the elimination of these preservatives. The main way of removing preservatives from the environment is biodegradation carried out by microorganisms. Biodegradation is a metabolism-dependent process of decomposition of xenobiotics into simpler compounds, taking place with the participation of extracellular and/or intracellular enzymes. This process often transforms the pollutants into simpler compounds that are typically less toxic than the parent compounds. In some cases, biodegradation leads to the mineralization of organic compounds and their degradation into carbon dioxide, water, and/or other inorganic products.

In the scientific literature, few works describe the potential of bacteria and fungi to effectively eliminate triclocarban, chloroxylenol, methylisothiazolinone and benzalkonium chloride. It should be noted, that the process of elimination of xenobiotics, in some cases, is not synonymous with their degradation and detoxification. Table 4 shows the biodegradation of the discussed biocides with the use of microorganisms.

4.1. Triclocarban

Research studies on microbial degradation of triclocarban are numerous. The usage of sewage sludge, including of TCC residues in agriculture poses a serious risk to the environment. The application of microorganisms over composting biosolids could reduce the environmental risks of the use of sewage sludge as fertilizer. Biodegradation of TCC via the composting of biosolids under high ventilation resulted in the reduction of xenobiotic concentrations by 83.1% during 16 days [147]. The immobilized microbial cells of Pseudomonas fluorescens (MC46) on biochar could be used for the effective purification of the sewage from TCC. The yield of the process carried out by immobilized P. fluorescens cells was much higher (79.80%) compared to the elimination of TCC by free P. fluorescens cells (42.12%) [148]. Similar results were obtained by Taweetanawanit et al. confirming that TCC was eliminated more efficiently by microorganisms entrapped in barium alginate [149]. Moreover, researchers observed 3,4-dichloroaniline (34DCA), 4-chloroaniline (4CA), and aniline as by-products emerging via hydrolysis, dehalogenation, hydroxylation and dechlorination, which were characterized by lower toxicity than the parent compound. Subsequently, aniline may be transformed through deoxygenation into catechol, and it is anticipated that catechol may thereafter undergo ring cleavage [149]. The same bacterial strain was used for bioaugmentation of TCC-contaminated soil, with an elimination efficiency of 74-76%. P. fluorescence was able to remove TCC as a sole

carbon source leading to its detoxification [150]. 34DCA, 4CA and 4-chlorocatechol were reported to be the major metabolites present as a result of bacterial degradation of TCC in Sphingomonas sp. YL-JM2C. The formed metabolites and parent compounds were too toxic for the tested strain and inhibited further biodegradation stopping at the level of 35% [151]. Three strains of Ochrobacterium sp., (MC22, TCC-1, and TCC-2) were also capable of triclocarban biotransformation under aerobic and anaerobic conditions [152–155]. Under aerobic conditions strain MC22 was able to degrade of TCC (initial concentration 9.40 mg L^{-1}) as a sole carbon and energy source with the efficiency of 78% within 6 days and produce two intermediates 34DCA and 4CA, which were detoxified [152]. Similar metabolites were observed in strains TCC-1 and TCC-2, however, these microorganisms were characterized by higher tolerance to upper concentrations of TCC [153–155]. Anaerobic degradation of TCC by Ochrobacterium sp. was conducted with acetate as an electron donor. During transformation, 4CA and DCA were formed in all three strains. Only MC22 produced additionally aniline [152–154]. Moreover, Yun et al. identified a protein accountable for TCC hydrolysis- amidase TccA [153].

4.2. Chloroxylenol

Despite several reports describing the ability of microorganisms to eliminate chloroxylenol, there is little research devoted to the identification of intermediate products formed during biodegradation and analyzing the mechanisms responsible for the course of these processes. Nowak et al. identified two fungal species capable of degrading chloroxylenol [127]. Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373 degraded PCMX with similar efficiencies through different degradation pathways. C. elegans removed 70% of PCMX during 120 h of incubation at an initial PCMX concentration of 25 mg L^{-1} via generation of two metabolites by dehalogenation, aromatic ring hydroxylation, and methyl group oxidation of the parent compound. T. versicolor demonstrated 79% removal of PCMX during 120 h of incubation via ring-opening during hydroxylation, dehalogenation, and oxidation, leading to the formation of three metabolites. The authors suggested that the two different enzyme systems are involved in the initial step of chloroxylenol degradation: cytochrome P450 monooxygenases in C. elegans and laccases in T. versicolor. Furthermore, the metabolites generated by tested microorganisms have lower toxicity than the parent compound. Among the microorganisms demonstrating the ability to eliminate PCMX, the microscopic fungus Aspergillus niger was also described, in which over 99% PCMX loss (initial substrate content - 2 mg L^{-1}) was shown after 7 days of incubation [156]. Choi and Oh demonstrated that the removal efficiency of chloroxylenol by activated sludge depended on the initial concentration of xenobiotic [157]. PCMX at concentration 5 mg L^{-1} was eliminated over two months with a yield below 50%. In this research, the authors analysed the impact of PCMX on bacterial community structure and isolated two bacterial strains probably able to PCMX degradation: Sphingobium and Luteolibacter. Based on the literature data, the authors suggested that the biodegradation of PCMX occurs via dehalogenation and ring hydroxylation.

4.3. Methylisothiazolinone

Little is known about the biodegradation of methylisothiazolinone by microorganisms. Most often, only the initial stages of the biotransformation of this preservative are known. The few microorganisms that exhibit the ability to metabolize MIT include mainly various species of filamentous fungi [20,158]. The ability of the ligninolytic fungus Phanerochaete chrysosporium to biodegrade this biocide during a 48-hour incubation in liquid culture under aerobic conditions was described. The tested microorganism was able to completely eliminate MIT at a concentration of 50 μg L^{-1} and 30 mg L^{-1} within 12 h [20]. Identified metabolites, formed during the degradation of MIT by P. chrysosporium were mono and dihydroxylated methylisothiazolinon and N-methylmalonamic acid. The presence of hydroxylated derivatives indicates the involvement of hydroxylating enzymes in

the biotransformation process. However, measurements of the activity of laccase, manganese peroxidase, lignin peroxidase, and cytochrome P450 did not confirm the involvement of these enzymes. It is noteworthy that the resulting MIT derivatives are less toxic than the parent compound against D. magna [20]. The process of biodegradation of MIT by three strains of filamentous fungi: Trichoderma longibrachiatum FB01, Aspergillus niger FB14, and Fusarium solani FB07 occurs differently. Short-chain organic acids such as tartaric acid, 2-oxobutanoic acid and acetic acid (T. longibrachiatum), malonic acid, 2-oxobutanoic acid, lactic acid, metoxiacetic acid, and acetic acid (A. niger) and malonic acid, 2-oxobutanoic acid, propanoic acid and acetic acid (F. solani) have been identified as metabolites of this preservative. The tested fungi were able to eliminate MIT in 16 h [158]. In both studies, stimulation of the growth of the tested microorganisms was observed, which probably use this compound as a source of carbon and energy. So far, another pathway for the biodegradation of MIT by the microalgae Scendesmus sp. LX1 has been described. The algae completely removed MIT over 4 days and led to the cleavage of the ring by methylation and carboxylation [159].

4.4. Benzalkonium chloride

Several reports have already described biologically mediated BAC degradation under laboratory conditions. The first report demonstrated the decomposition of BAC by 20 strains of bacteria *Burkholderia cepacia*. After an incubation period of 7 days, about 42.6% of BAC was eliminated. Benzyldimethylamine and benzylmethylamine were reported to be the metabolites present at the initial step of BAC degradation as a result of the cleavage of the C alkyl-N bond. The authors identified two enzymes potentially responsible for C-N bond cleavage: amine oxidase and Rieske-type oxygenase. Moreover, 8 catabolic enzymes involved in benzyldimethylamine degradation were identified, and complete degradation of the alkyl group of BAC was noted [160]. The isolation of BAC-degrading microorganisms from a wide range of ecosystems has been described. Ertekin et al. isolated a strain highly resistant to BAC at a minimal inhibitory concentration of 1024 mg L^{-1} [161]. Identified *Pseudomonas sp*. BIOMIG1 was able to eliminate BAC within 3 days by leading to complete mineralization. Evaluation of immobilization as a better method for BAC elimination was described by Bergero et al. [162]. Comparison of BAC biodegradation by planktonic cells of *Aeromonas hydrophila* MFB03 isolated from industrial WWTPs with its degradation by Ca-alginate encapsulated cells showed that immobilization increased the efficiency of elimination and after 48 h led to the utilization of 90% BAC as a sole carbon and energy source. Thanks to physical protection, immobilized cells are more resistant to BAC than free cells. Similar results were obtained for *Pseudomonas putida* ATCC 12633 [163]. Moreover, the use of microbial consortium formed by these two strains and encapsulated in Ca-alginate is the most efficient method for BAC removal [162]. N,N-dimethylbenzylamine was observed as the result of C-alkyl-N bond cleavage of BAC-C16 by two isolates from marine sediments, *Bacillus niabensis*, and *Thalassospira sp*. These bacteria were able to degrade up to 90% BAC, during 7 days [164]. Oh et al. studied the biodegradation of BAC as a sole carbon and energy substrate using a microbial community stemming from estuarine sediment and a member of the genus *Pseudomonas* [165]. Within 12 h, 80% of BAC degradation was observed in a bioreactor inoculated with mixed cultures without the detection of biotransformation products. In order to obtain energy, *P. nitroreducens*, with the use of amine oxidases causes dealkylation of BAC and the formation of two aldehyde products- dodecanal and tetradecanal aldehydes. Obtained metabolites are characterized by lower toxicity than the parent compound. Aerobic hydroxylation of BAC catalyzed by monooxygenase possibly occurs in an enriched community of *Pseudomonas spp*. The cleavage of the Calkyl-N bond leads to the formation of benzyldimethylamine. The authors suggested that benzyldimethylamine could be biotransformed by debenzylation to benzoic acid and dimethylamine. These transformations lead to the reduction of acute toxicity (Microtox) [166]. Algal degradation of BAC via pure cultures has been explored in seawater microalgae *Tetrasemis suecica*. The tested organisms were able to

successfully eliminate BAC-C12 and BAC-C14 from seawater and produced water, with rates of about 100% and 54% within 14 days of incubation, respectively. Furthermore, twelve isomeric intermediates, which are characterized by a lower tendency to be adsorbed into sediments than the parent compounds were found. The authors suggested that the chemical reactions involved in biodegradation pathways were multiple hydroxylations followed by dehydration. Hydroxylated-BAC-C12 and dihydroxylated-BAC-C14 were the most intense by-products formed during BAC-C12 and BAC-C14 transformation, respectively [167].

Table 4. Microbial degradation of cosmetic preservatives.

Microorganism used	Initial concentration of preservative	Removal [%]	Time taken	Metabolites	References
TCC					
Microbial consortium	975.4 μg kg^{-1}	83.1	16 d	not analysed	[147]
Pseudomonas fluorescens MC46 (immobilized cells)	10 mg L^{-1}	70.14-79.18	24 h	3,4-dichloroaniline; 4-chloroaniline; aniline; catechol	[148]
Pseudomonas fluorescens MC46 (free cells)	10 mg L^{-1}	42.12	24 h	3,4-dichloroaniline; 4-chloroaniline; aniline; catechol	[148]
Pseudomonas fluorescens MC46 (immobilized cells)	5 mg L^{-1}	73.97 ± 0.03	8 h	Not analysed	[149]
	10 mg L^{-1}	78.26 ± 0.14	8 h	3,4-dichloroaniline; 4-chloroaniline; aniline;	
	20 mg L^{-1}	50.98 ± 0.27	8 h	Not analysed	
	30 mg L^{-1}	27.05 ± 0.71	8 h	Not analysed	
	40 mg L^{-1}	10.54 ± 0.10	8 h	Not analysed	
	50 mg L^{-1}	7.88 ± 0.66	8 h	Not analysed	
Pseudomonas fluorescens MC46 (free cells)	5 mg L^{-1}	54.52 ± 0.06	8 h	Not analysed	[149]
	10 mg L^{-1}	44.73 ± 0.20	8 h	3,4-dichloroaniline; 4-chloroaniline; aniline;	
	20 mg L^{-1}	22.45 ± 0.27	8 h	Not analysed	
	30 mg L^{-1}	16.98 ± 0.13	8 h	Not analysed	
	40 mg L^{-1}	6.74 ± 0.01	8 h	Not analysed	
	50 mg L^{-1}	4.30 ± 0.02	8 h	Not analysed	
Pseudomonas fluorescens MC46	9. 5 mg L^{-1}	67 ± 2	6 h	Not analysed	[150]
Sphingomonas sp. YL-JM2C	4 mg L^{-1}	35	5 d	3,4-dichloroaniline; 4-chloroaniline; 4-chlorocatechol;	[151]
Ochrobactrum sp. TCC-2	5 mg L^{-1}	56.70 ± 1.50	48 h	Not analysed	[155]
Ochrobactrum sp. MC22 (aerobic conditions)	9.40 mg L^{-1}	78 ± 4.9	6 d	3,4-dichloroaniline; 4-chloroaniline;	[152]
Ochrobactrum sp. MC22 (anaerobic conditions)	9.40 mg L^{-1}	50%	14 d	3,4-dichloroaniline; 4-chloroaniline; aniline;	[152]
Ochrobactrum sp. TCC-2 (aerobic conditions)	31.7 μM	96.88 ± 0.05	24 h	4-chloroaniline; 3,4-Dichloroaniline;	[153]
Ochrobactrum sp. TCC-2 (anaerobic conditions)	31.7 μM	72.70 ± 2.90	24 h	4-chloroaniline; 3,4-Dichloroaniline;	[153]

PCMX					
Cunninghamella elegans IM 1785/21GP	25 mg L^{-1}	70	120 h	2,6-dimethylbenzene-1,4-diol, di-TMS; 2,5-dihydroxy-3-methylbenzaldehyde, di-TMS;	[127]
Trametes versicolor IM 373	25 mg L^{-1}	79	120 h	4,6-dioxohex-2-enoic acid, TMS; 5-methyl-6-oxohexa-2,4-dienoic acid, TMS; 3-chloro-2,4-dimethylhexa-2,4-dienedioic acid, di-TMS;	[127]
Aspergillus niger	2 mg L^{-1}	99	7 d	Not analysed	[156]
Klebsiella pneumoniae D2 (free cells)	8 mg L^{-1}	55.7	24 h	Not analysed	[168]
Klebsiella pneumoniae D2 (immobilized cells)	8 mg L^{-1}	88.3	24 h	Not analysed	[168]
Activated sludge	0.5 mg L^{-1}	39.4 ± 17.3	72 h	Not analysed	[157]
Activated sludge	5 mg L^{-1}	49.4 ± 15	72 h	Not analysed	[157]
MIT					
Pchanerochaete chrysosporium	50 μg L^{-1} and 30 mg L^{-1}	100	12 h	monohydroxylated MIT; dihydroxylated MIT; N-methylmalonamic acid;	[20]
Trichoderma longibrachiatum FB01	10 g L^{-1}	100	16 h	tartaric acid; 2-oxobutanoic acid; acetic acid;	[158]
Aspergillus niger FB14	10 g L^{-1}	100	16 h	malonic acid; 2-oxobutanoic acid; lactic acid; metoxiacetic acid; acetic acid;	[158]
Fusarium solani FB07	10 g L^{-1}	100	16 h	malonic acid; 2-oxobutanoic acid; propanoic acid; acetic acid;	[158]
BAC					
20 strains of *Burkholderia cepacia*	34-64 mg L^{-1}	4.7 ± 2.4 – 42.6 ± 12.3	7 d	benzyldimethylamine; benzylmethylamine	[160]
Pseudomonas sp. BIOMIG1	200 μM	62.5	3 d	mineralization	[161]
Aeromonas hydrophila MFB03 (immobilized cells)	25-210 mg L^{-1}	90	48 h	Not analysed	[162]
Aeromonas hydrophila MFB03 (free cells)	50 mg L^{-1}	74.2 ± 2.3 – 80.4 ± 0.6	48 h	Not analysed	[162]
Pseudomonas putida ATCC 12633 (immobilized cells)	50 mg L^{-1}	74 ± 4.70	48 h	Not analysed	[162]
Pseudomonas putida ATCC 12633 (immobilized cells)	105-315 mg L^{-1}	90	24 h	Not analysed	[163]

Bacillus niabensis	2 mg mL^{-1}	Up to 90	7 d	N,N-dimethylbenzylamine	[164]
Thalassospira sp	4 mg mL^{-1}	Up to 90	7 d	N,N-dimethylbenzylamine	[164]
Microbial community	50 mg L^{-1}	80	12 h	Not detected	[165]
Microbial community	50 mg L^{-1}	100	24 h	benzyldimethylamine	[166]
Tetrasemis suecica	5 mg L^{-1}	100	3-6 d	OH-BAC-C12; 2OH-BAC-C14	[167]

5. Conclusions

This review demonstrates that, due to their broad application in many products and no effective removal in wastewater treatment plants, triclocarban, chloroxylenol, methylisothiazolinone, and benzalkonium chloride are often detected as emerging contaminants in sewage as well as in receiving environments including surface water, sediments, and soils. Dischargers from WWTPs were identified as the major source of these xenobiotics in the natural environment, albeit biosolid-amended soils and reclaimed water for irrigation may too be important sources of preservatives in the environment. The concentrations of biocides residues were ranging from ng L^{-1} to ug L^{-1} in WWTP and surface waters and from ng g^{-1} to ug g^{-1} in sediments and soils. Among all discussed preservatives, TCC and BAC were the most frequently described. The factors influencing their occurrence include population size, consumption, social level, seasons, and wastewater treatment technology. The occurrence and prevalence of discussed micropollutants in the environment are of increasing interest, owing to their toxicity potential on non-target organisms such as aquatic and terrestrial ones. The disadvantageous effects include neurotoxicity, genotoxicity, embryotoxicity, growth inhibition, abnormality in motility, lifespan, hatching, and endocrine disrupting effects. Several microorganisms such as strains of bacteria, fungi, and microalgae as pure and mixed cultures, free or immobilized cells were found to be capable of degrading preservatives. With the availability of sensitive chromatographic and mass spectrometric methods, it was possible to identify and characterize formed metabolites. A decrease or increase in the toxicity of emerging products compared to parent compounds were also noted.

Here, we recommend the following key areas for research:

- Because of the market development, extensive use, and continuous discharge of personal care products, there is a need for more detailed data on the environmental occurrence mainly for chloroxylenol and methylisothiazolinone.
- Toxicological studies should be considered on chronic effects of environmental concentrations of single preservatives and their metabolites, as well as a mixture of pollutants toward aquatic and soil organisms.
- Mechanisms of microbial biodegradation of preservatives and their metabolites should also be better understood, which will make it possible to design a treatment technology that is both effective and affordable for limiting the release of pollutants.

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Occurrence of methylisothiazolinone in water and soil samples in Poland and its biodegradation by Phanerochaete chrysosporium

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Occurrence of methylisothiazolinone in water and soil samples in Poland and its biodegradation by *Phanerochaete chrysosporium*

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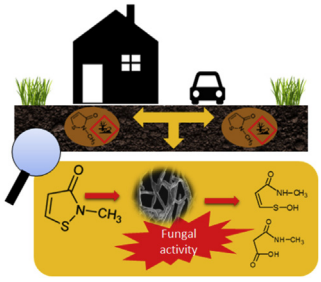
HIGHLIGHTS

- Methylisothiazolinone (MIT) occurs in soil and sewage samples in Poland.
- The ability of *P. chrysosporium* DSM 1556 to eliminate MIT was shown.
- Three metabolites of methylisothiazolinone were detected in *P. chrysosporium* cultures.
- Lower toxicity of MIT metabolites compared to the parent compound were observed.

GRAPHICAL ABSTRACT



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ABSTRACT

Methylisothiazolinone is a commonly used biocide that is released into natural environments. In this work, the ability of the fungal strain *Phanerochaete chrysosporium* DSM 1556 to biotransform this compound was evaluated. The tested strain was able to remove MIT (at concentrations 50 μg L^{-1} and 30 mg L^{-1}) from the growth medium with the efficiency 90% after the first 6 h and 100% after 12 h of incubation. Moreover, for the first time, qualitative LC-MS/MS and GC-MS analysis showed monohydroxylated and dihydroxylated methylisothiazolinone and N-methylmalonamic acid as the main products of fungal biodegradation. The ecological toxicity of the tested biocide and its derivatives was also evaluated by using an acute toxicity test with *Daphnia magna*. An approximately 90% decrease in the toxicity of metabolites formed in the *P. chrysosporium* culture was noticed. The concentration of MIT in soil and water samples collected in Poland was assessed for the first time. The analysis showed that the selected locations in Poland are contaminated by MIT in the range from 1.04–10.08 μg L^{-1}.

1. Introduction

Personal care products (PCPs) include a wide group of compounds used as additives in shampoos, soaps, toothpastes and many other products (Brausch and Rand, 2011). They act as preservatives, fragrances, surfactants and UV filters. Isothiazolinones are among the most often isolated biocides. Due to their strong antimicrobial activity, isothiazolinones are used in many industries, for example, in the paper and textile industries, as well as in households (Rafoth et al., 2007). One of the primary representatives of this group of compounds is methylisothiazolinone [2-methyl-4-isothiazolin-3-one] (MIT, MI), which is used to limit the growth of

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fungi, bacteria and algae (Capkin et al., 2017). Among isothiazolinones, MIT is the most common additive in cosmetics. Due to its strong allergenic properties, the maximum concentration of MIT in cosmetics has been set at 100 ppm. However, there are no restrictions the use of on industrial and household chemicals (Baranowska and Wojciechowska, 2013; Lundov et al., 2011).

The growing consumption of personal care products and the constantly developing industry contribute to the release of harmful substances into the natural environment. This threat is exacerbated by the fact that popular biocides, such as MIT, are leached from the paints used to cover facades and roofs of buildings by rainfall and can be brought into the soil or discharged into surface waters. Moreover, climate change and increased rainfall intensity may exacerbate this problem (Kresmann et al., 2018; Bollmann et al., 2017). Although MIT is not subject to bioaccumulation and persistence in the environment, due to its continuous release, it is referred to as a pseudo-persistent compound (Daughton, 2005).

The main goals in environmental protection are reducing the emission of harmful waste, introducing new methods of wastewater treatment, monitoring the level of contamination and understanding the transformation of PCPs in the natural environment (Sun et al., 2014b; Diamond et al., 2015; Wieck et al., 2016). The release of MIT and its derivatives into the environment is limited by wastewater treatment using appropriate methods with physical, chemical or biological agents. The physico-chemical methods of MIT elimination include photocatalytic oxidation, electrochemical degradation and ozonation (Kandavelu et al., 2004, 2016; Li et al., 2016). Kandavelu et al. (2004) carried out a study on the photocatalytic degradation of an isothiazolinones mixture to examine the possibility of decomposing these compounds, soon after applying paints on solid surfaces. The isothiazolinone mixture containing CMI (chloromethylisothiazolinone) and MIT in the total concentration $8{,}75 \times 10^{-4}$ M was photodegraded in an aqueous solution with the use of catalytic powder such as TiO_2 and ZnO. The obtained results indicated that the degradation process was faster under the UV-lamps than under the sunset or fluorescent lamps. Moreover, the reaction proceeded faster in the presence of the oxygen atmosphere and H_2O_2 than in the air atmosphere. The degradation process was accompanied by the transformation of MI and CMI into CO_2, SO_4^{2-}, NO_3^- and HCl. The possibility of electrochemical degradation of isothiazolinones was evaluated among others by Kandavelu et al. (2016). Researchers investigated the potential of the electrochemical degradation of MIT and CMI, at the born-doped diamond (BDD) anode in an aqueous solution, containing Na_2SO_4. The obtained results showed that the hydroxyl radicals (·OH) generated by the surface of BDD were responsible for the degradation of tested compounds. The resulting degradation products were the same as those produced during the photodegradation process (Kandavelu et al., 2016). Another physico-chemical method of MIT degradation is ozonation. The kinetics and mechanisms involved in the transformation of MIT during ozonation were investigated by Li et al. (2016). The obtained results indicated that ozonation is a useful method of degrading MIT in water, during which the following reactions take place: oxidation of the sulfur atom, hydrolysis, addition of the carbon-carbon double bond, oxidation of the α-carbon and decarboxylation. Moreover, Li et al. (2016) showed that ozone can decrease the toxicity of MIT by transforming the sulfur atom into a sulfate ion.

The traditional physico-chemical way of degrading and detoxifying MIT and other toxic compounds can be ineffective and uneconomical when the contaminant concentration is low. An alternative method is biodegradation using a microorganism able to eliminate many compounds (Andrade et al., 2013; Zawadzka et al., 2015; Janicki et al., 2016). The existing knowledge about the biodegradation of MIT is limited. The mechanisms of its biotransformation, potential derivatives and the biotoxic effects of these processes have not been investigated sufficiently. Moreover, currently available biological methods are disturbed by MIT that affects the wastewater treatment process by completely inhibiting the nitrification (Amat et al., 2015). The ability of mesophilic fungi to grow in the presence of MIT and to degrade the xenobiotic were evaluated by Gomes et al. (2018). Fungal strains were isolated from a water-based wall paint and its components in a paint factory. The potential of the tested fungi to degrade MIT during submerged fermentation with the addition 10 mg L^{-1} of MIT was evaluated. The obtained results indicated that *Aspergillus niger* FB14 was able to transform MIT to malonic, lactic, acetic, 2-oxobutiric and metoxiacetic acids. After the incubation of *Fusarium solani* FB07 with MIT, acetic, propionic, malonic, propanoic and 2-oxobutylic acids were identified. The detected intermediates of MIT degradation in *Trichoderma longibrachiatum* FB01 included tartaric, acetic, 2-oxobutyric acids.

There have been many reports on the toxicity of MIT in recent years. MIT is characterized as neurotoxic, allergenic, genotoxic and ecotoxic (Du et al., 2002; He et al., 2006; Kresmann et al., 2018). He et al. (2006) investigated the neurotoxicity potential of MIT in vitro, showing that 3 μM MIT caused the death of approximately 35% of nerve cells, whereas longer exposure to lower concentrations of the xenobiotic limited the development of cortical neurons. Moreover, MIT is presented in many works as a new allergen causing allergic contact dermatitis (Isaksson et al., 2004; Thyssen et al., 2006; García-Gavín et al., 2010; Flury et al., 2018). In one of the studies, which assessed the risk of MIT to consumers, there were observed patients with skin disorders caused by the use of wet intimate hygiene wipes and make-up removers containing methylisothiazolinone (García-Gavín et al., 2010). Kresmann et al. (2018) conducted a study that was aimed at evaluating the toxicity of terbutryn, octylinone and methylisothiazolinone to aquatic organisms. It was proven that all tested preservatives were characterized by acute toxicity towards *Daphnia magna* and rainbow trout cells. The sensitivity of *Scenedesmus* sp. LX1 microalgae to various concentrations of MIT was also assessed. It was shown that xenobiotics inhibit the growth of microalgae (Wang et al., 2018, 2019).

The white rot fungus, *Phanerochaete chrysosporium*, belongs to the *Basidiomycetes* family. These fungi are characterized by the ability to produce numerous lignolytic enzymes, including manganese peroxidase, lignin peroxidase and laccase, which are involved in the biodegradation of natural lignocellulosic substrates decomposing of contaminants including phenols, dyes, chlorinated insecticides, pesticides, polychlorinated biphenyls and many others (Wariishi et al., 1991; Singh and Chen, 2008; Hu et al., 2016). Moreover, there are chosen reports indicating the involvement of cytochrome P450 in oxidation significant environmental contaminant (Subramanian and Yadav, 2009; Kasai et al., 2010). Numerous papers describe the use of *P. chrysosporium* for the treatment of textile wastewater due to the excellent ability of this species to discolour and detoxify dyes (Erkurt et al., 2007; Bosco et al., 2016; Ansari et al., 2016; Kiram et al., 2019). Kalčíková et al. (2014) studied white rot fungi and their extracellular enzymes in terms of their use for the purification and detoxification of leachate from landfills. The obtained results clearly indicate that the tested microorganism is able to eliminate impurities and to significantly reduce their toxicity.

In this study, the ability of *Phanerochaete chrysosporium* DSM 1156 to degrade methylisothiazolinone at environmental concentrations has been tested for the first time. The metabolites formed during this process have been identified, and the toxicity of the test compound and the resulting metabolites has been analyzed. Additionally, the environmental concentrations of MIT in Poland have been estimated for the first time.

2. Materials and methods

2.1. Chemicals

2-Methyl-4-isothiazolin-3-one (purity ≥95,0%) was purchased from Sigma-Aldrich (USA). The other chemicals were acquired from POCh (Gliwice, Poland) and Sigma-Aldrich (USA). Stock solutions of MIT were prepared in ethanol at a concentration of 6 mg mL^{-1}.

2.2. Environmental sample preparation

The presence of MIT in the natural environment was assessed on the basis of water and soil sample analysis. Sampling was performed at different locations in Poland, including 2 sites near the Polish coast (Chłapowo and Łeba), 3 sites at carwashes (Poddębice, Tomaszów Mazowiecki and Radom), 2 sites in home gardens (Świecie and Radom), 1 site in the Vistula River near a paper factory (Świecie) and 1 site at a waste water treatment plant (Łódź). The locations were chosen because of possible contamination by various compounds from the group of personal care products.

Surface water samples were collected from the mainstream at a depth of approximately 20–50 cm below the water table and then stored in bottles at −22 °C until analyzed. Wastewater samples were collected at the place of sewage inflow to the sewage treatment plant. They were taken in three equal time intervals and then stored in bottles at −22 °C until analysis. Soil samples were taken from a depth of 20 cm, from three different places within 1 m^2 and then stored in bottles at −22 °C until analysis. All the samples were prepared in four independent replications. Before the LC-MS/MS analysis, they were weighed and flooded with deionized water.

2.3. Preparation of microorganism precultures

The *Phanerochaete chrysosporium* DSM 1556 used in this study was purchased from the DSMZ collection (Germany). Spores from 14-day-old cultures incubated on ZT agar slants (glucose 4 g; yeast extract 4 g; agar 25 g; malt extract 12°Blg; up to 1 L; pH 7.0) at 28 °C were used to inoculate 30 mL of Sabouraud medium (Difco, USA), supplemented with 2% glucose, in 100 mL Erlenmeyer flasks. Using Thoma cell counting chamber the number of spores was determined to be $3,5 \times 10^6$ cells mL^{-1}. The inoculum was incubated on a rotary shaker (120 rpm) at 28 °C for 24 h. Then, the preculture was transferred to fresh Sabouraud medium with 2% glucose and incubated for 24 h under the same conditions.

2.4. Methylisothiazolinone elimination and dry weight estimation

The 18 mL of fresh Lobos medium (K_2HPO_4 4.35 g; KH_2PO_4 1.7 g; NH_4Cl 2.1 g; $MgSO_4$ 0.2 g; $FeSO_4 \times 7H_2O$ 0.01 g; $CaCl_2 \times 2H_2O$ 0.03 g; up to 1 L; pH 6.8) with 1% glucose and with or without (control sample) the addition of the xenobiotic was inoculated with 2 mL preculture. Abiotic controls were performed without the addition of microorganisms. In all experiments, the initial concentrations of MIT were 50 μg L^{-1} and 30 mg L^{-1}. All the samples were incubated on a rotary shaker (120 rpm) at 28 °C for 48 h. For dry weight estimation the mycelium of *P. chrysosporium* from the tested samples and the biotic controls was separated by filtration, washed two times in distilled water and dried at 100 °C to obtain constant mass.

2.5. MIT removal by P. chrysosporium post-culture medium

The preculture prepared as described in Section 2.3. was separated by filtration through Sartorious membrane filters (0.25 μm pore size). Then 2 mL supernatant was added to 18 mL fresh Lobos medium supplemented with MIT (50 μg L^{-1} and 30 mg L^{-1}). Biotic and abiotic controls were prepared as in Section 2.4. All the samples were incubated on a rotary shaker (120 rpm) at 28 °C for 48 h.

2.6. MIT elimination by autoclaved preculture of P. chrysosporium

The preculture prepared as described in Section 2.3. was autoclaved at 121 °C for 20 min. Next, 18 mL fresh Lobos medium was inoculated with autoclaved preculture. Biotic and abiotic controls were prepared as in Section 2.4. All the samples were incubated on a rotary shaker (120 rpm) at 28 °C for 48 h.

2.7. The influence of cytochrome P450 inhibitors on MIT elimination

The 2 mL preculture from Section 2.3. was added to 18 mL fresh Lobos medium. Then, four different inhibitors of cytochrome P450 (proadifen, metyrapone, 1-aminobenzotriazole, sodium azide) were added at concentrations which had no negative influence on the growth of mycelium (0.1, 0.1, 2.5, 0.5 mM). All the samples were incubated on a rotary shaker (120 rpm) at 28 °C, for 2.5 h. Subsequently, a suitable amount of the xenobiotic was added. Further, the biotic control, growth control with the addition of inhibitors and abiotic control were prepared. The samples were incubated in the same conditions as in the previous section.

2.8. Determination of ligninolytic enzymes activity

Extracellular ligninolytic enzyme activities were measured in post-culture medium obtained after centrifugation for 10 min at 10.000 rpm of the samples prepared like in section 2.4. Manganase peroxidase (MnP) activity was assayed spectrophotometrically at 590 nm. The reaction mixture contained 0.1 mM of succinate-lactate buffer (pH 4.5), 0.05 mM of H_2O_2, 0.07 mM of 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH), 0.98 mM *para*-Dimethylaminobenzaldehyde (DMAB), 0.3 mM $MnSO_4$ and the tested sample (Ngo and Lenhoff, 1980). Lignin peroxidase (LiP) activity was also measured spectrophotometrically at 310 nm. The reaction mixture containing 2 mM veratryl alcohol solution in tartrate buffer, 0.4 mM H_2O_2 and tested sample (Collins et al., 1996). Laccase activity was assayed by measuring the oxidation of 2,7′-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt (ABTS) (Niku-Paavola et al., 1990). The enzymatic activities were expressed as nkat L^{-1}.

2.9. MIT extraction and LC-MS/MS analysis

The samples after incubation and the environmental samples were disintegrated with the use of a Mixer Mill MM400 (Retsh, Germany). The obtained homogenates were shaken for 2 min on a vortex mixer with acetonitrile (1:1, v/v). According to the modified QuEChERS protocol in the next step, 0.25 g of disodium hydrogen citrate, 0.5 g of sodium chloride, 0.5 g trisodium citrate and 2 g of anhydrous magnesium sulfate were added to each sample, and then the samples were vortexed for 2 min. The test tubes were centrifuged for 5 min at 3000 rpm, and the supernatant was frozen at −20 °C. Before the analysis the frozen samples were thawed, centrifuged for 15 min at 12.000 rpm and cleaned with syringe filters (Sartorius, Poland). Finally, the supernatants were diluted in a mixture of methanol:water (5:95, v/v) supplemented with 0.1% formic acid and 5 mM ammonium formate and analyzed on LC-MS/MS.

Quantitative analyses of MIT content in environmental samples and MIT elimination by *P. chrysosporium* were performed using an AB Sciex 4500 QTRAP mass spectrometer (AB Sciex, USA) with an Eksigent microLC 200 System (Eksigent, USA). Qualitative analyse of methylisothiazolinone metabolites produced by *P. chrysosporium*

were carried out with the same equipment as quantitative analyses. The separation of MIT and its fungal metabolites was carried out on the Eksigent C18 (0.5 mm × 50 mm x 3 μm, 120 Å) column at 50 °C. Mobile phase A was water supplemented with 0.1% formic acid and 5 mM ammonium formate and mobile phase B was methanol supplemented with 0.1% formic acid and 5 mM ammonium formate. In the quantitative analysis, the gradient of eluent A was started at 30% and maintained until 0.7 min, then eluent A was increased to 50% at 0.9 min and maintained until 1.3 min; next, eluent A was decreased to 20% and maintained until 1.7 min, before returning to the initial conditions, which were maintained until 2.5 min. The flow rate was 50 μL/min and the injection volume was 10 μL. The detection of the methylisothiazolinone was conducted using MS/MS acquisition in the multiple-reaction monitoring (MRM) and positive ionization mode. The monitored MRM pairs were *m/z* 115.9 > 101.0 and 115.9 > 98.9. The collision energies were 31 V and 23 V, respectively, declustering potential was 61 V and entrance potential 10 V. The parameters of the microESI ion source were set as: IonSpray voltage: 4500 V, Curtain gas: 25, IonSource gas 1: 35, IonSource gas 2: 35 and temperature: 500 °C.

Eight-point calibration curves were prepared for MIT in the range from 0.01 to 10 ng mL^{-1}. The linearity ranges of calibration curves for MIT were 0.25–10 ng mL^{-1} in methanol:water (5:95, v/v) matrixes. The accuracies at each MIT concentration ranged from 94.93% to 105.04%. The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.0176 ng mL^{-1} and 0.0841 ng mL^{-1}, respectively. MIT recovery [%] from different matrixes was established in the range from 60.4% ± 0.71–82% ± 11.03. The extraction efficiency for individual matrixes is shown in Table 1. During all analyses, the blanks were below the detection level.

The qualitative analysis of methylisothiazolinone metabolites produced by *P. chrysosporium* were performed with the use of LC-MS/MS and GC-MS techniques. The samples for qualitative analysis on LC-MS/MS were prepared in the same manner as the samples for quantitative analysis. The detection of MIT metabolites was performed on an MS/MS detector working in the full-scan MS and precursor ion (Prec) modes. The full-scan MS experiment was operated in the *m/z* range of 50–300. The second method was based on the monitoring of the precursor ion at *m/z* 90.9 within the *m/z* range of 50–300. EPI scans over the *m/z* range of 50–300 were used to collect mass spectra of possible MIT fungal metabolites. Finally, the qualitative LC-MS/MS method involving multiple reaction monitoring (MRM) was prepared. The monitored MRM pairs were *m/z* 132.9–115.9, 132.9–105.0, 148.9–105.0, 148.9–134.9, 116.1–89.0 and 116.1–73.0. Information Dependent Acquisition (IDA) criteria were set to select two peaks above 5000 counts, with an exclusion rule after three occurrences for 20 s with dynamic background subtraction. The chromatographic conditions were the same as those used for MIT quantitation except that the total flow rate was set to 30 μL per min. The optimized ion source parameters were: CUR: 25, IS: 4500 V, TEMP: 500 °C, GS1: 30, GS2: 25. Data analysis was performed with the Analyst™ software version 1.6.2 (AB Sciex, Framingham, MA, USA).

Table 1
The extraction efficiency from different matrixes. Each result represents an average ± SD (n = 4 collected from four independent experiments).

Sample	Extraction efficiency [%] ± SD
Universal soil I	60.9 ± 0.71
Universal soil II	71.7 ± 2.12
Standard sea water	80.6 ± 0.28
Standard fresh water	76.3 ± 9.76
Tap water	77.2 ± 8.2
Deionized water	77.1 ± 0.42
Sabouraud medium	82 ± 11.03
Lobos medium supplemented with 1% glucose	77.7 ± 2.97

The qualitative analysis on GC-MS was performed according to the method described by Schettgen et al. (2017). Filtered culture medium was freeze dried and then redissolved in 1 mL of acetonitrile in an ultrasonic bath for 5 min. Next, pentafluorobenzylbromide (PFBBr) solution (100 μL) and 10 mg of potassium carbonate were added. The samples were placed in an oven at 60 °C for 16 h. In the next step, 3 mL of N-acetyl-cysteine at a concentration of 10 g L^{-1} and 1 mL of n-hexane were added and then vortexed. After centrifugation, the n-hexane layer was obtained, which was then analyzed by GC-MS. Metabolites were detected using an Agilent Technologies 7890A Gas Chromatography system equipped with a 5975C Triple-Axis Detector. Separations were performed on a methyl polysiloxane HP 5MS column (30 m × 0.25 mm). Helium at 1.2 mL min^{-1} was used as a carrier gas. The following temperature gradient was applied: the initial temperature was set at 80 °C for 2 min, increased to 300 °C at 20 °C min^{-1}, and held for 3 min. The mass range of qualitative analysis included 100–400 *m/z*.

2.10. Toxicity study

The ecological toxicity effects of MIT and its fungal metabolites were analyzed by using an acute toxicity test with *Daphnia magna*. The biotest was conducted with the Daphtoxkit F magna commercial test (Microbiotests, Inc., Mariakerke-Gent, Belgium) in accordance with the instructions attached by the producer. Fungal cultures incubated with MIT (30 mg L^{-1}) were prepared according to the procedure describing in Section 2.4. After incubation, the cultures were filtered with the use of membrane filters (0.25 μm pore size). The obtained supernatants were diluted tenfold and the MIT stock solution, in appropriate concentrations, was added to fresh water. The toxicity of MIT and its metabolites was calculated as a percentage of immobile larvae after 48 h of incubation.

2.11. Statistical analysis

All results are shown as the mean values ± standard deviation (SD). The Mann-Whitney *U* test was used to determine the statistical significance of the results, and differences at $P < 0.05$ were considered significant. Statistical analyses were performed using Statistica 13 (StatSoft, Poland).

3. Results and discussion

3.1. Occurrence of MIT in the environment

Isothiazolinones are effective preservatives used to control the growth of microorganisms such as fungi and bacteria. Considering their wide application, there are many pathways that allow isothiazolinones to enter water or soil environments. Despite their mass production and widespread use, few scientific studies regarding the occurrence of these substances in the natural environment are available at this point (Bollmann et al., 2017; Fewings and Menné, 1999; Nielsen, 1994).

Due to the minimal amount of literature data, selected environmental samples were examined for the presence of MIT. The described method, using advanced techniques of liquid chromatography coupled with tandem mass spectrometry, enabled detection of one of the most commonly used representatives of isothiazolinones – methylisothiazolinone - in large amounts in samples from various regions of Poland. The MIT content in water and soil samples was determined on the basis of a calibration curve and a comparison of retention times with a reference substance. The obtained results are shown in Table 2.

Most of the xenobiotic was found in soil samples taken from the

Table 2
Concentrations of MIT in environmental samples collected in Poland. Each result represents an average ± SD (n = 4 collected from four independent experiments).

Sample	Concentration of identified compound [μg L^{-1}/μg Kg^{-1}]
Chłapowo (sea water sample)	Below LOQ
Chłapowo (beach sand sample)	4.48 ± 1.04
Łeba (sea water sample)	Below LOQ
Łeba (beach sand sample)	2.19 ± 0.47
Vistula River (Świecie)	Below LOQ
Świecie (soil sample from a garden)	1.04 ± 0.06
Poddębice (soil sample from a carwash)	10.8 ± 1.07
Tomaszów Mazowiecki (soil sample from a carwash)	5.92 ± 0.56
Radom (soil sample from a garden)	1.91 ± 0.12
Radom (soil sample from a carwash)	2.5 ± 0.31
Łódź (untreated sewage)	1.21 ± 0.46

car washes in Poddębice and Tomaszów Mazowiecki. It was probably caused by the use of high concentrations of biocides to protect the water system against algae and fungi. In addition, some of the water that is used to wash cars, despite the sewage system, flows directly into the soil. High levels of MIT were also observed near the Polish coast, and more specifically in sand samples. In summer frequent use of sun protection cosmetics, which contain preservatives, may cause the presence of high concentrations of this biocide. Seasonal variation of contamination was observed by Loraine and Pettigrove (2006). The highest concentration of benzophenone-3 (UV-filter) in raw drinking water in California was observed in dry season. Also results presented by Sun et al. (2014a) showed that an increased use of anti-inflammatory agents in cold season causes the occurrence of high concentrations of pharmaceuticals in waste water in Xiamen, China. Slightly lower concentrations of the xenobiotic were found in the garden soil of a home garden. MIT presence in these samples may have been caused by the leaching of MIT from the paints that cover the houses. Bester et al. (2014) showed that, as a result of MIT runoff from the walls of buildings, its concentration in the environment can reach up to 30.000 μg L^{-1}. Thus, the presented results confirm the effect of the use biocides on the biocidal pollution of the environment. The concentration of the test compound in raw sewage was 1.21 μg L^{-1} ± 0.46. Rafoth et al. (2007) analyzed the water samples for the presence of five main representatives of isothiazolinones. In the sewage samples, they detected the presence of MIT, CMI (chloromethylisothiazolinone) and BIT (benzoisothiazolinone). BIT was the most frequent and most abundant biocide, and its concentrations were from 1.7 to 3.2 μg L^{-1} (Rafoth et al., 2007). It should be emphasized that high concentrations of biocides can be dangerous for microorganisms living in sewage treatment plants; as a result, the effectiveness of wastewater treatment may be reduced.

No contaminants in water samples were detected with the use of the LC-MS/MS method. The contamination of other Polish rivers by preservatives was described by Baranowska and Wojciechowska (2013). The authors developed new liquid chromatography methods, for the detection of sodium benzoate, benzyl alcohol and CMI at the concentrations of 2.68–3.12, 35.1 and 7.89–11.57 μg L^{-1}, respectively.

3.2. Effect of MIT on the growth of P. chrysosporium

In the next stage of this study, the growth of the *Phanerochaete chrysosporium* strain in the presence of MIT was evaluated (Fig. 1.). The results presented in Section 3.1. and literature data (Bester et al., 2014) enabled to select the appropriate environmental concentration of MIT, which was used for further analysis. MIT at the concentrations of 50 μg L^{-1} and 30 mg L^{-1} had no statistically significant effect on the growth of mycelium for the first 12 h. After this time, growth stimulation by the biocide was observed ($p < 0.05$).

Available literature data also describe high tolerance to MIT exhibited by various microorganisms. Minimum inhibitory concentrations (MIC) of five biocides were shown for two fungi, *Aspergillus niger* and *Saccharomyces cerevisiae*. The study revealed that MIT was the least efficient among all tested biocides, with MIC values of 166 mg L^{-1} and 60 mg L^{-1} for *A. niger* and *S. cerevisiae*, respectively (Williams, 2007). Similar research was carried out by the team of Diehl and Chapman (1999) for *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* ATCC 13388 and *Pseudomonas fluorescens* ATCC 13525. MIC values for these strains were 16, 13.8, and 17.5 μg mL^{-1}, respectively. Gomes et al. (2018) tested 30 strains of microscopic filamentous fungi. Among them, 13 strains were sensitive to MIT at the concentration of 10 g L^{-1}. The remaining strains were able to grow even at the concentrations of 30–40 g L^{-1}.

3.3. Elimination of MIT by P. chrysosporium

The preliminary stages of our research included the screening of microscopic filamentous fungi towards the elimination of MIT (unpublished data). Among the tested microorganisms, *P. chrysosporium* demonstrated the ability to efficiently remove MIT after 12 days of incubation and was selected for further analysis. Samples for quantitative LC-MS/MS evaluation were collected after 0, 6, 12, 24 and 48 h of fungal cultures with the addition of MIT. Abiotic and biotic controls were introduced, as a control system and were incubated under the same conditions. The obtained results indicated that the tested fungal strain possesses the ability to eliminate the biocide with high efficiency. At the initial concentrations of 50 μg L^{-1} and 30 mg L^{-1} MIT, *P. chrysosporium* removed approximately 90% in the first 6 h and 100% after 12 h (Fig. 2). To exclude the suspicion that the elimination of MIT was associated with the passive adsorption of the tested preservative to the cell wall, an experiment with autoclaved mycelium was conducted. The results showed that the substrate recovery was high, approximately 77–100% for each time point, which indicated the fungal metabolic activity during the elimination process. We also examined the ability of the post-culture medium to eliminate MIT. After the incubation of MIT with supernatants, we observed that the amount of the recovered substrate decreased along with the length of incubation ($p < 0.05$).

There is little literature data describing microbial elimination of biocides from the isothiazolinones group. Gomes et al. (2018) showed that fungal strains that were able to grow in the presence of MIT caused a complete elimination of the substrate. The fungal strains were isolated from a water-based wall paint and its components in a paint factory. The potential of the tested fungi to degrade MIT during submerged fermentation with the addition of 10 mg L^{-1} MIT was evaluated. After 16 h of incubation at 28 °C on a rotary shaker three strains *T. longibrachiatum*, *F. solani*, *A. niger*

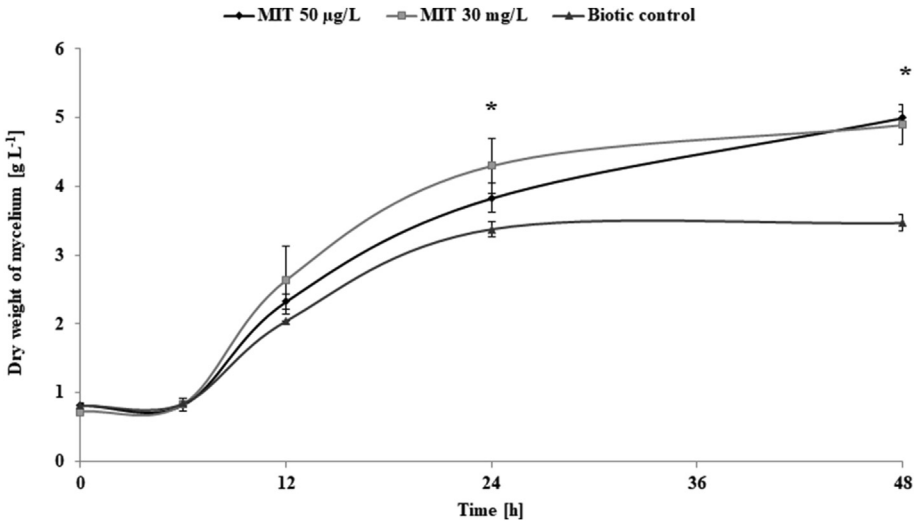

Fig. 1. Effect of MIT concentrations (50 µg L^{-1} and 30 mg L^{-1}) on the growth of *P. chrysosporium*. Each result represents an average ± SD (n = 4 collected from four independent experiments). Statistical analysis was performed using the Mann-Whitney *U* test with *p < 0.05.

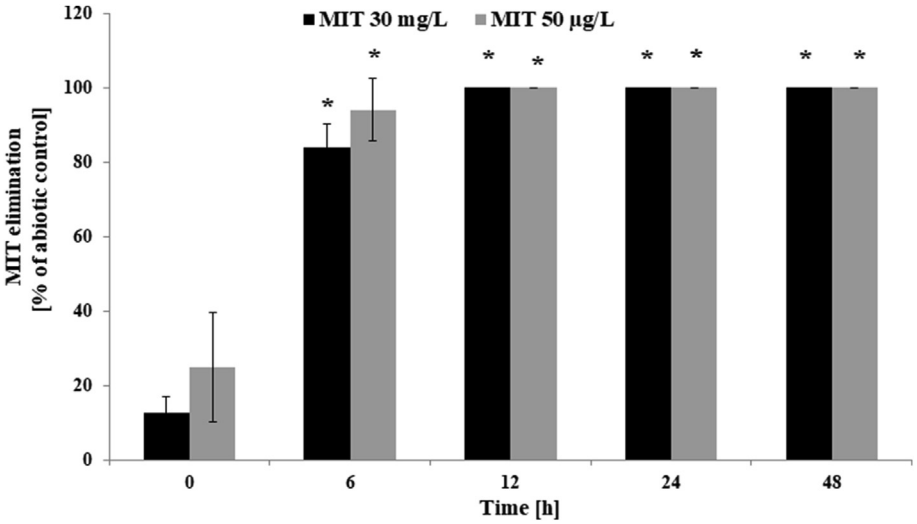

Fig. 2. MIT elimination in *P. chrysosporium* cultures incubated with the addition of MIT at the concentrations 50 µg L^{-1} and 30 mg L^{-1}. Each result represents an average ± SD (n = 4 collected from four independent experiments). Statistical analysis was performed using the Mann-Whitney *U* test with *p < 0.05.

eliminated 100% of MIT. On the other hand, numerous papers describe the possibility of using *P. chrysosporium* to degrade a wide range of toxic environmental pollutants (Hu et al., 2016; Singh and Chen, 2008). Chigu et al. (2010) describe the ability of *P. chrysosporium* to metabolize anthracene and anthrone at a concentration of 25 mM to form hydroxylated derivatives within 6 days of incubation. Andrade et al. (2013) evaluate the removal of red congo dye (30 mg L^{-1}) by *P. chrysosporium* in a batch reactor during 15 days and evaluate the influence of glucose and wheat bras as co-substrates on the process. The results show that the presence of glucose causes the highest efficiency of dye elimination with a yield of 97% without adding an external source of nitrogen. The published data also indicate that *P. chrysosporium* is capable of complete mineralization of pentachlorophenol at a concentration of 1.1 mg L^{-1} under nitrogen-limiting conditions within 28 h (Mileski et al., 1988). *P. chrysosporium* has also been reported to degrade diuron (6 µg L^{-1}) in the corn cob liquid medium under the nitrogen-limited conditions. Within 10 days of incubation, the tested strain was able to eliminate 94% of the herbicide (Coelho-Moreira et al., 2013).

3.4. Qualitative analysis of MIT metabolites produced in *P. chrysosporium* cultures

Samples for qualitative analysis of MIT metabolites were collected after 0, 6, 12, 24 and 48 h culture incubation. The corresponding abiotic and biotic controls were used as reference samples. The search for possible MIT metabolites produced by *P. chrysosporium* was carried out using full-scan MS and precursor ion scan IDA LC-MS/MS experiments. The obtained spectra allowed the creation of a final LC-MS/MS method based on a predicted MRM mode.

Hydroxylated metabolites of methylisothiazolinone with the retention time (Rt) of 0.77 and 0.92 min were identified in the fungal culture on the basis of the molecular ions [M+H]+ at *m/z* 148.9 and 132.9, which was shifted by approximately 33 and 17 Da, towards the mass of the parent compound. The mass spectrum of the monohydroxylated metabolite of MIT (Fig. 3 A), with the molecular ion $[M+H]^+$ at *m/z* 132.9, revealed the fragments at *m/z* 114.9, 105.0, 90.9 and 66.9. The second detected metabolite with the molecular ion $[M+H]^+$ at 148.9 was identified as a

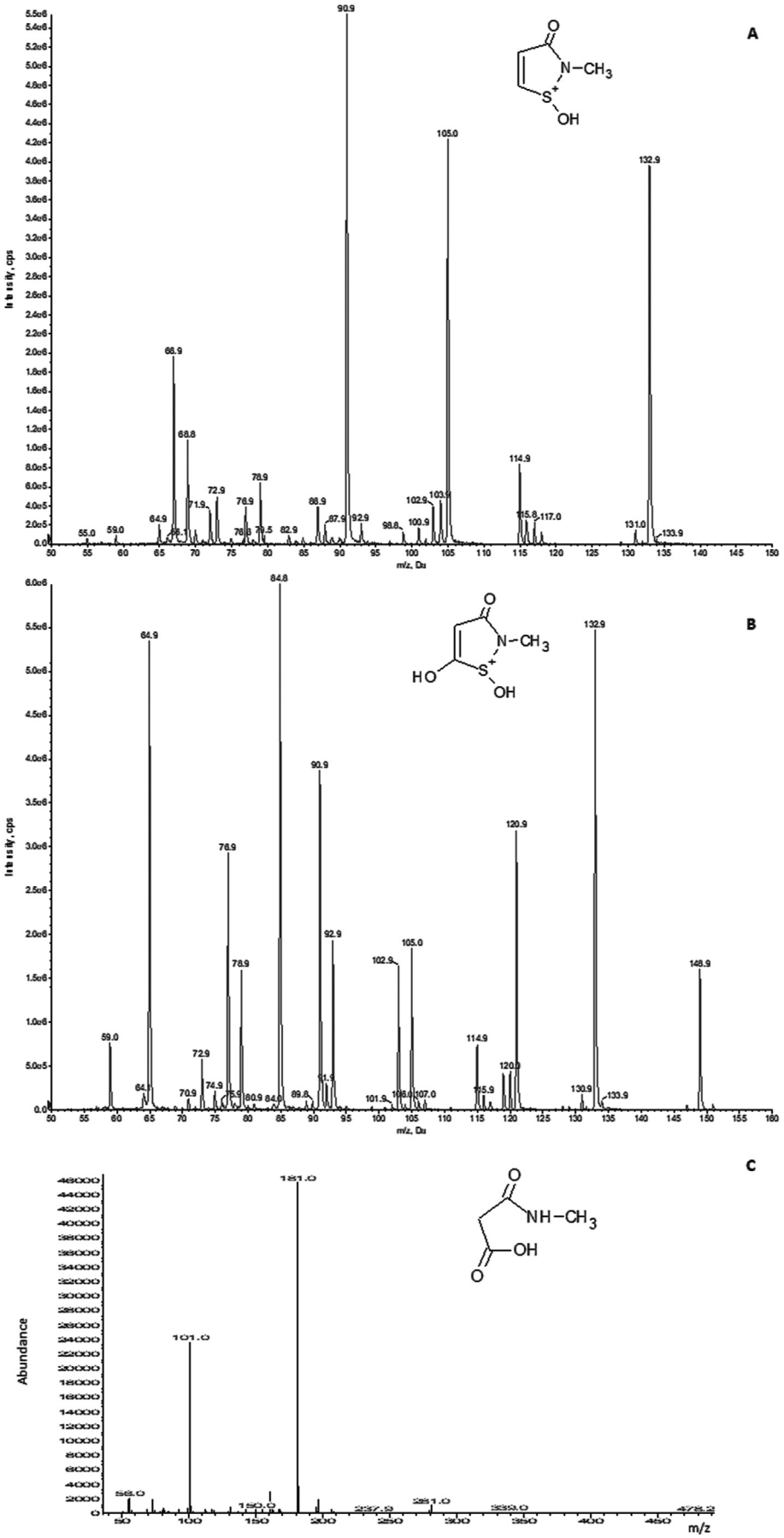

Fig. 3. Mass spectra of methylisothiazolinone metabolites produced by *P. chrysosporium*. LC-MS/MS mass spectra of monohydroxylated and dihydroxylated MIT (A,B), GC-MS mass spectrum of N-methylmalonamic acid (C).

dihydroxylated derivative of MIT on the basis of revealed fragments at *m*/*z* 132.9, 120.9, 105.0, 90.9, 84.8 and 64.9 (Fig. 3 B). The highest amount of the hydroxylated metabolites of methylisothiazolinone were detected after 6 h of incubation, and its content slightly decreased from 12 h of cultivation.

GC-MS analysis of MIT metabolites was also carried out, followed by a derivatization process. The applied protocol with pentafluorobenzylbromide (PFBBr) and potassium carbonate is a typical procedure used in the quantitative and qualitative analysis of highly polar compounds in variable biological matrices (Schettgen and Kraus, 2017; Schettgen et al., 2017). The GC-MS chromatograms allowed the detection of one MIT metabolite with Rt of 11.01 min. A comparison of the mass spectrum of the detected metabolite with the data obtained by (Schettgen and Kraus, 2017; Schettgen et al., 2017) enabled the identification of the metabolite as N-methymalonamic acid. Its mass spectrum revealed the major fragments at *m*/*z* 181 and *m*/*z* 101, characteristic peaks of the derivatizing agent (PFBBr) and NMMA, respectively (Fig. 3C). The metabolite was detected for the first time after 12 h of incubation, and the analysis of its peak area showed an increase in its amount within the fungal culture. GC-MS results were compared with LC-MS/MS data. A decrease in the amount of the first metabolite was correlated with an increased amount of the second metabolite.

There is little literature data describing microbial degradation of isothiazolinones. The ability of fungal biotransformation of methylisothiazolinone was evaluated by Gomes et al. (2018). After the incubation of *Aspergillus niger* FB14, *Trichoderma longibrachiatum* FB01 and *Fusarium solani* FB07, organic acids were identified. Tartaric, 2-oxybutanoic, acetic, malonic, lactic, methoxyacetic, and propanoic acids were detected in the cultivation medium. Moreover, the mammalian metabolism of MIT was demonstrated. Schettgen and Kraus (2017) examined the metabolism of MIT in human urine after oral dosage. A major product of MIT metabolism in humans was N-methylmalonamic acid (NMMA). In this paper, NMMA and hydroxylated intermediates have been described for the first time as the products of the microbial metabolism of MIT.

3.5. Mechanisms of methylisothiazolinone biodegradation

P. chrysosporium produces several extracellular enzymes including laccase, lignin peroxidase and manganese-dependent peroxidase, which are able to degrade a variety of environmental pollutants (Cameron et al., 2000). The activity of ligninolytic enzymes was evaluated because they may be involved in MIT degradation. Laccase activity was detected throughout the process and the highest activity was noted between 6 and 24 h of incubation. In the samples containing MIT a maximum activity was observed in 6 h and reached 41.85 nkat L^{-1}. MnP and LiP activity was not detected under any conditions with or without MIT. Insignificant levels of laccase activity suggest that this enzyme cannot be responsible for MIT degradation. The low levels of laccase activity could have been caused by the fact that the process of MIT elimination in our study was very fast and after 6 h of incubation the efficiency of elimination reached 90%. All available publications present the results of laccase activity induction after a few days during the secondary metabolism. Guo et al. (2014) demonstrated the biodegradation of sulfomethoxazole by *P. chrysosporium* in the range 10–30 mg L^{-1} and evaluated the activity of ligninolytic enzymes. The results showed that the maximum value of laccase activity was detected after 7 days of incubation. The analysis of MIT degradation products indicated that this activity was mediated by enzymes. The resulting hydroxylated metabolites suggested the contribution of CYP450 in the biotransformation process. Cytochrome P450 enzymes are a large superfamily of monooxygenases that are commonly found in living organisms. They participate in many metabolic processes, among others in the process of antibiotic detoxification (Ortiz de Montellano, 2015). In order to investigate the contribution of cytochrome P450 to fungal metabolism of MIT, its elimination by *P. chrysosporium* was also examined in the presence of four inhibitors of cytochrome P450 (proadifen, metyrapone, 1-aminobenzotriazole, sodium azide). The obtained results show that despite the addition of CYP450 inhibitors to the *P. chrysosporium* cultures supplemented with MIT, after 48 h of incubation the xenobiotic was also completely eliminated. Some researchers suggest that the activity of CYP450 inhibitors may be regulated by different nutrient conditions. The influence of nitrogen content on nonylphenol (NP) degradation by *P. chrysosporium* was investigated by Subramanian and Yadav (2009). The results showed that the eukaryotic P450 enzyme inhibitor piperonyl butoxide exerted different effects on NP elimination under low nitrogen content. A similar phenomenon was observed in the our study. The lack of cytochrome P450 inhibition, despite the use of inhibitors in *P. chrysosporium* culture supplemented with MIT, might have been caused by low nitrogen content in Lobos medium. However, the presence of hydroxylated derivatives suggested the involvement of cytochrome P450 in biotransformation MIT by *P. chrysosporium.* Therefore, further research is needed in the future to clarify in detail the mechanisms involved in the process of MIT degradation by *P. chrysosporium.*

3.6. Toxicity of MIT and its derivatives

Personal care products include a group of contaminants most frequently detected in surface water all over the world (Peck, 2006), which is caused by their widespread use and the fact that, unlike pharmaceuticals, PCPs are intended for external use and are not metabolized (Ternes et al., 2004). Currently, researchers emphasize the need to broaden the knowledge about the ecological toxicity of compounds from the PCP group (Brausch and Rand, 2011; Kresmann et al., 2018). It is assumed that the high toxicity of MIT towards microorganisms is caused by the penetration of MIT into the interior of the cell and by the interaction with significant intracellular proteins and smaller biological molecules, i.e., glutathione (GSH) (Arning et al., 2009). It is known that MIT causes high contamination of the natural environment, which has a negative impact on living organisms. However, biocidal substances such as MIT may undergo numerous physical, chemical and biological changes. As a result of these changes, derivatives may be created, the impact of which on living organisms is unknown. Therefore, we evaluated the toxicity of post-culture supernatants and pure MIT solution to *Daphnia magna.* The results for MIT in four concentrations are presented in Table 3. The obtained results proved that among the tested concentrations of MIT, only 30 mg L^{-1} was toxic to *Daphnia magna*, after 48 h. Therefore, the *P. chrysosporium* cultures with addition 30 mg L^{-1} MIT were chosen to evaluate the detoxification of the tested compound.

Supernatants from the *P. chrysosporium* culture with MIT (30 mg L^{-1}) at three time points, tenfold diluted in fresh water, and adequate abiotic controls were used to evaluate the toxicity of MIT and its metabolites (Fig. 4). The mortality of *D. magna* after 48 h of exposure to abiotic controls at all tested time points was 100%. The

Table 3
Toxicity of pure MIT solution in four concentrations against *Daphnia magna.*

Concentration of MIT [mg L^{-1}]	Mortality of *Daphnia magna* after 48 h [%]
50	100
5	100
0.5	20
0.25	0

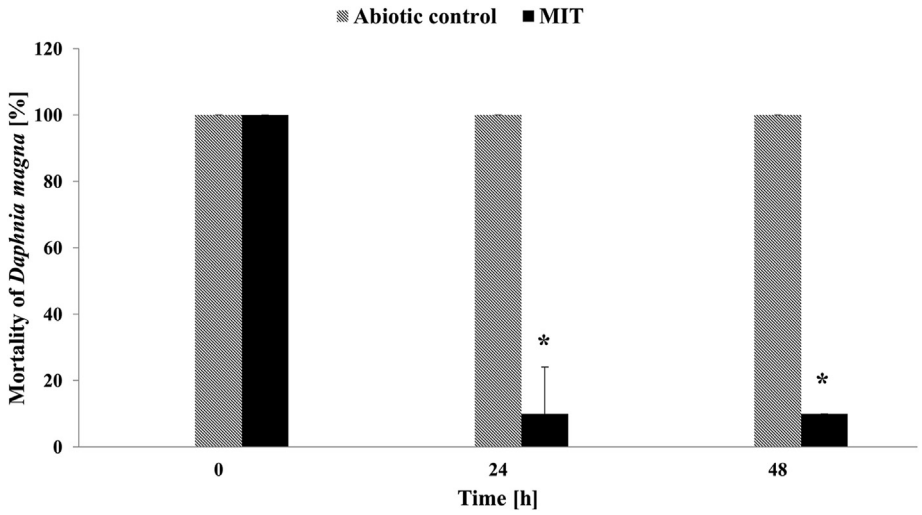

Fig. 4. Toxicity of MIT (30 mg L^{-1}) and its metabolites formed in *P. chrysosporium* cultures. Each result represents an average ± SD (n = 4 collected from four independent experiments). Statistical analysis was performed using the Mann-Whitney *U* test with *p < 0.05.

toxicity of Lobos medium was also evaluated and was shown to cause 10% mortality. A decrease approximately 90% in the toxicity of supernatants from the test sample, was observed (Fig. 4). It was proved that the metabolites formed by fungi were not characterized by higher toxicity than the parent compound. Furthermore, the data clearly demonstrate the reduction of MIT toxicity due to metabolic changes of *P. chrysosporium*.

Available studies confirm the acute toxicity of MIT to aquatic organisms. Kresmann et al. (2018) assessed the toxicity of three PCP representatives. Among all tested compounds, octanone (EC_{50} 150 μg L^{-1}) was found to be the most toxic. MIT was in the second position, with an LC_{50} dose 510 μg L^{-1} for *Daphnia magna* after 48 h of exposure. A series of tests carried out by the team of Capkin et al. (2017) showed that MIT is mutagenic and causes histopathological effects on rainbow trout. Moreover, among the tested preservatives, MIT was characterized by the highest genotoxic activity at 8.9 ± 1.8 mg L^{-1}. MIT at a concentration of 15 μM adversely affects development and regeneration processes in planaria species (Van Huizen et al., 2017). Exposure to 75 μM of MIT causes disturbances in tissue regeneration and wound healing in *Xenopus laevis* tadpoles, which may affect the viability of the developing organism (Santos et al., 2016).

4. Conclusion

Knowledge regarding the occurrence and fate of MIT in natural environments is limited. Furthermore, there is little literature data about the transformation of MIT by environmental microorganisms. In this work, the ability of *P. chrysosporium* to eliminate and biodegrade MIT was assessed. The obtained results indicated that the tested strain demonstrated tolerance to the presence of the xenobiotic. Moreover, for the first time, LC-MS/MS analysis showed that *P. chrysosporium* removed nearly 90% of MIT from the growth medium during the first 6 h and 100% after 12 h of incubation. This process was accompanied with the formation of three metabolites: monohydroxylated methylisothiazolinone, dihydroxylated methylisothiazolinone and N-methylmalonamic acid. The toxicity of MIT and the reduction of MIT toxicity due to metabolic changes of *P. chrysosporium* were demonstrated. Additionally, the environmental concentrations of MIT in Poland were evaluated for the first time.

Declaration of competing interest

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

CRediT authorship contribution statement

Marta Nowak: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Katarzyna Zawadzka:** Conceptualization, Investigation, Writing - review & editing, Visualization. **Katarzyna Lisowska:** Conceptualization, Writing - review & editing, Supervision, Project administration.

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Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways

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Biodegradation of Chloroxylenol by *Cunninghamella elegans* IM 1785/21GP and *Trametes versicolor* IM 373: Insight into Ecotoxicity and Metabolic Pathways

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Abstract: Chloroxylenol (PCMX) is applied as a preservative and disinfectant in personal care products, currently recommended for use to inactivate the SARS-CoV-2 virus. Its intensive application leads to the release of PCMX into the environment, which can have a harmful impact on aquatic and soil biotas. The aim of this study was to assess the mechanism of chloroxylenol biodegradation by the fungal strains *Cunninghamella elegans* IM 1785/21GP and *Trametes versicolor* IM 373, and investigate the ecotoxicity of emerging by-products. The residues of PCMX and formed metabolites were analysed using GC-MS. The elimination of PCMX in the cultures of tested microorganisms was above 70%. Five fungal by-products were detected for the first time. Identified intermediates were performed by dechlorination, hydroxylation, and oxidation reactions catalysed by cytochrome P450 enzymes and laccase. A real-time quantitative PCR analysis confirmed an increase in CYP450 genes expression in *C. elegans* cells. In the case of *T. versicolor*, spectrophotometric measurement of the oxidation of 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) showed a significant rise in laccase activity during PCMX elimination. Furthermore, with the use of bioindicators from different ecosystems (Daphtoxkit F and Phytotoxkit), it was revealed that the biodegradation process of PCMX had a detoxifying nature.

Keywords: biodegradation; chloroxylenol; cytochrome P450; detoxification; environmental xenobiotics; filamentous fungi; laccase

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1. Introduction

Xenobiotics occurring in the natural environment at low concentrations are referred to as anthropogenic micropollutants (MPs), which are a wide and heterogeneous group of organic compounds that enter the natural environment as a result of human activities. Despite their low concentrations, they can disrupt ecosystem function [1]. One of the representatives of these compounds is chloroxylenol (4-chloro-3,5-dimethylphenol, para-chloro-meta-xylenol, PCMX), which is an antimicrobial agent applied in disinfectant products, such as hand washing liquids, liquid soaps, Dettol and as a preservative in human care and cosmetic products [2]. Chloroxylenol has unique antimicrobial activity and is effective against bacteria, fungi, algae, and viruses. Currently, due to the global pandemic caused by a new kind of coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), many cosmetics and household chemicals have been enriched with disinfectants. The National Environment Agency (NEA) in Singapore has compiled a list of active substances and concentrations effective against the virus. One of the active ingredients is chloroxylenol at a concentration of 0.12% [3–5].

The widespread use of such products may result in the disruption of wastewater treatment unit operations and the release of MPs into the natural environment. Wastewater treatment plants (WWTPs) with residues of xenobiotics are identified as the major route responsible for contamination with micropollutants. A maximum concentration (404 μg L^{-1}) was detected in sewage treatment plants in China [6]. Despite good removal (>95%) in WWTPs, its concentration in effluent was reported to be approximately 19 to 3010 ng L^{-1} in Europe and 1700 ng L^{-1} in North America [7–10]. Incomplete removal of preservatives in WWTPs and subsequent discharge of effluents result in the presence of MPs residues in the receiving environments. Recent research regarding the occurrence of PCMX in surface waters was conducted by Dsikowitzky et al. in Jakarta city (Indonesia), where PCMX was detected in the range of 20–1200 ng L^{-1} [11,12]. PCMX was also observed in European rivers at a frequency of 20–90%, at maximum concentrations ranging from 254–358 ng L^{-1} [8,9,13].

Chloroxylenol in humans, after the long dermal exposition, is a skin and eye irritant and can modify the blood cellular composition [14]. In terms of ecotoxicity, published studies regarding the effect of PCMX on environmental species are very scarce. PCMX residue in the environment poses toxicological risks to various organisms from low to high trophic levels in aquatic environments. Recent studies on PCMX ecotoxicity have shown genotoxicity, histopathology, embryonic mortality, neurotoxicity, morphology changes and the modulation of endocrine functions in aquatic organisms. Moreover, PCMX may cause changes in the community structure of the microorganisms in the soil [15,16]. Available studies regarding the toxicological impact of PCMX on various organisms strongly suggest its potential to cause environmental health risks.

The harmful impact of chloroxylenol on the natural environment has caused the development of effective approaches for its elimination from WWTPs with the use of physicochemical and biological methods. For the elimination of chlorophenols, including PCMX, many common physicochemical approaches have been applied, including an advanced oxidation process with the use of ozonation, ultraviolet irradiation and thermally activated persulfate [17–21]. Despite the high efficiency of elimination and short-term treatment, there are some disadvantages, including the cost, the use of numerous chemical solvents, and the formation of harmful by-products. Biological methods, using microorganisms are inexpensive and are considered a major method of PCMX conversion in the natural environment. The biodegradation activity of natural microbial communities plays a key role in eliminating MPs, which is why increasing interest in understanding the metabolic pathways of xenobiotic degradation has been observed in recent years [22]. Products of microbial biodegradation of MPs might be more active or more toxic than parent compounds. Moreover, a mixture of different by-products may exhibit a higher toxicity than each metabolite alone. For these reasons, it is important to study how PCMX is transformed and check the toxicity of its by-products. There are many literature data describing the ability of microorganisms to eliminate chloroaromatic compounds; unfortunately, studies about PCMX are very scarce. Brahma et al. [23] isolated and characterized PCMX-resistant bacteria, which effectively eliminated the preservative with the formation of fewer toxic products. Moreover, several fungal species, especially those belonging to *Aspergillus* sp., are described as good PCMX degraders. As reported by Ghanem et al. [24], degradation rates of 99.72% of PCMX (2 mg L^{-1}) by *Aspergillus niger* were observed within seven days. Information about PCMX microbial degradation pathways is still unknown. However, mammalian metabolism of PCMX was studied by Thomas et al. [25], where cytochrome P450 monooxygenases removed PCMX, mainly by side chain oxidation and aromatic hydroxylation. Moreover, *ipso* substitution was evaluated as a minor degradation pathway. Much more research concerns the biodegradation pathways of other chloroaromatic compounds. Kang et al. [26,27] reported recombinant enzyme complexes cloned from *Arthrobacter chlorophenolicus* and *Escherichia coli* which catalysed the elimination of 4-chlorophenol. The side chain monooxygenation during the degradation of chlorotoluenes by toluene dioxygenase from *Pseudomonas putida* and by chlorobenzene dioxygenase from

Burkholderia sp. was observed by the team Lehning et al. [28]. Likewise, the ability of *Comamonas testosterone* to degrade chlorotoluenes was investigated. The increase in the activity of catechol 1,2-dioxygenase, responsible for the *ortho* cleavage pathway, was observed [29]. The metabolism of chlorobenzenes via hydroxylation, where chlorine atoms were substituted by hydroxyl groups, and via the *ortho* ring cleavage pathway, were noted in the *Bacillus subtilis* strain [30]. Furthermore, the enzymes of the *ortho* cleavage pathway were involved in the metabolism of toluene by *Burkholderia fungorum* [31]. In contrast, during the biodegradation of 2-chlorotoluene by *Rhodococcus* sp., 4-chlorophenol by mixed microbial community via *meta* cleavage pathway were involved [32,33]. With respect to the fungi, the ability to biodegrade chloroaromatic compounds was widely investigated. The involvement of cytochrome P450 monooxygenases catalysing the degradation of chlorophenols through hydroxylation and dehalogenation was observed [34]. The removal of 2,4-dichlorophenol by *Thielavia* sp. was conducted by the formation of hydroxylated and oxidized metabolites [35]. Furthermore, ligninolytic enzymes were found to play a key function in the degradation of chlorophenols. Kordon et al. [36] reported the total dechlorination of monochlorinated hydrobiphenyl by the laccase of *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*.

In this study, we focused on the ability of the fungi *Cunninghamella elegans* IM 1785/21GP and *Trametes versicolor* IM 373 to remove chloroxylenol and proposed its degradation by-products. Furthermore, we suggested enzymes involved in this process and determined the toxicity of PCMX untreated and treated with *C. elegans* and *T. versicolor* strains.

2. Results and Discussion

2.1. Growth and Chloroxylenol Elimination

The conducted analyses revealed a high tolerance of both strains towards high concentrations of chloroxylenol after 120 h of incubation (Figure S1). Only at the highest concentration of PCMX (50 mg L^{-1}) was a statistically significant inhibition of *T. versicolor* (77%) and *C. elegans* (86%) growth observed. Tolerance to chloroxylenol seems to be microorganism- and PCMX concentration-specific, although the majority of reports point to the impact of this preservative on the inhibition of growth. In the work by Gomaa et al., the percent inhibition of growth of *Candida albicans* rose from 3.9% to 100% with increasing the PCMX concentration from 10–70 ppm [37]. An inverse relationship between the PCMX concentration and fungal growth was reported for *Aspergillus niger* [24]. In the study of Mohammed and Al-Jibouri, most of the tested fungal growth was significantly inhibited by chloroxylenol at a concentration of 1.25%, but in the case of *Aspergillus flavus*, *Mucor* and *Fusarium*, a higher tolerance was observed [38].

Among the available scientific literature, researchers mention the physicochemical losses of PCMX rather than biodegradation. For treating PCMX-bearing waste streams, several advanced oxidation processes (AOPs) have been identified, including UV/O3, sonochemical, and electrochemical methods. Scientists have also focused on the elimination of PCMX from WWTPs by sorption and coagulation. Many of these approaches have outstanding short-term treatment efficiencies, but all of them have significant limitations and disadvantages, such as costly infrastructure and higher by-product toxicity [20,21,39]. Available literature data concerning the biological treatment of PCMX in WWTPs suggest that PCMX in the conventional activated sludge system is not decreased to satisfactory levels that avert the ecological risk to receiving bodies of water [40]. The described methods apply mainly to processes taking place under controlled conditions, e.g., in sewage treatment plants. However, we do not know much about the processes occurring in natural conditions with the participation of microorganisms. *Cunninghamella elegans* and *Trametes versicolor* are well known for their ability to eliminate different hazardous pollutants, including drugs, pesticides, dyes and personal care products [41–44]. Despite the fact that chloroxylenol is a micropollutant occurring in the natural environment, there is still little information available about its elimination and fate. An initial quantitative analysis showed that the elimination of the preservative was closely correlated with the growth of mycelium

(Figure S1). Both tested species were able to efficiently remove PCMX from culture medium supplemented with the preservative concentration in the range 5–25 mg L^{-1}, where the inhibition of growth was not observed. The level of elimination in these cultures ranged from 63% to 79% ($p < 0.05$). The highest residue of PCMX (100%), with reference to the abiotic control, was in the samples with the initial concentration of 50 mg L^{-1}, where the highest growth limitation of the tested fungi was noted. The variant containing 25 mg L^{-1} chloroxylenol was selected for the next stages of experiments, because PCMX at the chosen concentration was eliminated by the tested fungi with the highest efficiency.

The intensity of *C. elegans* and *T. versicolor* growth in the presence of the chosen PCMX concentration is demonstrated in Figure 1. Biotic controls were incubated as reference samples for the same time periods. After 120 h of cultivation, the tested concentration (25 mg L^{-1}) of PCMX did not cause significant differences in the amounts of *T. versicolor* and *C. elegans* biomass between samples with PCMX and controls.

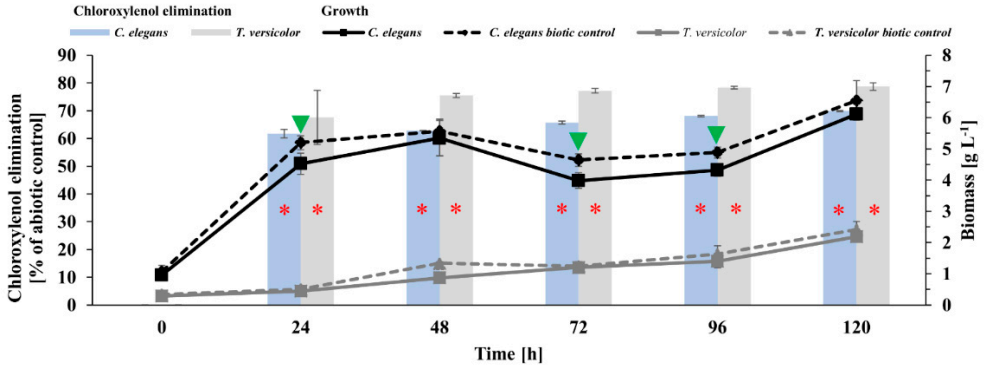


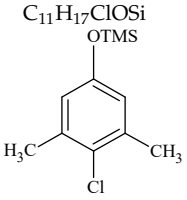
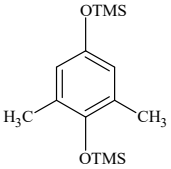
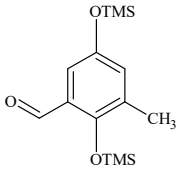
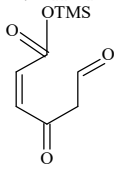
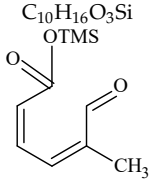
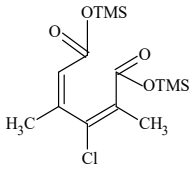
Figure 1. Kinetics of chloroxylenol elimination and the growth of *C. elegans* and *T. versicolor* cultures incubated with the addition of PCMX at a concentration of 25 mg L^{-1} on Czapek-Dox medium supplemented with 20 g L^{-1} glucose. Each result represents an average ± SD ($n = 6$). Statistical analyses were performed using the Mann–Whitney U test (* $p < 0.05$—a statistically significant increase in PCMX elimination relative to the abiotic control; ▼ $p < 0.05$—a statistically significant decrease in biomass in the sample with PCMX relative to the biotic control).

The dynamics of preservative elimination by *C. elegans* and *T. versicolor* cultures (Figure 1) showed a rapid decrease in the amount of the xenobiotic. The removal of PCMX (25 mg L^{-1}) started 24 h after its supplementation, and only 33% and 39% of PCMX were detected in the *T. versicolor* and *C. elegans* extracts, respectively. Among the tested strains, *T. versicolor* was characterized by higher effectiveness than *C. elegans*. After 120 h, the residues of the preservative in those cultures were 21% and 30%, respectively. Some research has also focused on identifying microorganisms effective towards PCMX biodegradation, for instance, a study conducted for PCMX elimination by the fungi *Aspergillus* spp. An efficient degradation level was obtained by *Aspergillus niger*, which conducted an elimination process for 7 days with a yield of 99.72% at 2 mg L^{-1} PCMX. An increase in the PCMX concentration from 2 mg L^{-1} to 20 mg L^{-1} led to a decrease in the degradation yield by approximately 8% [24]. The team of Ghanem et al. also assessed the ability of *Aspergillus terreus* and *Aspergillus versicolor* to eliminate PCMX [24]. The studied strains degraded only 55.62% and 45.62% of PCMX (2 mg L^{-1}). In another study regarding the optimization of the PCMX elimination process with the use of different methods of immobilization of *Klebsiella pneumoniae* 2D, the results revealed that immobilization on polyurethane foam pieces was the most effective in degrading PCMX, showing the highest percentage of elimination (88.3%) after 24 h of incubation. The elimination results obtained for free cells reached 52% [45]. A microbial population in the activated sludge was tested for the possibility of eliminating PCMX, which occurred at different concentrations. The obtained results showed that the level of biodegradation significantly depended on the PCMX input to the sewage. Eighty-seven percent removal was achieved for 0.5 mg L^{-1} and 44% for 5 mg L^{-1} [40].

2.2. Identification of PCMX Biodegradation Metabolites

Despite the few reports on the elimination of chloroxylenol by microorganisms, no data are currently available on what and how microorganisms degrade PCMX. Therefore, the present study focuses not only on the ability of *C. elegans* and *T. versicolor* to eliminate PCMX, but also on PCMX detoxification by formed metabolites and enzymatic mechanisms involved in this process. Qualitative analyses of PCMX metabolites formed by *T. versicolor* and *C. elegans* were carried out in 24 h incubation periods until the end of the experiment. After extract derivatization with BSTFA, the samples were analysed with GC-MS. None of the intermediates were detected in the biotic and abiotic controls. The first step of qualitative analysis was the determination of the fragmentation pattern of BSTFA-derivatized chloroxylenol. It was identified based on a molecular ion $[M]^+$ at *m/z* 228 for trimethylsilylation (TMS) derivatives and a retention time (Rt) of 7.323 min. The fragment ion at *m/z* 213 was observed after the loss of methyl $[M-15]^+$ from the trimethylsilyl group. The ion at *m/z* 177 was obtained due to the loss of methyl groups and further loss of the chloride atom from the molecular ion of chloroxylenol. The ion at *m/z* 73, which corresponded to the TMS derivative fragment ion $[(CH_3)_3Si]^+$, suggested that the hydroxyl group in the molecule was efficiently derivatized. In the next step, a search for chloroxylenol by-products formed by *C. elegans* and *T. versicolor* was carried out. The structure of the detected compounds was assigned from the fragmentation pattern and *m/z* values obtained. In our research, PCMX metabolites formed during the biotransformation process conducted by microorganisms were identified (Table 1). PCMX biotransformation by *C. elegans* probably occurred via dehalogenation, aromatic ring hydroxylation and methyl group oxidation, with the formation of two metabolites, which were identified as 2,6-dimethylbenzene-1,4-diol, di-TMS (MC1), and 2,5-dihydroxy-3-methylbenzaldehyde, di-TMS (MC2). The results of the analysis of its peak area are presented in Supplementary Materials (Figure S3). The compound MC1 showed a molecular ion $[M]^+$ at *m/z* 282 for TMS derivatives and an Rt of 8.67 min with fragment ions at *m/z* 267 (M^+-CH_3), 223 (M^+-$2CH_3$), 193 (M^+-OTMS) and 73 (TMS). The metabolite was detected for the first time after 24 h of incubation, and the analysis of its peak area showed an increase in its amount within the fungal culture. The compound MC2 had an Rt of 8.733 min, a molecular ion $[M]^+$ at *m/z* 296 for TMS derivatives, and characteristic fragment ions at *m/z* 281, 252, 179 and 73, and was detected at 24 h of incubation only. In the case of the *T. versicolor* strain, three acids formed by ring-opening of the parent compound during dehalogenation; hydroxylation and oxidation were detected. Peak MT1 with an Rt of 6.435 min was supposed to be 4,6-dioxohex-2-enoic acid, TMS, which showed positive ion chemical ionization at $[M]^+$ at *m/z* 214 for TMS derivatives and fragmentation ions at *m/z* 143, 129, 75, and 73. The second identified product, MT2 (Rt 6.696 min), was observed to be 5-methyl-6-oxohexa-2,4-dienoic acid, TMS with a molecular ion $[M]^+$ at *m/z* 212 and ion fragments at *m/z* 197, 183, 169, and 73. The molecular weight of the compound with Rt 15.501 min (MT3) was $[M]^+$ at *m/z* 348. Based on the molecular weight and its fragmentation ions (259, 217, 147, 73), the compound was identified as 3-chloro-2,4-dimethylhexa-2,4-dienedioic acid, di-TMS. The metabolite MT1 was detected for the first time after 48 h of incubation and MT2 and MT3 after 24 h of incubation. The analysis of the peak area of *T. versicolor* metabolites showed an increase in their amount within the fungal culture, with maximum concentrations on the last day of incubation. The proposed chemical structure of the detected derivatives is shown in Figure 2.

Table 1. Gas chromatography-mass spectrometry results of qualitative analysis of chloroxylenol degradation by *C. elegans* and *T. versicolor*.

ID	RT (min)	Proposed Compound Name	Chemical Formula/Structure	Molecular Mass (Da)	Mass Spectrum *m/z* (10 Largest Ions Relative Abundance)	Proposed Fragmentation Pattern EI-MS *m/z* [Ion]
Parent compound	7.323	4-chloro-3,5-dimethylphenol, TMS	$C_{11}H_{17}ClOSi$ 	228.79	73 (21.4), 91 (12), 93 (16), 177 (17.6), 213 (99.9), 214 (17.5), 215 (36.7), 228 (61.9), 229 (11.6), 230 (22.7)	228 [$C_{11}H_{17}ClOSi^+$] 215 [$C_{10}H_{16}ClOSi^+$] 213 [$C_{10}H_{14}ClOSi^+$] 177 [$C_{10}H_{13}OSi^+$] m/z 73 [$C_3H_9Si^+$]
Cunninghamella elegans						
MC1	8.670	2,6-dimethylbenzene-1,4-diol, di-TMS	$C_{14}H_{26}O_2Si_2$ 	282.1	45 (11.1), 73 (67,9), 126 (13), 193 (60.9), 194 (11), 223 (72.1), 224 (16.1), 267 (99.9), 268 (23.7), 282 (24.5)	282 [$C_{14}H_{26}O_2Si_2^+$] 267 [$C_{13}H_{23}O_2Si_2^+$] 223 [$C_{10}H_{15}O_2Si_2^+$] 193 [$C_{11}H_{17}OSi^+$] 73 [$C_3H_9Si^+$]
MC2	8.733	2,5-dihydroxy-3-methylbenzaldehyde, di-TMS	$C_{14}H_{24}O_3Si_2$ 	296.1	45 (10.3), 73 (99.9), 74 (8.6), 75 (20.8), 81 (12.4), 164 (12.3), 179 (26.8), 252 (12.4), 281 (13.8), 296 (12.8)	296 [$C_{14}H_{24}O_3Si_2^+$] 281 [$C_{13}H_{21}O_3Si_2^+$] 252 [$C_{12}H_{20}O_2Si_2^+$] 179 [$C_{10}H_{15}OSi^+$] 73 [$C_3H_9Si^+$]
Trametes versicolor						
MT1	6.435	4,6-dioxohex-2-enoic acid, TMS	$C_9H_{14}O_4Si$ 	214.76	28 (20.3), 45 (19), 56 (18.2), 73 (99.9), 75 (54.9), 129 (30.3), 130 (23.1), 143 (46.7), 157 (44), 214 (33.9)	214 [$C_9H_{14}O_4Si^+$] 143 [$C_6H_7O_4^+$] 129 [$C_5H_9O_2Si^+$] 75 [$C_2H_7OSi^+$] 73 [$C_3H_9Si^+$]
MT2	6.696	5-methyl-6-oxohexa-2,4-dienoic acid, TMS	$C_{10}H_{16}O_3Si$ 	212.36	73 (99.9), 75 (76.2), 127 (17.7), 141 (17.7), 169 (93.3), 170 (32.7), 183 (18.6), 184 (25), 197 (19.6), 212 (25.1)	212 [$C_{10}H_{16}O_3Si^+$] 197 [$C_9H_{13}O_3Si^+$] 183 [$C_8H_{11}O_3Si^+$] 169 [$C_7H_9O_3Si^+$] 73 [$C_3H_9Si^+$]
MT3	15.501	3-chloro-2,4-dimethylhexa-2,4-dienedioic acid, di-TMS	$C_{14}H_{25}ClO_4Si_2$ 	348.70	73 (99.9), 103 (21.5), 129 (18), 147 (31.8), 157 (9.9), 217 (49), 218 (10.1), 228 (24), 259 (40.4), 348 (14.5)	348 [$C_{14}H_{25}ClO_4Si_2^+$] 259 [$C_{11}H_{16}ClO_3Si^+$] 217 [$C_9H_{21}O_2Si_2^+$] 147 [$C_5H_{15}OSi_2^+$] 73 [$C_3H_9Si^+$]

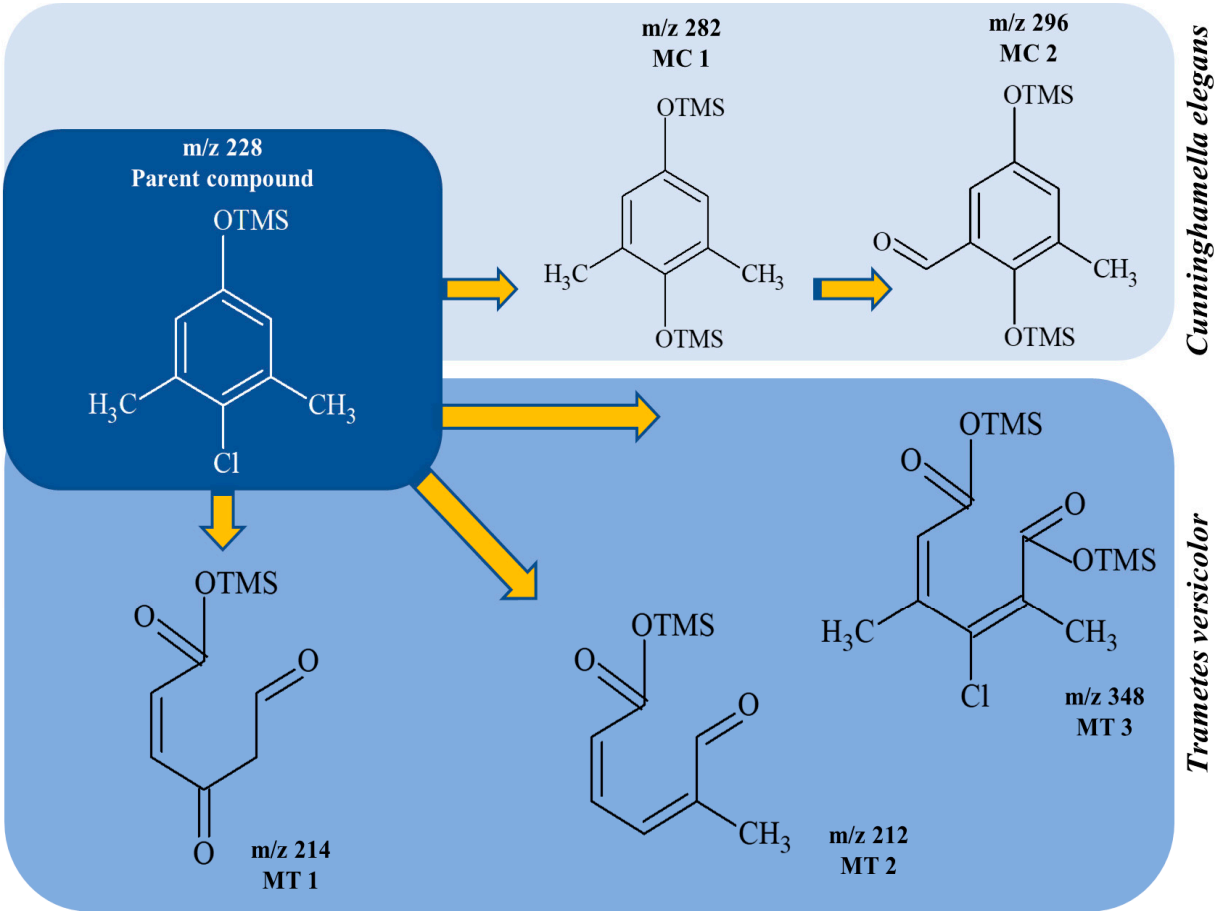


Figure 2. The chemical structure of chloroxylenol by-products formed by the tested strain.

Due to the lack of information about microbial biotransformation of PCMX, we compared our results to reports on mammalian metabolism and physicochemical degradation. Among the methods of chloroxylenol removal and complete mineralization, the most popular is degradation with the use of AOPs. Li et al. proposed the degradation pathway of PCMX in UV or UV/persulfate systems [20]. The use of these methods leads to aromatic ring-opening reactions, dechlorination and oxidation, resulting in the formation of short-chain carboxylic acids and total mineralization. Similar results were obtained by Sun et al., who conducted PCMX degradation by thermally activated persulfate in aqueous solution [21]. Chloroxylenol biotransformation through the substitution of a chlorine atom by a hydroxyl group (as in MC1) was described for mammalian liver microsomes [25]. It was also detected that side chain oxidation (methyl group hydroxylation) led to the production of mono- and dehydroxylated metabolites. The authors suggested that PCMX hydroxylation was a common biotransformation process conducted by cytochrome P450 in higher organisms. Some reports have documented the ability of fungi to degrade several halogenated pollutants, i.e., alkylphenols and chlorophenols. The degradation process proceeded mainly via hydroxylation and halogenation reactions. Mtibaá et al. evaluated the ability of the ascomycetes *Thielavia* sp. HJ22 to remove phenolic xenobiotics, i.e., 2,4-dichlorophenol was found to be metabolized by the formation of hydroxylated and oxidized derivatives [35].

2.3. Mechanisms of Chloroxylenol Biotransformation

Cunninghamella sp. has the ability to biotransform pharmaceuticals and other xenobiotics by oxidation and conjugation reactions, forming compounds similar to those found in humans and other mammals. These fungi have been widely investigated as xenobiotic

metabolism models in mammals [43]. In our work, we analysed the activity of CYP450 and cytochrome reductase (CRP) genes to evaluate the participation of these monooxygenase systems in the biodegradation of PCMX by *C. elegans*. We also studied the influence of CYP450 inhibitors on the elimination process yield. Among the tested strains, CYP450 was involved in the metabolism of chloroxylenol only in the case of *C. elegans*. The profile of by-products emerging in the *C. elegans* culture suggested the involvement of these enzymes. Three CYP450 inhibitors, sodium azide, 1-aminobenzotriazole and proadifen, caused a significant inhibition of PCMX degradation reactions ($p < 0.05$) (Figure S2). After a 120 h incubation of the fungus with inhibitors, the remaining amount of PCMX reached approximately 96, 82, and 82%, respectively. In the case of the cultures incubated with metyrapone, no statistically significant suppression was observed; nonetheless, a high residue (79%) of PCMX compared to the abiotic control was also detected. A less visible inhibition of elimination was noted in the cultures of *T. versicolor* incubated with CYP450 inhibitors and PCMX. In this case, the efficiency of PCMX elimination reached 65, 60, 76 and 66% in the samples, with the addition of sodium azide, 1-aminobenzotriazole, metyrapone and proadifen, respectively, compared to the abiotic control (Figure S2). These results suggest that the CYP450 enzyme system might not have been involved in the elimination process in the fungus *T. versicolor*. To confirm the participation of the CYP450 enzyme system in the PCMX elimination process in *C. elegans* cultures, the real-time quantitative PCR of CYP450s and CRP genes was also performed (Figure 3). An increase in CYP450s and CRP genes expression was observed after 6 h of cultivation, and its maximum was reached after 24 h of incubation. Then, a decrease in their expression levels was noted, while in the biotic sample, no changes in the expression during the whole time period of the experiment were detected. The greatest expression of CYP450s and CRP genes in 24 h of incubation was correlated with the high elimination of PCMX during the first 24 h in the *C. elegans* culture. Furthermore, a less visible increase in the efficiency of elimination after 24 h to the end of the experiment corresponded with a decrease in CYP450s and CRP gene expression. The MC1 metabolite (Table 1) was probably formed analogically to the formation of metabolites with rat, mouse, and human microsomes. CYP450 enzymes can remove para-substituents from phenols with an *ipso* substitution pathway, which involves the formation of a p-benzoquinone derivative with the loss of a halide ion, and then reduced to the final hydroquinone component [25]. Similar studies were conducted by Zawadzka et al. for *C. elegans* fungi, which were incubated with carvedilol. An increase in the levels of CYP450s and CRP genes and inhibition of the xenobiotic elimination by supplementation with CYP450 inhibitors were also shown. Furthermore, the profile of carvedilol biotransformation fungal products (hydroxylated and conjugative metabolites) indicated that CYP450 enzymes were involved in the process [42]. Monooxygenases can also play a role in the degradation of chlorophenols through successive hydroxylation and dehalogenation reactions, according to Arora and Bae [34]. The formation of hydroxylated by-products during the biodegradation process of 2,4-dichlorophenol in *Thielavia* sp. HJ22 is also catalysed by cytochrome P450 monooxygenases [35].

White-rot fungi (WRF), including *T. versicolor*, are mostly used in the degradation of a wide variety of pollutants, as well as those found in personal care products. WRF contain nonspecific oxidative extracellular enzymes like laccase, which belongs to the class of multicopper oxidases [46]. This group of enzymes in fungi catalyses the degradation process by oxidatively cleaving the aromatic ring present in the lignin structure of wood, giving fungi the ability to degrade toxic xenobiotics. Laccases can catalyse the oxidation of various substrates, including phenols [47]. The next stage of the study involved evaluating the influence of PCMX on ligninolytic enzyme activity and the contribution of these enzymes to the elimination of the preservative. The production of laccase by *T. versicolor* occurred in 24 h and reached its maximum at 72 h (156.4 U L^{-1}) for the cultures supplied with PCMX. At the same time period, the activity of laccase in the control samples was 70.4 U L^{-1} (Figure 4). In the cultures older than 72 h, the enzyme activity decreased, and in 120 h of incubation, laccase activity was detected until 84.9 U L^{-1}. Laccase activity stimulation

by different xenobiotics is well-documented. Mougin et al. reported increased laccase activity in a *T. versicolor* culture supplemented with different compounds of industrial origin (e.g., aniline, nonylphenol, diquox or 9-fluorenone) [48]. Increased activity of this enzyme during the biodegradation of bisphenol A and diclofenac by *T. versicolor* was noted by Yang et al. [49]. By-products formed in *T. versicolor* cultures suggest the involvement of the ligninolytic enzyme in the biodegradation process of PCMX. Unfortunately, very little is known about the pathways of PCMX and other chlorophenols transformation by filamentous fungi, which play an important role in ecosystems. Our results present a total dechlorination of PCMX, which may be caused by oxidative release of chloride. Some previous studies reported that laccase plays a key function in the enzymatic oxidation of contaminants including phenolic compounds. A similar reaction was described by Kordon et al., who revealed total dechlorination of monochlorinated hydroxybiphenyl by the laccase of *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*, which led to the formation of less toxic derivatives [36]. Navada and Kulal reported chloramphenicol dehalogenation and oxidation catalysed by laccase, resulting in the formation of chloramphenicol aldehyde, which was non-toxic to microorganisms [50]. The formation of MT1, MT2, and MT3 products (Table 1) could also be obtained through two pathways: direct oxidation of a product catalysed by laccase, and reductive dehalogenation of a product catalysed primarily by CYP450 monooxygenases, followed by an oxidative reaction catalysed by laccase. Further research will be needed to fully elucidate the mechanisms involved in the process of PCMX biodegradation by filamentous fungi.

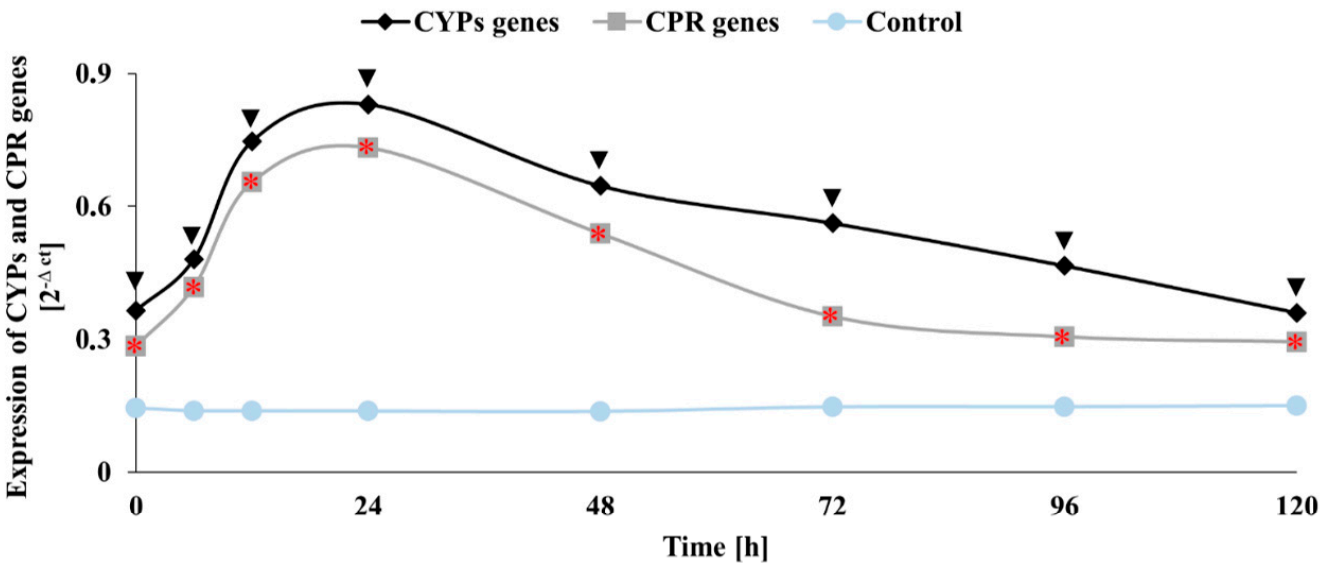


Figure 3. The expression of cytochrome P450 and cytochrome P450 reductase genes in the cells of *C. elegans* incubated with the addition of PCMX at a concentration of 25 mg L^{-1} compared to the control without the xenobiotic. Each result represents an average ± SD (n = 3). Statistical analysis was performed using the Mann–Whitney U test (▼ $p < 0.05$—a statistically significant increase in CYP450s genes relative to the biotic control; * $p < 0.05$—a statistically significant increase of CRP genes relative to the biotic control).

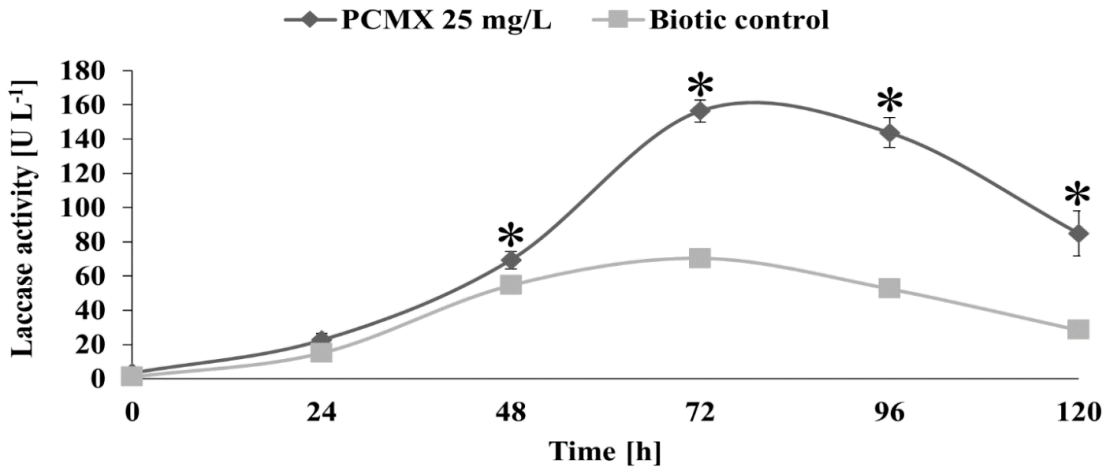


Figure 4. Laccase activity in *T. versicolor* cultures incubated with the addition of PCMX at a concentration of 25 mg L^{-1} for 120 h. Each result represents an average ± SD (n = 3). Statistical analysis was performed using the Mann–Whitney U test (* $p < 0.05$—a statistically significant increase in laccase activity relative to the control).

2.4. Toxicity Assessment

Chloroxylenol is a well-known antimicrobial chemical with a long history of use, however information about its ecotoxicology is very scarce. Moreover, the fate of PCMX in the natural environment is unknown. Therefore, the ecotoxicity estimation of PCMX and its by-products formed by microorganisms in ecosystems is necessary. Metabolites formed by biodegradation are a major concern of ecotoxicology because of a potential harmful effect on organisms that is lower or higher than that of the parent compound. However, microbial biodegradation of toxic chemicals involving dechlorination, hydroxylation, oxidation, and conjugation reactions leads to the detoxification of the substrate [16,51]. To evaluate the toxicity changes during the biotransformation of PCMX by *C. elegans* and *T. versicolor*, Daphtoxkit F and Phytotoxkit bioassays were applied. The ecotoxic effects of filtrates from fungal cultures were assessed using bioindicator species from different ecosystems, i.e., *Daphnia magna* as a freshwater species and *Lepidium sativum* and *Sorghum saccharatum* as soil, water and waste contaminant indicators. The results of the toxicological studies are presented in Table 2. Moreover, the 48 h LC_{50} (half maximal lethal concentration) of PCMX for crustaceans and 72 h EC_{50} (half maximal effective concentration) of PCMX for plants were evaluated. The LC_{50} value for *D. magna* was estimated at 8.78 mg L^{-1}, and the EC_{50} values for *L. sativum* and *S. saccharatum* were established at 30.79 mg L^{-1} and 15.4 mg L^{-1}, respectively. Slight toxicity of the biotic controls of both fungi was observed for all tested organisms. The toxicity of the abiotic control was constant during the time course of the experiment. Czapek–Dox medium did not have a toxic effect on the tested organisms. A comparison of the percentage effect for the tested samples from 0 h of incubation and xenobiotic-treated fungal samples from 120 h indicated a reduction in toxicity. Biotransformation of PCMX by *C. elegans* caused a decrease in toxicity from 84 ± 8.7% to 45.2 ± 5.2% and from 91.1%. ± 6.4% to 37.7 ± 9.5% and from 100% to 20 ± 16.3% in *L. sativum*, *S. saccharatum* and *D. magna*, respectively. Likewise, in the case of *T. versicolor* culture, the biodegradation process led to a reduction in the inhibition of root growth by three times and more than two times in *L. sativum* and *S. saccharatum* biotests, respectively. The mortality of *D. magna* after 48 h of incubation with postculture liquids from *T. versicolor* achieved 100% (0-day of incubation) and 40 ± 23.1% (5-day of incubation), which meant a 2.5-fold reduction in toxicity. Similar results had been obtained in our previous report, where we did not observe an increase in the toxicity of methylisothiazolinone derivatives after incubation with *Phanerochaete chrysosporium* [52]. Additionally, Zawadzka et al. showed a decrease in the toxicity of metabolites of carvedilol formed by *Cunninghamella echinulate* [51]. Sun et al. evaluated PCMX degradation by AOPs,

which led to the formation of more toxic compounds during the initial stages of oxidation. However, the authors pointed out that the next stages of degradation by thermally activated persulfate resulted in the formation of less toxic intermediates than those formed by the parent compound [21].

Table 2. Toxicity analysis of *C. elegans* and *T. versicolor* cultures with chloroxylenol in the Daphtoxkit F and phytotoxicity assays. Each result represents an average ± SD (n = 4). Statistical analysis was performed using the Mann–Whitney U test with * $p < 0.05$.

	Percentage Effect [%]					
	***L. sativum* 72 h**		***S. saccharatum* 72 h**		***D. magna* 48 h**	
Sample	**0 h**	**120 h**	**0 h**	**120 h**	**0 h**	**120 h**
C. elegans + PCMX	84.0 ± 8.7 *	45.2 ± 5.2 *	91.5 ± 6.4 *	37.7 ± 9.5	100.0 ± 0.0 *	20.0 ± 16.3
C. elegans	20.5 ± 12.1	36.1 ± 7.5 *	20.5 ± 15.5	25.0 ± 13.6	5.0 ± 10.0	20.0 ± 23.1
T. versicolor + PCMX	100.0 ± 0.0 *	32.9 ± 8.4	100.0 ± 0.0 *	41.5 ± 12.9	100.0 ± 0.0 *	40.0 ± 23.1 *
T. versicolor	35.2 ± 16.1 *	38.8 ± 24.3 *	41.5 ± 18.9	45.3 ± 20.8	15.0 ± 19.2	20.0 ± 23.1
Abiotic control	81.3 ± 11.4 *	77.6 ± 19.6 *	94.3 ± 7.2 *	84.9 ± 18.5 *	90.0 ± 11.6 *	100.0 ± 0.0 *

3. Materials and Methods

3.1. Chemicals

Chloroxylenol, metyrapone, proadifen hydrochloride, 1-aminobenzotriazole, sodium azide, 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Sigma-Aldrich (Poznań, Poland). High purity grade ethyl acetate and 96% ethanol were purchased from POCH S.A. (Gliwice, Poland). All PCR reagents came from Applied Biosystems (Foster City, CA, USA). All the other chemicals were from Sigma-Aldrich (Darmstadt, Germany), POCH S.A. (Gliwice, Poland) and Chempur (Piekary Śląskie, Poland).

3.2. Microorganisms and Culture Conditions

The strains *Cunninghamella elegans* IM 1785/21GP and *Trametes versicolor* IM 373 originated from the Microorganisms Collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz, Poland. Spores from 14-day-old ZT slants [52] were used to inoculate 20 mL Sabouraud medium (BD Difco, Franklin Lakes, NJ, USA) supplemented with 2% glucose in 100 mL Erlenmeyer flasks. Cultivation was carried out on a rotary shaker (140 rpm) at 28 °C. After 1 d of incubation, the preculture of *C. elegans* was transferred to fresh medium at a ratio of 1:2 and incubated for the next 24 h. The preculture of *T. versicolor* was carried out for 3 days. The homogenous precultures of *C. elegans* and *T. versicolor*, prepared as presented above, were transferred into modified Czapek–Dox medium ($NaNO_3$ 1 g; KH_2PO_4 1 g; KCl 0.5 g; $MgSO_4 \times 7H_2O$ 0.5 g; $FeSO_4 \times 7H_2O$ 0.01 g; glucose 20 g up to 1 L; pH 6.6) at a ratio of 1:9. The concentrations of PCMX in the culture medium were 5, 10, 25, and 50 mg L^{-1}. To evaluate the elimination kinetics of PCMX, the expression of cytochrome P450 and cytochrome P450 reductase genes, laccase activity and toxicity, only one concentration (25 mg L^{-1}) of PCMX was used. Biotic controls (without the xenobiotic) and abiotic controls (without fungal inoculum) were also prepared. Moreover, proadifen, metyrapone, 1-aminobenzotriazole and sodium azide, nonselective CYP450 inhibitors, were added to some cultures at concentrations of 1, 2.5, 4 and 1 mM for *C. elegans* and 0.5, 1, 1, and 0.1 mM for *T. versicolor*. CYP450 inhibitors and PCMX stocks were prepared in 96% ethanol except sodium azide, which was diluted in distilled water. The cultures were incubated on a rotary shaker (120 rpm for *C. elegans* and 140 rpm for *T. versicolor*) at 28 °C. Samples were collected over 5 days at different time intervals. For biomass assessment, the mycelia were separated by Whatman filter paper number 1 (Sigma-Aldrich, Darmstadt, Germany), washed twice with distilled water and dried at 100 °C, until a constant weight was achieved.

3.3. Quantitative Analysis of PCMX

The whole content of each *C. elegans* and *T. versicolor* culture after incubation was homogenized mechanically with the use of a Mixer Mill MM400 (Retsch, Haan, Germany) with glass beads (1 mm diameter). Next, 20 mL of each homogenate was extracted for 10 min with the same volume of ethyl acetate. The collected organic phases were dried over anhydrous Na_2SO_4. The residues after evaporation to dryness under reduced pressure at 40 °C were dissolved in 2 mL ultrapure ethyl acetate, diluted, and transferred to chromatography vials for quantitative analyses. Extraction efficiency was 87 ± 5.2%. The PCMX concentration was determined using a Hewlett-Packard HP 6890 gas chromatograph and HP 5973 mass spectrometer. An HP 5 MS capillary column (Agilent, 30 m × 250 µm, film thickness 0.25 µm) was used for the chromatographic separation of PCMX. The injection volume was 1.6 µL. The inlet was set to split mode with a split ratio of 10:1, and the split flow was 12 mL min^{-1}. The temperature was kept at 280 °C. The carrier gas was helium at a steady flow rate of 1.2 mL min^{-1}. The temperature of the column was maintained at 80 °C for 2 min, and then it was increased at a rate of 20 °C min^{-1} to 300 °C and maintained for 3 min. The total time of analysis was 16 min. Selective ion monitoring (SIM) mode was targeted at quantifying ions at *m/z* 121 and qualifier ions at *m/z* 156. A quantitative analysis was performed using a calibration curve within the working range from 2.5 to 50 µg L^{-1} PCMX.

3.4. Qualitative Analysis of PCMX Elimination

For the identification of chloroxylenol metabolites, the obtained extracts were dissolved in 1 mL of ethyl acetate and evaporated to dryness. Next, 50 µL of BSTFA was appended and warmed up to 60 °C for 60 min, according to the method described by Krupiński et al. and Nowak et al. [53,54]. Then, the samples were supplemented with 450 µL of ethyl acetate and diluted. GC-MS analysis was conducted in scan mode, with the mass range set from 25 amu to 450 amu. The injection volume was 1.6 µL. All PCMX by-products were identified on the basis of the mass spectra analysis.

3.5. Analysis of Cytochrome P450 and Cytochrome P450 Reductase Genes

C. elegans cultures with and without chloroxylenol at a concentration of 25 mg L^{-1} were collected for 5 days for the analysis of CYP450 and CRP gene expression according to the method described in a previous publication [42]. The InviTrap Spin Universal Kit (Stratec Molecular, Berlin, Germany) was used for total RNA isolation. A Picodrop spectrophotometer was used to assess the total RNA concentration. An Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) were used to verify the quality of isolated total RNA.

cDNA from extracted total RNA of *C. elegans* was obtained with the TaqMan® RNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. Primers for RT-PCR were constructed on the basis of the Lisowska et al. paper [45]. TAMRA dye and FAM reporter dye were used to label the 3′ and 5′ ends of the sequence, respectively. The ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to quantify the cytochrome P450, cytochrome P450 reductase and GAPDH genes. All samples were incubated at 50 °C for 2 min and at 95 °C for 10 min and then cycled at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min for 40 cycles. Fluorescence emission data were collected, and mRNA levels were quantified using a critical threshold (Ct) value. Relative gene expression levels were obtained using ΔCt standard $2^{-\Delta ct}$ calculations [55].

3.6. Determination of Laccase Activity

Laccase activity in liquid cultures was determined during 5 days of cultivation using supernatant obtained after culture centrifugation for 10 min at 10,000 rpm. Laccase activity was assayed according to the method described earlier by Góralczyk-Bińkowska et al., with slight modifications [56]. The measurement of ABTS oxidation was monitored spec-

trophotometrically at 420 nm. The reaction mixture (2 mL) contained 1800 µL citrate phosphate buffer (pH 4.5), 100 µL of the tested sample and 100 µL of 10 mM ABTS. The sample without culture supernatant was used as a reference blank. The concentration of the enzyme needed to oxidize 1 M substrate per minute was specified as one unit of laccase activity (U).

3.7. Toxicity Assays

Daphnia magna Daphtoxkit F magna (Microbiotests, Inc., Mariakerke-Gent, Belgium) was used according to the manufacturer's procedure and ISO 6341 standard. *D. magna* ephippia were incubated in standard fresh water for 72 h at 20 °C at 6000 l×. The motile larvae were subjected to an acute toxicity test. The cultures of *C. elegans* and *T. versicolor* with or without PCMX (control) were separated by filtration. The supernatant and abiotic samples containing chloroxylenol at the same concentration as the biotic samples were sterilized by filtration through sterile Sartorius membrane filters (0.25 µm pore size). The obtained supernatants and Czapek–Dox medium were diluted twice in standard fresh water. PCMX stock solution, at appropriate concentrations, was added to fresh water to evaluate the LC_{50}. The standard fresh water was used as a growth control. The toxicity of PCMX and its metabolites was calculated as a percentage of dead larvae after 48 h of incubation.

The assessment of *C. elegans* and *T. versicolor* postculture liquids and PCMX phytotoxicity was performed using *Lepidium sativum* and *Sorgum saccharatum* seeds. The evaluation of root growth was conducted with a Phytotoxkit (Microbiotests, Inc., Mariakerke–Gent, Belgium) in accordance with the instructions and ISO 18763 standard. Briefly, commercially available seeds were rinsed with sterile deionized water and placed in test plates containing a layer of filter paper moisturized with 20 mL of the tested sample prepared as described above. A stock solution of PCMX was diluted with sterile deionized water, which was used as a control. The test plates were covered with the bottom part of the plate and placed in an incubator in a vertical position for 72 h at 25 °C (±1 °C) in darkness. At the end of the incubation period, the percentage of root length inhibition was calculated, and the EC_{50} was evaluated.

3.8. Data Analysis

Statistica 13.3 software was used to do the statistical analyses (StatSoft, Kraków, Poland). Sample variability was given as a standard deviation (±SD). The Mann–Whitney U test was used to investigate the statistical significance. Values of $p < 0.05$ were considered significant.

4. Conclusions

The ability of *C. elegans* and *T. versicolor* to remove 70% and 79% of PCMX within 120 h of incubation, was shown. Moreover, five metabolites of PCMX formed by oxidation, hydroxylation, and dehalogenation reactions, were identified. The involvement of two different groups of enzymes, including intracellular CYP450 and extracellular laccase, was revealed. This report also demonstrated the detoxification of PCMX by the tested microorganisms via the reduction of the xenobiotic concentration and the formation of less toxic metabolites. The results of the present study indicate how PCMX could be metabolized in the natural environment and show the possibility of using the tested fungi as potential tools for environmental bioremediation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22094360/s1, Figure S1: Chloroxylenol elimination and the growth of *C. elegans* and *T. versicolor* cultures after 120 h of incubation with PCMX at concentrations of 5, 10, 25 and 50 mg L^{-1}. Figure S2: Effect of cytochrome P450 inhibitors on chloroxylenol elimination by *C. elegans* and *T. versicolor*., Figure S3: The peak area of detected metabolites of PCMX produced by *C. elegans* (A) and *T. versicolor* (B) during 120 h of incubation.

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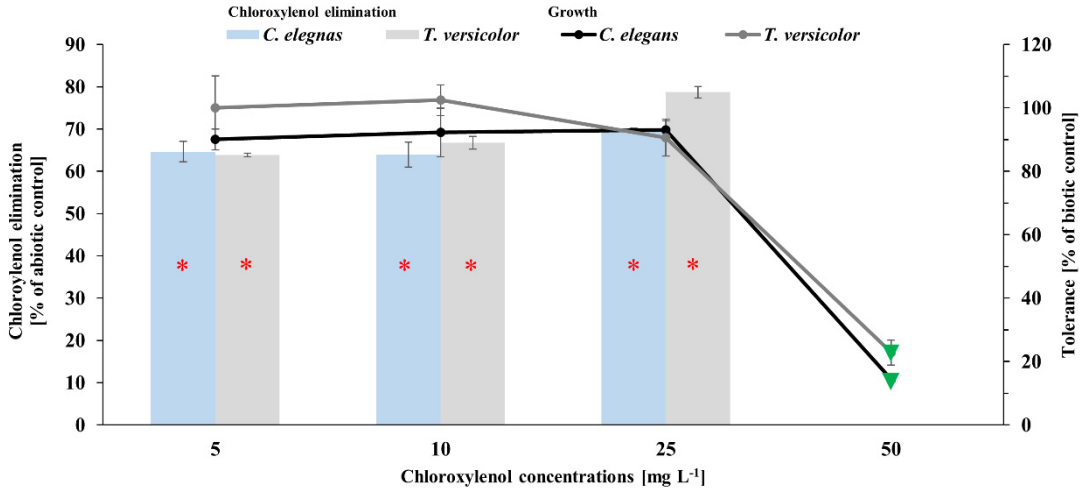

Figure S1. Chloroxylenol elimination and the growth of *C. elegans* and *T. versicolor* cultures after 120 h of incubation with PCMX at concentrations of 5, 10, 25 and 50 mg L^{-1}. Each result represents an average ±SD (n = 6). Statistical analyses were performed using the Mann-Whitney U test (*p < 0.05 – a statistically significant increase in PCMX elimination relative to the abiotic control; ▼ p < 0.05 – a statistically significant decrease in biomass in the sample with PCMX relative to the biotic control).

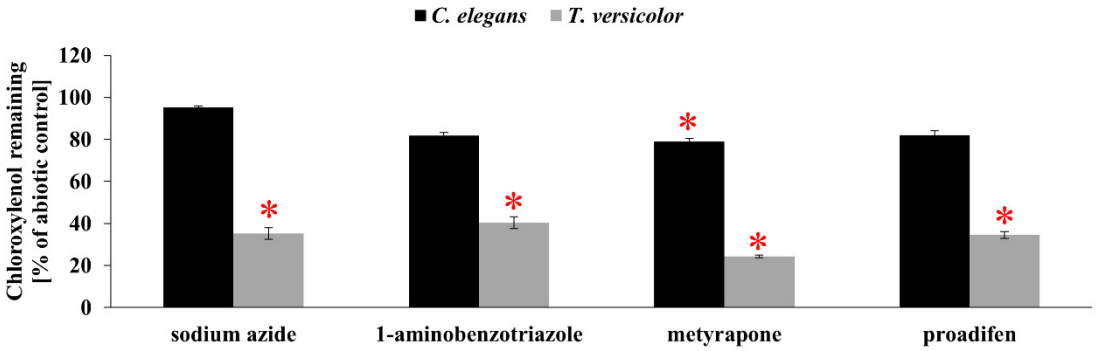

Figure S2. Effect of cytochrome P450 inhibitors on chloroxylenol elimination by *C. elegans* and *T. versicolor*. Each result represents an average ± SD (n = 6). Statistical analysis was performed using the Mann-Whitney U test with *p < 0.05.

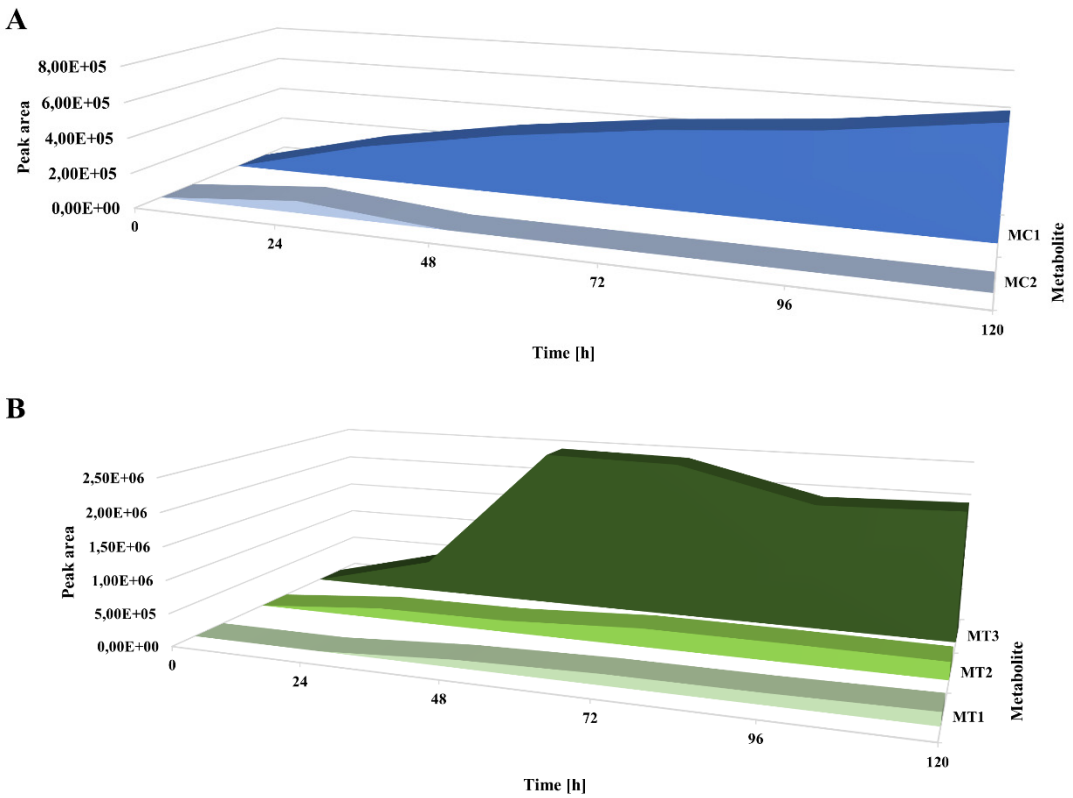

Figure S3. The peak area of detected metabolites of PCMX produced by *C. elegans* (**A**) and *T. versicolor* (**B**) during 120 h of incubation.

In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria

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In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria

Marta Nowak-Lange, Katarzyna Niedziałkowska, Przemysław Bernat & Katarzyna Lisowska✉

Methylisothiazolinone (MIT) and chloroxylenol (PCMX) are popular disinfectants often used in personal care products (PCPs). The unregulated discharge of these micropollutants into the environment, as well as the use of sewage sludge as fertilizer and reclaimed water in agriculture, poses a serious threat to ecosystems. However, research into their ecotoxicity towards nontarget organisms is very limited. In the present study, for the first time, the ecotoxicity of biocides to *Pseudomonas putida*, *Pseudomonas moorei*, *Sphingomonas mali*, and *Bacillus subtilis* was examined. The toxicity of MIT and PCMX was evaluated using the microdilution method, and their influence on the viability of bacterial cells was investigated by the AlamarBlue® test. The ability of the tested bacteria to form biofilms was examined by a microtiter plate assay. Intracellular reactive oxygen species (ROS) production was measured with CM-H2DCFDA. The effect of MIT and PCMX on phytohormone indole-3-acetic acid (IAA) production was determined by spectrophotometry and LC–MS/MS techniques. The permeability of bacterial cell membranes was studied using the SYTOX Green assay. Changes in the phospholipid profile were analysed using LC–MS/MS. The minimal inhibitory concentrations (MICs) values ranged from 3.907 to 15.625 mg L^{-1} for MIT and 62.5 to 250 mg L^{-1} for PCMX, indicating that MIT was more toxic. With increasing concentrations of MIT and PCMX, the cell viability, biofilm formation ability and phytohormone synthesis were maximally inhibited. Moreover, the growth of bacterial cell membrane permeability and a significantly increased content of ROS were observed, indicating that the exposure caused serious oxidative stress and homeostasis disorders. Additionally, modifications in the phospholipid profile were observed in response to the presence of sublethal concentrations of the chemicals. These results prove that the environmental threat posed by MIT and PCMX must be carefully monitored, especially as their use in PCPs is still growing.

Abbreviations

AB	AlamarBlue
ATP	Adenosine triphosphate
CE	Collision energy
CUR	Curtain gas
DP	Declustering potential
EPI	Enhanced product ion
ESI	Electrospray ionization
GS	Gas
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IDA	Information-dependent acquisition
IS	Ion source

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LC−MS/MS	Liquid chromatography-tandem mass spectrometry
MHB	Mueller–Hinton broth
MIC	Minimal inhibitory concentration
MIT	Methylisothiazolinone
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
NB	Nutrient broth
PBS	Phosphate buffer saline
PCP	Personal care products
PCMX	Chloroxylenol
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGPR	Plant growth-promoting rhizobacteria
PL	Phospholipid
ROS	Reactive oxygen species
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
TEM	Temperature
WWTP	Wastewater treatment plant

Currently, much attention is given to the pollution of the natural environment with toxic compounds of anthropogenic origin. Mass production and application of synthetic chemicals increase this threat. One of the groups of pollutants is micropollutants, which are not covered by existing statutes due to their low accumulation in the environment (i.e., ng L^{-1} up to µg L^{-1}). These contaminants have been introduced into ecosystems for many years, but current advances in analytical processes have allowed the detection of their presence (despite their low quantities in the environment)[1]. Among these micropollutants, personal care products (PCPs) and household chemicals are important emerging contaminants. PCPs form an integral part of the daily lives of humans and include skincare products, hair care formulations, toothpastes, soaps, sunscreens and perfumes. These chemicals are pervasive in the environment due to their incomplete elimination by conventional biological wastewater treatment systems. Although most PCPs are considered readily biodegradable in environmental matrices, their threat to the environment is not due to persistency but rather to their biological activity together with their continuous emission, which characterizes them as "emerging contaminants" or "prospective pollutants"[2,3].

Chloroxylenol (PCMX, 4-chloro-3,5-dimethylphenol, p-chloro-m-xylenol) and methylisothiazolinone (MIT, 2-methyl-4-isothiazolin-3-one) are broad-spectrum antimicrobial agents that are used extensively in industrial, consumer and health care products, including cosmetics, household chemicals and disinfection products such as preservatives or disinfectant agents[4,5]. PCMX is a to strong antimicrobial capable of reducing populations of bacteria, such as *Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris,* and *Salmonella typhi*, and fungi, such as *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* and *Candida parapsilosis*. Moreover, the virucidal activity of PCMX towards Ebola virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been confirmed, making it widely used for disinfection during the SARS-CoV-2 pandemic[6–11]. Generally, it is assumed that the mechanism of action of chloroxylenol is similar to that of other phenolic and halophenolic antibacterial agents, particularly those perturbing cell membranes and causing cell leakage[4,12]. MIT, which is the best representative of preservatives from the isothiazolinones group, is a powerful biocide and has been described to be able to diffuse across the bacterial cell membrane and the cell wall of fungi. The main mechanism of the inhibitory effect is based on the presence of a reducing sulfur in the MIT molecule. Sulfur is able to react with nucleophilic groups in cellular components and inactivate thiols in cellular proteins to form disulfide bonds (-S-S-). By blocking several specific enzymes, isothiazolinones effectively stop respiration and inhibit the synthesis and utilization of adenosine triphosphate (ATP), which inhibits cellular activity and ultimately causes bacterial and fungal cell death. Moreover, ROS accumulate because the vital pathways in cellular metabolism are disrupted; this accumulation leads to cellular death[13–17].

Considering the large presence of MIT and PCMX in everyday products, their entry into water or soil systems is inevitable, and their presence may lead to alterations in the edaphic environment. Moreover, because popular antimicrobial agents such as triclosan and triclocarban have been banned by the Food and Drug Administration of the United States, it is anticipated that alternative chemicals, such as MIT and PCMX, which are commonly perceived as less harmful, will be used in PCPs in higher quantities. Therefore, the concentrations of MIT and PCMX in the natural environment should be continually surveyed[18]. The occurrence and fate of MIT and PCMX in wastewater treatment plant (WWTP) systems have frequently been detected at the level of nano- and micrograms per litre[19–25]. Discharge of wastewater after incomplete removal of preservatives from WWTPs leads to the accumulation of residue in the receiving environment. PCMX was detected in the range of 20–1200 ng L^{-1} in surface waters in Jakarta city[26,27]. There are few data in the literature about the occurrence of MIT in surface water. Paijens et al.[28] describes the contamination of Paris wastewater by MIT at a concentration of 14 ng L^{-1}.

Because preservatives, such as MIT and PCMX, are designed to elicit biological effects, the potential exists for these compounds to affect nontarget organisms. The toxic effects of MIT and PCMX have been explored previously, mainly on aquatic organisms. In zebrafish and rainbow trout, prolonged PCMX and MIT exposure cause hatching delays or inhibition, altered gene expression, embryonic mortality, morphological abnormalities, neurotoxicity and DNA damage to erythrocytes[29,30]. Moreover, Lee et al.[31] revealed that exposure to MIT caused modulation of genes involved in thyroid hormone regulation. PCMX also modulated antioxidant enzymatic

activity, produced changes in swimming speed and caused neurotoxic and mitochondrial malfunction in the estuarine rotifer *Brachionuskoreanus*[32].

The possible influence of PCPs on terrestrial fauna, especially soil-dwelling organisms, has not been adequately examined. Soil is frequently the final reservoir for most pollutants that penetrate the environment. The application of sewage sludge or the use of reclaimed water as an important water resource in the field is becoming more controversial. A great benefit of this practice is the conversion of precious components such as plant nutrients and organic matter. However, biosolids and recycled water, which are sources of harmful xenobiotics, can reach agricultural lands, and the chemicals can persist in soil for prolonged periods of time[33,34]. Moreover, new data in the literature suggest that, due to their biocidal activity, preservatives from the isothiazolinone group could be used as a new class of insecticides for the control of pests, which makes them a new source of environmental contamination[35]. Literature data show that MIT is present in soil in Poland at concentrations ranging from 1.04 to 10.8 μg kg^{-1}[24]. Soils work as a large bioreactor for degrading pollutants and simplifying nutrient transformation. Moreover, soils play a vital role in ecosystems as a habitat for organisms and plants. Microorganisms are one type of living form that are abundant in soil and are referred to as soil microbiomes. Soil contains a wide variety of microorganisms, including bacteria, archaea, fungi, algae, and nematodes, that can be beneficial or pathogenic. Beneficial microbes in soil are essential for maintaining soil ecosystem health by breaking down organic matter, recycling elements, and promoting plant growth[36]. Among the variably distributed heterotrophic microflora, populations of bacteria belonging to different species make up approximately 15% of the overall microbial populations[37]. Undoubtedly, soil is a notable sink antimicrobial agents, which has a negative impact on indigenous microbes by killing specific groups of soil microbial flora[38,39]. Since it is known that MIT and PCMX have antibacterial activity, they could be expected to exert deleterious effects on bacteria in the soil. The antibacterial effects of MIT and PCMX have been primarily studied on pure cultures of human pathogenic bacteria[40–43]. Similar to other xenobiotics, the negative impact of these preservatives on environmentally significant bacteria is gradually increasing, but it is still poorly investigated.

The present study, for the first time, investigated the ecotoxicity of these two preservatives on species belonging to the three different genera *Pseudomonas*, *Sphingomonas* and *Bacillus*, which are representative genera of beneficial soil bacteria. The species belonging to these genera are well known for their multifaceted functions ranging from producing highly beneficial phytohormones, such as indole acetic acid, gibberellins and sphingan, to remediating many types of environmental contamination. These microbes have also been noted to reduce stress factors, such as salinity, heavy metals, and drougth, leading to improved plant growth in agricultural soil[37,44,45].

This study was designed to investigate the tolerance of the bacterial species *Pseudomonas putida* (DSM 291), *Pseudomonas moorei* (DSM 12647), *Sphingomonas mali* (DSM 10565), and *Bacillus subtilis* (DSM 3657) to MIT and PCMX, reactive oxygen species production, changes in the phospholipid profile, biofilm formation and indole-3 acetic acid production. The objectives of the present work were to gain insight into the toxic effects of MIT and PCMX on beneficial soil bacteria and to evaluate the ecological risks they pose to soil environments.

Results and discussion

Determination of bacterial sensitivity and viability towards MIT and PCMX. The rich diversity and abundance of soil microflora and their activity determine the good quality and fertility of the soil. Although chemicals such as antibiotics, personal care products and plant protection products are an integral part of human life, their impact on nontarget organisms poses a serious threat to the functioning of the entire environment, including soil ecosystems. Disrupting the number or ratio of microbes in the soil can potentially inhibit the recycling and transformation processes of elements or pollutants. Studies on biocide toxicity on soil bacteria and other microorganisms are limited and generally focused on biodegradation of these pollutants or antibacterial activity in the context of using them in the final consumer products[46–48]. In the present study, for the first time, the ecotoxicology potential of MIT and PCMX has been evaluated towards gram-negative *P. putida*, *P. moorei*, and *S. mali* and gram-positive *B. subtilis* bacterial strains by determining their growth after treatment with various concentrations of MIT and PCMX. The results are presented as the percent of biotic control growth (control of bacterial growth without the addition of xenobiotics). Moreover, based on the level of bacterial growth, the MIC was measured. The effects of MIT and PCMX concentrations on bacterial growth and MIC values are illustrated in Fig. 1. The tested preservatives showed differential antimicrobial activity. For the first time, it was observed that the MIC values of preservatives towards soil microorganisms ranged from 3.907 to 15.625 mg L^{-1} for MIT and from 62.5 to 250 mg L^{-1} for PCMX, which clearly indicated that MIT was the most ecotoxic compound. The incubation of *P. putida* with MIT or PCMX at concentrations of 0.12225 mg L^{-1} and 0.977 mg L^{-1}, respectively, caused statistically significant stimulation of growth. A similar effect was observed for *P. moorei* after incubation with MIT (concentration range of 0.0305625–0.2445 mg L^{-1}) and *B. subtilis* after incubation with PCMX (concentration range of 1.954–15.625 mg L^{-1}) with $p < 0.05$. It is known that some beneficial soil bacteria, such as *Pseudomonas* sp. and *Bacillus* sp., which are characterized as plant growth-promoting rhizobacteria (PGPR), can degrade various contaminants in soil and use them as a source of energy that stimulates bacterial growth[49,50]. Thus, it could not be excluded that MIT and PCMX, in low concentration ranges, are used as sources of carbon and energy.

Both strains of *Pseudomonas* were similarly susceptible to the action of MIT and PCMX. The first significant inhibition of growth was observed at an MIT concentration of 0.977 mg L^{-1}, which caused approximately 80% growth inhibition of these bacteria. A further increase in the concentration led to a complete inhibition of growth. In the case of PCMX, an increased sensitivity of *Pseudomonas* was observed at concentrations above 31.25 mg L^{-1}, where the xenobiotic reduced bacterial growth by more than 50% and 60% for *P. putida* and *P. moorei*, respectively. *S. mali* and *B. subtilis* were the most resistant microorganisms to the tested concentrations of

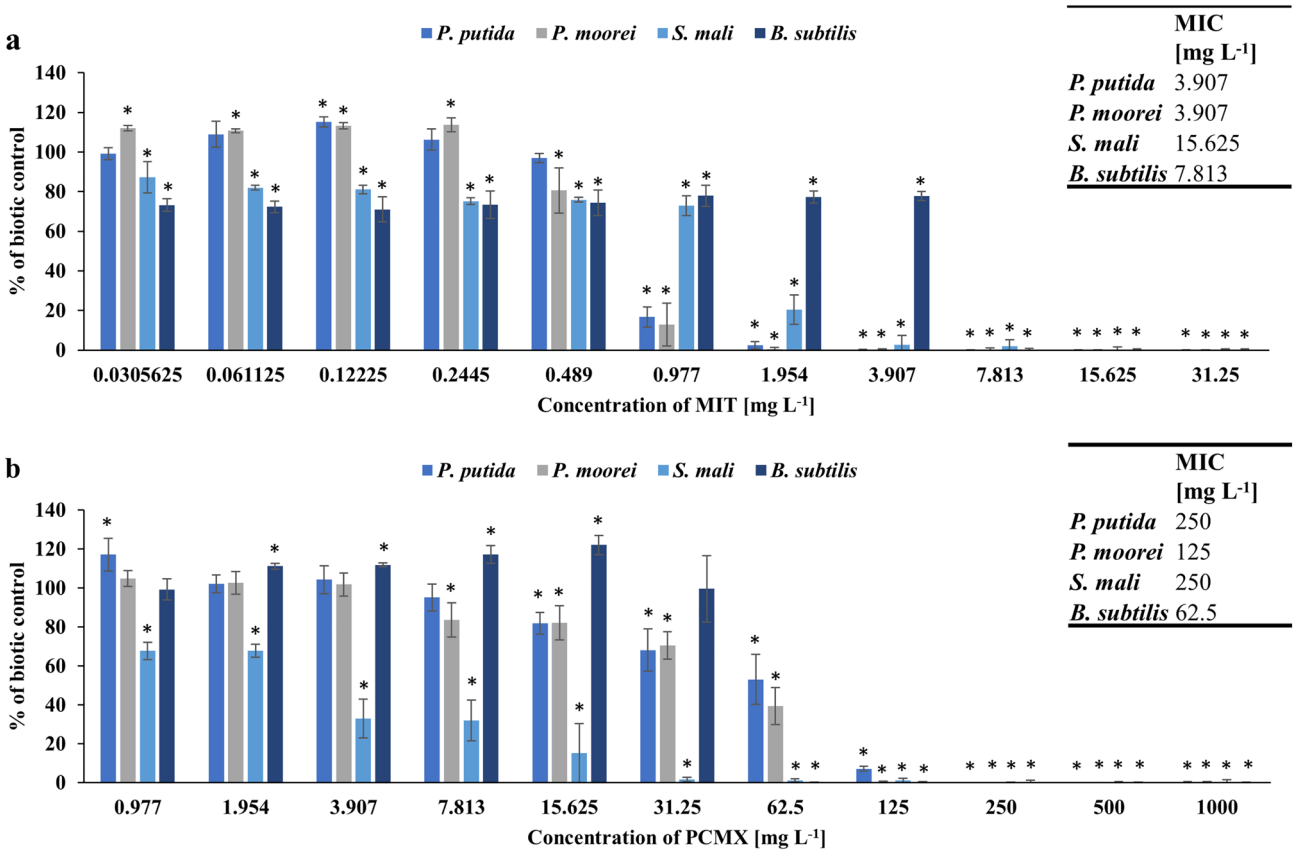

Figure 1. The growth of the tested strains incubated with the addition of methylisothiazolinone (**a**) or chloroxylenol (**b**). Data are expressed as the average percentage of bacterial growth (compared to biotic control) ± SD (n = 8). The presented results were analysed using the Mann−Whitney U test with *p < 0.05.

MIT. In both cases, the concentration of 0.977 mg L^{-1} inhibited bacterial growth by approximately 23%. However, in contrast to that of *B. subtilis*, *S. mali* growth was highly reduced at the next tested concentration (1.954 mg L^{-1}).

The toxicity of the investigated compounds, at five significant concentrations, towards bacterial species was also examined using an AlamarBlue® (AB) assay. The AB assay is widely applied in studies for monitoring microbial viability based on measuring the difference in the fluorescence intensity of the nonfluorescent, blue dye resazurin and the highly fluorescent reduced form resofurin. This redox reaction is a result of a metabolic pathway and cell respiration of metabolically active bacteria[51,52]. The results are presented as the percent of biotic control. The results presented in Fig. 2 indicate that the inhibition of AB dye reduction could be highly correlated with the inhibition of bacterial species growth, which might suggest that the inhibition of cell growth is caused by the inhibition of metabolic activity by xenobiotics.

There are no available studies on the ecotoxicity of MIT and PCMX towards beneficial soil bacteria. Moreover, literature data about the influence of other personal care products on soil bacteria are very scarce. The antibacterial activity of the described micropollutants was evaluated mainly for microorganisms being the target of treatment. Despite the fact that these preservatives are very popular in the chemical industry, there are no recent studies describing their antimicrobial activity. For instance, Collier et al.[16] evaluated the antimicrobial activity of compounds belonging to the isothiazolinone group towards *Schizosaccharomyces pombe* and *Escherichia coli*. MIC values for MIT achieved 245 mg L^{-1} for gram-negative rods and 41 mg L^{-1} for yeast. Lear et al.[53] evaluated the MIC for PCMX towards industrial strains of *Pseudomonas* sp. The obtained values ranged from 200 mg L^{-1} to even above 1000 mg L^{-1}. Although those studies were conducted on industrial or clinical strains, their results are similar to those obtained in our work. The differential tolerance of the tested bacteria to biocides can lead to a shift in the bacterial community composition and variation in the total number of bacteria. Several studies have indicated that the presence of some pharmaceuticals and personal care products in agricultural soil, at concentrations exceeding the tolerance of microorganisms, can reduce the diversity of the microbial community and completely change its structure. Such changes could disturb the proper functioning and health of soil[34,54,55].

Inhibition of biofilm formation. According to the study by[56,57], biofilm formation by beneficial soil bacteria plays an important role in plant health. Bacterial biofilms on the surface of plants and soil can stimulate plant growth and protect against pathogenic microorganisms and stressful environmental conditions, such as contamination by pesticides, antibiotics and heavy metals. Bacteria in the biofilm structure are more resistant to stress factors than planktonic cells. Bacteria produce different signalling molecules, whose accumulation depends on cell density. Quorum sensing is a type of communication used to recognize the population density of

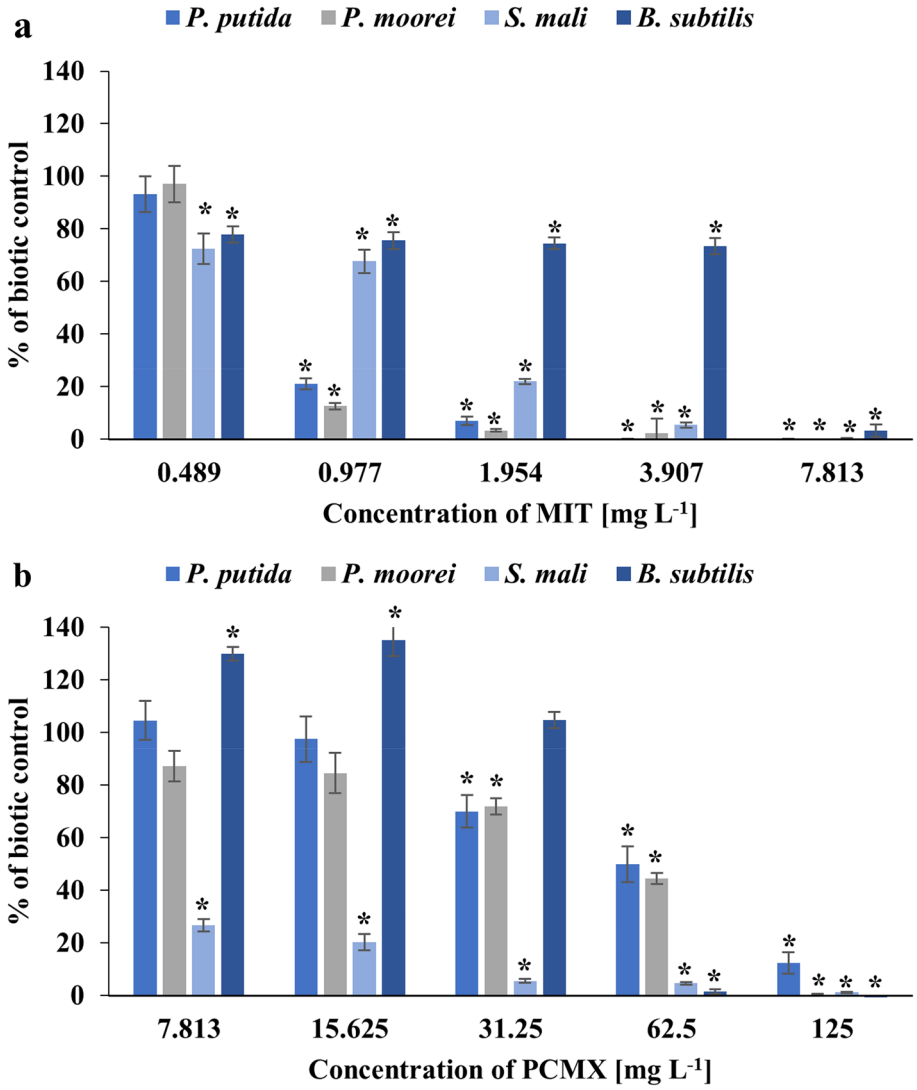

Figure 2. Reduction of resazurin by the tested strains incubated with the addition of methylisothiazolinone (**a**) or chloroxylenol (**b**). Data are expressed as the average percentage of bacterial growth (compared to biotic control) ± SD (n = 4). The presented results were analysed using the Mann−Whitney U test with *$p < 0.05$.

the same species, which probably participates in biofilm formation. Thus, the presence of toxic pollutants in the soil can disrupt biofilm formation by inhibiting cell growth, specific gene expression, chemical signal compound production and exopolysaccharide formation[58–60].

In the next step of this study, we compared the effects of MIT and PCMX on the tested soil bacterial strains using crystal violet in the microtiter plate assay (MPA). The inhibition of biofilm formation was measured by comparing biotic controls to tested samples supplemented with xenobiotics at concentrations of 0.489, 0.977, 1.954, 3.807, and 7.813 mg L^{-1} (MIT) and 7.813, 15.625, 31.25, 62.5 and 125 mg L^{-1} (PCMX). According to the study of Mathur et al., tested microorganisms were classified as high (*P. putida*, *P. moorei* and *B. subtilis*) and moderate (*S. mali*) biofilm producers[61]. A limited ability of the tested strains to form biofilms was observed in a dose-related manner with statistical significance (Fig. 3). For instance, the absorbance value in the biotic control of *P. putida* reached approximately 0.52, whereas the addition of MIT at a concentration of 0.977 mg L^{-1} reduced it to 0.26. In the samples with the addition of PCMX, a significant impact on biofilm formation was observed at a concentration of 15.625 mg L^{-1}. The biofilm formation ability of *P. moorei* was significantly inhibited by even the lowest concentration of MIT. Cells of *P. moorei* treated with PCMX at a concentration of 15.625 mg L^{-1} showed a decrease in biofilm formation. Moderate inhibition of biofilm production by *B. subtilis* was noted after the addition of MIT at a concentration of 1.954 mg L^{-1}. The *Bacillus* strain proved highly resistant to MIT, and a concentration as low as 7.813 mg L^{-1} caused an 80% decrease in absorbance. Likewise, high concentrations of PCMX did not inhibit biofilm formation by *B. subtilis*. Significant abolition of the ability to form biofilms was observed in the *B. subtilis* samples supplemented with PCMX at a concentration of 62.5 mg L^{-1}.

The use of reclaimed water or sewage sludge in agriculture, without the control of the concentration of micropollutants, could have a negative effect on the capability of bacteria to form biofilms on plant surfaces and

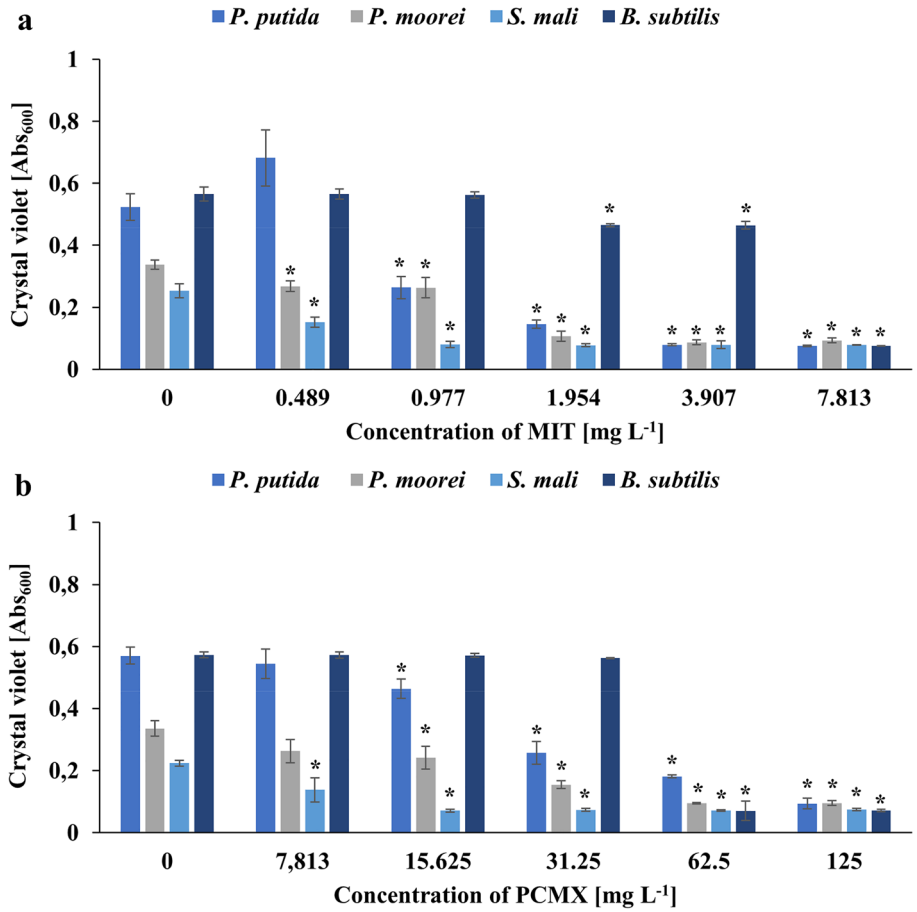

Figure 3. Effect of MIT (**a**) and PCMX (**b**) on biofilm formation by the tested strains. Data are expressed as the average of crystal violet absorbance ± SD (n = 4). The presented results were analysed using the Mann−Whitney U test with *p < 0.05.

subsequently lead to decreased protection of plants. Soil exposure to MIT and PCMX may disrupt rhizosphere ecosystems and make plants more susceptible to disease, resulting in poor growth and decreased agricultural yield[62].

Reactive oxygen species. Bacterial cells that have been exposed to oxidants, such as biocides, have mechanisms for recognizing and eliminating them. ROS are harmful species that react with many biological components, causing them to malfunction. Excess ROS generation occurs when bacterial cells are subjected to environmental stress, resulting in the initiation of cell necrosis or apoptosis by damage to nucleic acids and proteins or disturbed cell membrane integrity[63,64]. Therefore, to determine the presence of ROS in cells treated with MIT or PCMX, the intensity of 2′,7′-dichlorofluorescein (DCF) fluorescence was measured. As shown in Fig. 4, the ROS intensity in the positive control in comparison to the biotic control was significantly higher (more than 2.5 times). Strains treated with MIT or PCMX showed higher ROS levels than untreated cells. Regardless of the strain or type of xenobiotic, the ROS levels rose with increasing biocide concentrations until they reached the MIC. Despite the high concentrations of xenobiotics, the ROS levels decreased, which might have resulted from bacterial cell death caused by oxidative damage. Sharma et al. have also reported similar results for gram-negative *Pseudomonas fluorescens* and gram-positive *B. subtilis* exposed to copper nanoparticles. The authors observed a decrease in the ROS level above MICs and suggested that the reason for cell death was caused by higher oxidative stress at minimal bactericidal concentrations[65]. The maximum ROS intensity induced by MIT was 4.93 times higher than that of the biotic control and was observed in *S. mali* at 7.813 mg L^{-1} MIT. On the other hand, the maximum intensity induced by PCMX was observed in *P. putida* at a concentration of 125 mg L^{-1} and was 3.1 times higher than that of the control. The higher increase in fluorescence under all treatment concentrations of MIT confirmed that MIT is more toxic to the tested bacteria than PCMX. The obtained results acknowledge the induction of ROS generation in response to the administration of MIT and PCMX as a mechanism of killing bacteria.

The formation of ROS is a key measure for assessing oxidative stress. Toxicity studies of numerous environmental contaminations, at an angle of oxidative stress, have emerged as critical biomarkers for assessing the environmental impact of pollution. Matejczyk et al., evaluated the toxicity of diclofenac and its metabolites,

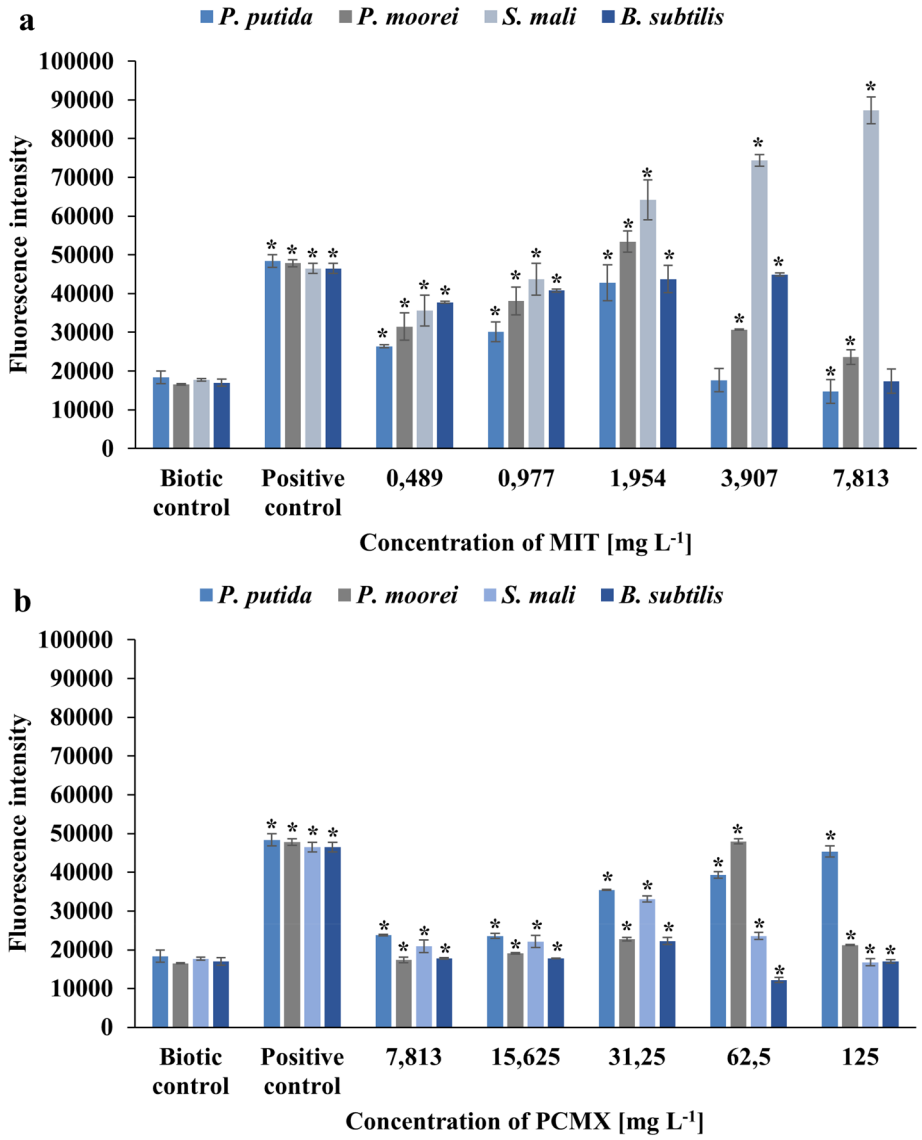

Figure 4. Fluorescence intensity representing the amount of intracellular ROS production in bacterial strains after their treatment with MIT (**a**) or PCMX (**b**). Data are expressed as the average fluorescence intensity ± SD (n = 3). The presented results were analysed using the Mann−Whitney U test with *p < 0.05.

mixed with various compounds, on *E. coli* cells in WWTPs. The occurrence of xenobiotics in the growth environment of bacteria led to the induction of oxidative stress and intensified ROS generation[66]. Zheng et al., identified the influence of perfluoroalkyl substances on damage caused by ROS in the soil microorganism *B. subtilis*. The obtained results showed that with increasing concentrations of toxic substances, a decrease in the level of ROS was observed. Moreover, the activities of superoxide dismutase and catalase, which are antioxidant enzymes involved in the antioxidant defence system, were analysed. The authors observed the opposite trend of enzyme activity to ROS levels and suggested that it might be a result of stability between the oxidant system and antioxidant system in microorganism cells[67].

The influence of MIT and PCMX on the production of phytohormones. Soil beneficial bacteria are known to synthesize phytohormones, which may affect the growth and health of plants. Auxins are phytohormones produced by soil bacteria, and their mechanisms of action involve controlling the individual stage of plant growth by stimulating of cell division and elongation, differentiating of tissue, and assisting in apical dominance. Among known auxins, indole-3-acetic acid is the most abundant hormone produced by beneficial soil bacteria. *Pseudomonas, Bacillus, Rhizobium, Enterobacter, Micrococcus, Azospirillum, Actinomycetes*, and *Kocuria* are popular strains of PGPR capable of producing IAA and other auxins[68,69]. The effectiveness of microbial stimulation of plant growth may be dependent in part on changes in the production of IAA caused by toxic substances occurring in the growth environment. To evaluate of the influence of different concentrations of MIT

Treatment	Concentration [mg L^{-1}]	Concentration of IAA [mg L^{-1}]			
		P. putida	*P. moorei*	*S. mali*	*B. subtilis*
Control	0	38.86 ± 1.18	54.30 ± 1.61	1.71 ± 0.32	1.18 ± 0.19
MIT	0.489	37.63 ± 2.22	33.05 ± 2.24*	0.93 ± 0.15*	1.55 ± 0.43
	0.977	16.24 ± 1.16*	2.95 ± 0.29*	0.56 ± 0.12*	1.28 ± 0.08
	1.954	3.75 ± 1.48*	2.60 ± 0.08*	0.08 ± 0.20*	0.82 ± 0.05*
PCMX	7.813	39.24 ± 1.95	44.23 ± 0.92*	0.30 ± 0.02*	1.37 ± 0.42
	31.25	41.15 ± 1.92*	39.25 ± 0.20*	0 ± 0.01*	0 ± 0.04*
	62.5	42.44 ± 1.05*	30.26 ± 1.75*	0 ± 0.07*	0 ± 0.004*

Table 1. Production of IAA by tested bacterial strains supplemented with MIT or PCMX after 48 h of incubation. Data are expressed as the average IAA concentration ± SD (n = 4). The presented results were analysed using the Mann–Whitney U test with *$p < 0.05$.

or PCMX on the production of auxin, the tested bacteria were incubated on nutrient broth (NB) or Mueller–Hinton broth (MHB) medium supplemented with L-tryptophan as a precursor. To quantify IAA production, the most common spectrophotometric method based on the colour reaction of the Salkowski reagent with auxins was used. The concentration of IAA was calculated on the basis of the standard curve after 48 h of incubation, when the highest concentration IAA was observed. Among untreated bacteria, *P. moorei* produced a maximum amount of 54.30 ± 1.61 mg L^{-1} of IAA. The next strain able to produce a high amount of IAA was *P. putida*, with an average value of 38.86 ± 1.18 mg L^{-1}. In the present study, neither *S. mali* nor *B. subtilis* was able to produce a high amount of phytohormones. The average concentration of IAA in the control samples was below 2 mg L^{-1}. After exposure of *P. putida, P. moorei, S. mali*, and *B. subtilis* to 0.489, 0.977, and 1.954 mg L^{-1} MIT, IAA production decreased in a dose-dependent manner (Table 1). Taking into consideration the production of auxin at a mean concentration (0.977 mg L^{-1}) of methylisothiazolinone by four bacterial strains, the maximum inhibition of IAA production (94.5%) was noted for *P. moorei*, followed by *S. mali* (67.2%) and *P. putida* (58.21%) in comparison to the control. Chloroxylenol exhibited less toxicity than MIT towards the tested strains. *P. putida* was able to secrete a larger amount of IAA with higher concentrations of the xenobiotic than in the control sample. IAA production was significantly inhibited in the case of the rest of the microorganisms, and PCMX at a mean concentration of 31.25 mg L^{-1} reduced IAA synthesis by 27.72, 100, and 100% for *P. moorei, S. mali,* and *B. subtilis*, respectively. The reduction in the synthesis of phytohormones at increasing concentrations of biocides could have been due to slower growth and disturbed physiological activity of microbial cells.

Because the method is based on the use of the Salkowski reagent, which can give a nonspecific colour reactions with other similar indolic compounds and could provide inaccurate information about quantities of IAA[69], we decided to additionally use liquid chromatography coupled with tandem mass spectrometry. This technique allowed for confirmation of the presence of the tested phytohormone in the study samples. Comparison of the mass spectra of the tested samples with that of the IAA standard confirmed the presence of indole-3-acetic acid. IAA obtained from the culture supernatants had a retention time of 1.12 min and produced a spectrum identical to that of standard IAA, with a parent ion *m/z* of 176 and fragments at *m/z* 158, 149, 130, 103 and 96.

The influence of MIT and PCMX on cellular membrane modification. The activity of many antibacterial agents is associated with the disturbance of bacterial cell membrane permeability. To investigate the possible mechanisms of the antibacterial activity of MIT and PCMX towards beneficial soil bacteria, their effect on the integrity of bacterial membranes was assessed with the fluorogenic dye SYTOX Green. This stain can penetrate only bacterial cells with the damaged plasma membrane and bind to nucleic acids, causing an increase in fluorescence intensity. We found that MIT and PCMX significantly changed the permeability of the cell membrane in all tested strains at concentrations above 0.489 mg L^{-1} and 15.625 mg L^{-1} for MIT and PCMX, respectively, compared to the biotic controls (Fig. 5). These results were closely correlated with bacterial growth inhibition by the tested preservatives.

Moreover, to confirm the effect of biocides on the components of cell membranes, the phospholipid (PL) profiles were evaluated. Phospholipids are the main components of the bacterial membrane, whose content is variable and depends on environmental conditions. The occurrence of toxic substances in the habitat of bacterial communities induces bacterial cells to adapt to potentially harmful surroundings modifying of membrane phospholipids for the regulation of membrane fluidity[70].

The changes in the PL profile were assessed by comparing the biotic control to the tested samples. Each bacterial strain was supplemented with biocides at a concentration below the MIC value, causing approximately 50% growth inhibition. Based on the mass spectra in negative ionization mode, the PL profile of each strain was identified. Despite high differentiation among microbial families, the bacterial membrane structure is highly conserved. Anionic phosphatidylglycerol (PG) and zwitterionic phosphatidylethanolamine (PE) are the major lipid elements of bacterial membranes[71,72]. Our results also indicated that the head groups of glycerophospholipids found in the control and treated samples were PE and PG. A comparison of the control samples with the treated cultures allowed us to observe the significant changes in the PL content only in the case of the *B. subtilis* cultures. After exposure to MIT, the amount of PE decreased to 31.67%, and the amount of PG increased to 66.5%. An analogous tendency was observed in the case of PCMX, but differences in the content of PE and PG between the tested samples and control were more significant (Table 2). Moreover, all the tested strains changed

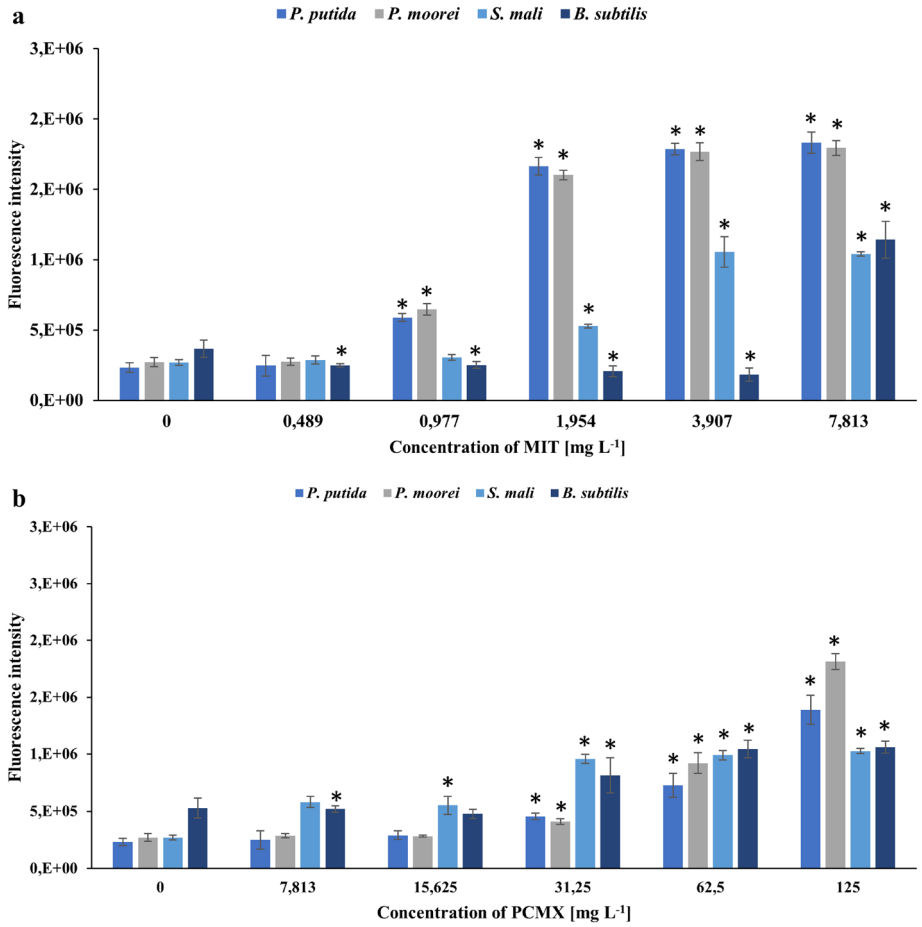

Figure 5. The effect of MIT (**a**) or PCMX (**b**) on the membrane permeabilization of the tested bacteria. Data are expressed as the average fluorescence intensity ± SD (n = 3). The presented results were analysed using the Mann−Whitney U test with *p < 0.05.

Phospholipid classes	Sample		
	P. putida	*P. putida* + MIT [0.7 mg L^{-1}]	*P. putida* + PCMX [62.5 mg L^{-1}]
PE	44.08 ± 2.2	44.89 ± 2.24	47.4 ± 2.37
PG	53.59 ± 2.68	52.82 ± 2.64	48.94 ± 2.45
PE/PG	0.82 ± 0.0	0.85 ± 0.0*	0.97 ± 0.00*
	P. moorei	*P. moorei* + MIT [0.7 mg L^{-1}]	*P. moorei* + PCMX [62.5 mg L^{-1}]
PE	45.62 ± 2.28	46.64 ± 2.33	47.58 ± 2.38
PG	50.62 ± 2.53	49.94 ± 2.5	49.46 ± 2.47
PE/PG	0.90 ± 0.0	0.93 ± 0.0*	0.96 ± 0.0*
	S. mali	*S. mali* + MIT [1.4 mg L^{-1}]	*S. mali* + PCMX [2.5 mg L^{-1}]
PE	35.88 ± 1.79	35.87 ± 1.79	39.38 ± 1.97
PG	62.37 ± 3.12	60.13 ± 3.0	58.01 ± 2.9
PE/PG	0.58 ± 0.0	0.6 ± 0.0*	0.68 ± 0.0*
	B. subtilis	*B. subtilis* + MIT [5.0 mg L^{-1}]	*B. subtilis* + PCMX [40.0 mg L^{-1}]
PE	35.73 ± 1.79	31.67 ± 1.58*	27.71 ± 1.39*
PG	62.71 ± 3.14	66.65 ± 3.33	71.09 ± 3.55*
PE/PG	0.57 ± 0.0	0.48 ± 0.0*	0.39 ± 0.0*

Table 2. Changes in the total amount of PLs bacterial cells after incubation with MIT or PCMX. Data are expressed as the average percentage ± SD (n = 3). The presented results were analysed using the Mann–Whitney U test with *p < 0.05.

their PE/PG ratio after being supplemented with MIT or PCMX. Changes in the PE/PG ratio can help stabilize the membrane by reducing its fluidity. Murzyn et al.[73] conducted computational analysis, which revealed that an increased quantity of PG, whose glycerol polar head is larger than that of ethanolamine, and a decrease in the PE content could lead to increased membrane stability and reduced fluidity. The modification of membrane composition as a process of adapting microorganisms to toxic contaminants is well documented[74–76]. Similar results were obtained by Bernat et al.[77], where the influence of iturin A lipopeptide on the phospholipid composition of *B. subtilis* was determined. According to the literature, PE is a dominant phospholipid in the inner membrane of gram-negative bacteria, while the membranes of gram-positive species are enriched in anionic phospholipids[78]. Additionally, in our research, an increased amount of PE was observed in the gram-positive *B. subtilis* strain. Moreover, gram-positive bacterial membranes were found to be more susceptible to the applied biocides. A quantitative analysis of PL species was also conducted. For *P. putida, P. moorei, S. mali,* and *B. subtilis*, 10, 10, 4, and 10 PE species, and 11, 10, 9, and 17 PG species were identified. The alkyl chains (two per phospholipid species) of PE and PG in the respective strains consisted of 12–18 carbon atoms. The examined phospholipids included saturated, mono-unsaturated, and di-unsaturated fatty acids. In gram-negative bacteria, the dominant PE species were 32:1, 36:2, and 38:2, and the dominant PG species were 30:2, 34:3, and 36:2. In the case of gram-positive bacteria, it was difficult to indicate prevailing species of PE because of high differentiation and a similar amount of each species. The dominant species of PG were 30:0, 31:0, and 32:0. The detailed difference in the quantity of PL species is shown in Fig. 6.

Briefly, in *P. putida* and *P. moorei* cells treated with PCMX, a slight increase in the major forms of both PE and PG was observed (*P. putida*: PE 32:1, PG 30:2; *P. moorei*: PE 32:1, PE 32:2, PE 34:2, PG 30:2. PG 32:1, PG 32:3). Upon addition of MIT to *Pseudomanas* cultures, a slight increase was observed in PG 30:2 (*P. putida*) and PE 32:1, PG 30:2 (*P. moorei).* Moreover, a slight decrease was observed in the main PL species of *P. moorei* (PE 32:2, PG 32:1). In *S. mali* cells after exposure to MIT, both PE (38:2) and PG 36:2 levels increased. However, the presence of PCMX in the growth medium led to an increase in the amount of PE 36:2 and a decrease in PE 38:2 and PG 36:2. The changes in levels in phospholipid species are characteristic of microorganisms exposed to xenobiotics such as antimicrobial compounds, organotin compounds, pharmaceuticals, steroids and alcohols[79–82]. The study conducted by Simon et al. showed that *P. putida* KT2440 exposed to n-butanol results in changes in PL species levels. The authors observed a small increase in the principal forms of both PG and PE after adding 0.5 g L^{-1} butanol. In contrast, the abundance of PE and PG was reduced at a greater butanol concentration of 3 g L^{-1}[81]. Additionally, Bernat et al.[79] noted the modification of the PL species profile in *Pseudomonas* sp. B-219 exposed to tributyltin.

Conclusion

In the present study, for the first time, disinfectant agents (MIT and PCMX) were tested against four soil bacteria, *P. putida, P. moorei, S. mali* and *B. subtilis*. The effects of the different concentrations of the studied compounds on the growth, viability, biofilm formation, ROS generation, IAA production and cell membrane modification of the tested strains were investigated. The results indicated that MIT and PCMX at increasing concentrations inhibited the growth of bacteria. MIT appeared to be more toxic to the tested organisms than PCMX. However, the toxicity of preservatives against bacterial cells depended on the bacterial species. Furthermore, reduced viability and inhibition of biofilm formation were clearly observed with increasing concentrations of each biocide. A significant increase in DCF fluorescence intensity suggested that the antioxidant system of microorganism cells at increasing concentrations of xenobiotics was unable to eliminate excess ROS. In addition, MIT and PCMX altered the metabolic pathway and disturbed the synthesis of the phytohormone indole-3-acetic acid. The presence of MIT and PCMX in the growth environment of bacteria resulted in changes in the phospholipid composition and increased cell membrane permeability. The ecotoxicity assessment conducted in this study indicated that MIT and PCMX pose a risk to soil bacteria. Due to the high use of personal care products and household chemicals that are preserved by MIT and PCMX, their unregulated discharge in the soil and aquatic environments may impact the useful bacterial population and the health of the natural environment.

Personal care products, among other preservatives, such as MIT and PCMX, have harmful effects on nontarget organisms. In the near future, continuous monitoring and measures of biocide levels must be undertaken and would be helpful for controlling and predicting their ecotoxic effects on the environment. Moreover, the ecotoxicological risks of MIT and PCMX to different terrestrial organisms need to be evaluated because of limited data in this field. Analysis of the influence of single compounds, as well as a mixture of these contaminants, is also needed. Finally, in vitro studies focus only on certain ecotoxicological mechanisms and aspects, without taking into account the effect on the populations of organisms and the ecosystem. Therefore, the toxicity activity of these preservatives needs to be analysed in in situ, in vivo, and in silico studies, such as towards microbial communities.

Materials and method

Bacterial cultures and chemicals. *Pseudomonas putida* (DSM 291), *Pseudomonas moorei* (DSM 12647), *Sphingomonas mali* (DSM 10565) and *Bacillus subtilis* (DSM 3657) were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (Germany). Methylisothiazolinone, chloroxylenol, 3-indoleacetic acid, iron(III) chloride, phospholipid standards and ammonium format were purchased from Merck (Poland). SYTOX™ Green Nucleic Acid Stain, General Oxidative Stress Indicator (CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) and AlamarBlue Cell Viability Reagent were obtained from Thermo Fisher Scientific (Poland). Difco™ nutrient broth and Mueller Hinton broth were purchased from Becton Dickinson (Poland). DMSO and phosphate-buffered saline (PBS) were obtained from BioShop (Canada). Methanol, ethyl acetate, hydrochloric acid, acetic acid, crystal violet and sodium sulfate

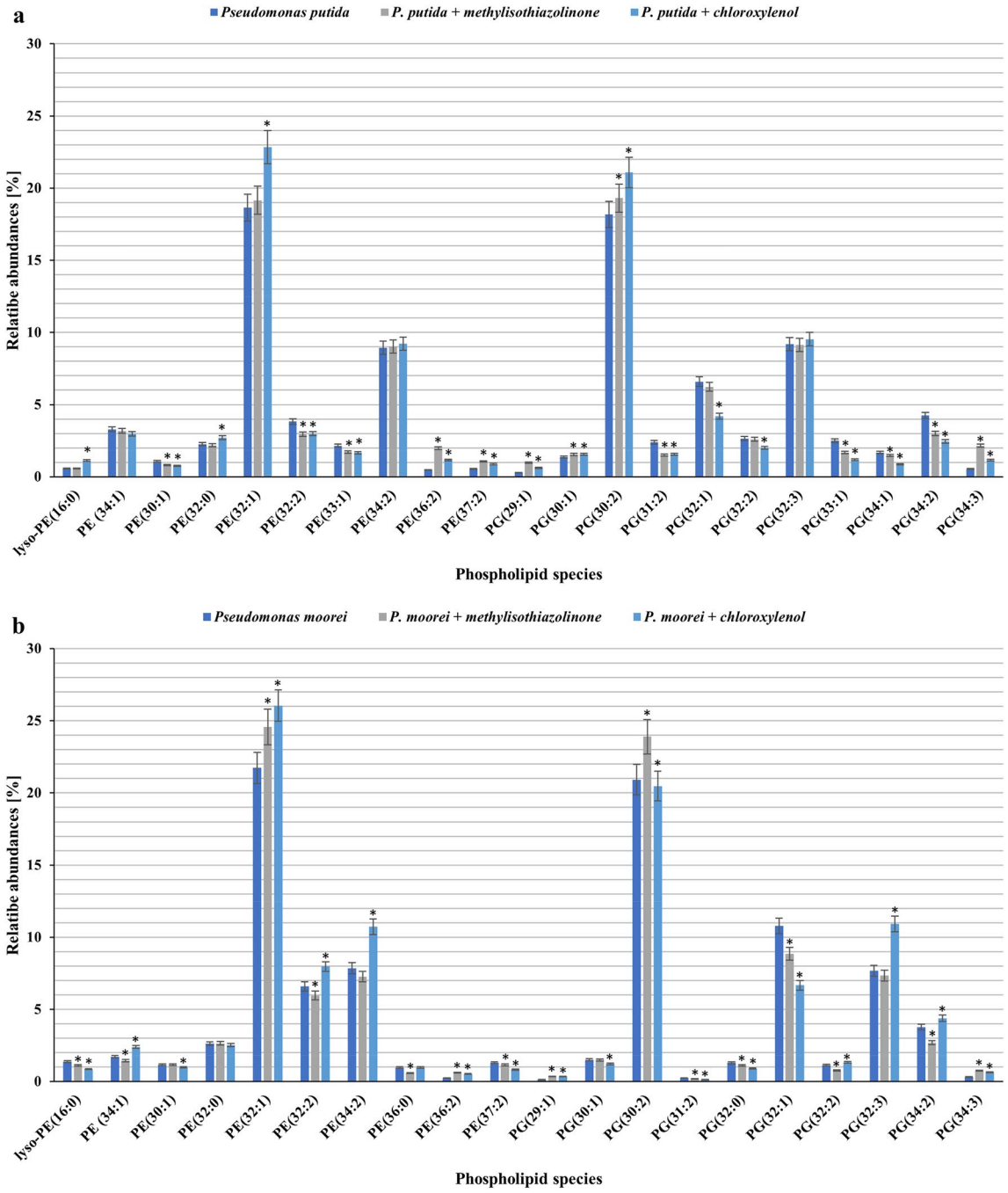

Figure 6. Changes in the contents of phospholipid molecular species determined in bacterial cells after incubation with MIT or PCMX [*P. putida* (**a**); *P. moorei* (**b**), *S. mali* (**c**), *B. subtilis* (**d**)]. Data are expressed as the average percentage ± SD (n = 3). The presented results were analysed using the Mann−Whitney U test with $^{*}p < 0.05$.

anhydrous were obtained from Chempur (Poland). All of the other reagents with a high analytical purity grade were purchased from Avantor (Poland).

Determination of bacterial sensitivity to MIT and PCMX. The toxicity potential of MIT and PCMX was determined using pure bacterial cultures according to the standard microdilution method of the Clinical and Laboratory Standards Institute[83]. All experiments were carried out in nutrient broth for *P. putida*, *P. moorei* and *S. mali* and Mueller Hinton broth for *B. subtilis*. The bacterial sensitivity towards MIT and PCMX was assessed over the concentration range from 0.0305625 to 31.25 mg L^{-1} and 0.977 to 1000 mg L^{-1} with two drug dilution steps. Stock solutions of MIT (62.5 µg mL^{-1}) and PCMX (2 mg mL^{-1}) were prepared in ethanol and diluted in adequate growth medium. The quantity of ethanol in the tested samples was below 5 µL, and toxic effects on the microorganisms were not observed. The growth of soil bacteria was carried out in 96-well

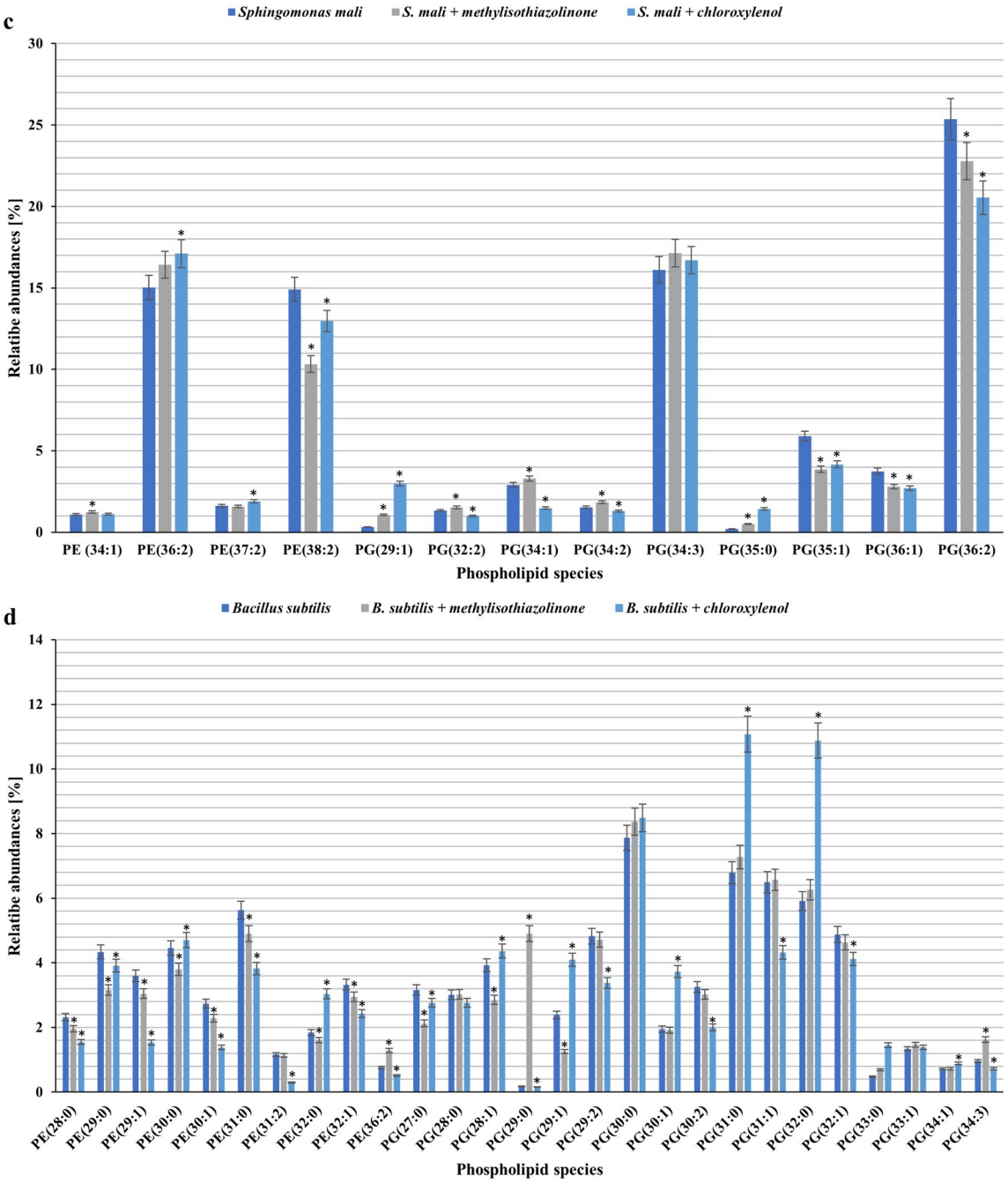

Figure 6. (continued)

cell culture plates by adding 100 µL suitable growth medium with or without the tested compounds and 100 µL bacterial inoculum prepared in NB or MHB to each of the tested wells. The final density of the tested strains was 1×10^6 CFU mL^{-1}. Simultaneously, the tested samples were led controls, abiotic controls, without the addition of microorganisms and with the addition of xenobiotics, biotic controls with the addition of microorganisms and without the addition of xenobiotics and controls of nonsupplemented medium. Adequate controls and tested samples were incubated for 24 h (*P. putida*, *P. moorei*) and 48 h (*S. mali* and *B. subtilis*) at 28 °C. The minimum inhibitory concentration (MIC) and bacterial susceptibility were determined based on optical density (λ = 630 nm) using a Multiskan TM FC Microplate Photometer spectrophotometer (Thermo Fisher Scientific). The results of the antibacterial effect of MIT and PCMX are shown as a percentage of the control samples, and MIC values are expressed in mg L^{-1}.

Determination of bacterial cell viability by AlamarBlue® assay. The AlamarBlue® dye used for the assessment of the viability of bacterial cells after incubation with MIT and PCMX was performed in accordance with the manufacturer's protocol and literature data[80]. Ten microliters of a fluorescent dye was added to the 96-well plates prepared as in sensitivity testing over the concentration range from 0.489 to 7.813 mg L^{-1} (MIT)

and 7.813 to 125 mg L^{-1} (PCMX). The adequate controls were made up the same as for the growth inhibition test with the addition of 10 µL dye. Then, the cells were incubated at a temperature of 28 °C for 3 h. The reduction of resazurin to resorufin was evaluated by fluorescent measurement at λ = 540 nm using a multimode microplate reader BMG LabTech FLUOstar Omega (BMG LABTECH GmbH, Germany). The results are shown as a percentage of the control sample.

Measurement of biofilm formation with a microtiter plate assay. The microtiter plate assay was carried out as previously described by Lee et al.[84]. Clear polystyrene 96-well plates with a flat bottom and untreated surface (Thermo Fisher Scientific, Poland) were prepared in the same way as in sensitivity testing at the concentration range from 0.489 to 7.813 mg L^{-1} (MIT) and 7.813 to 125 mg L^{-1} (PCMX) and with the biotic, abiotic and medium controls. After incubation and optical density measurements, media and unattached cells were removed. Next, the wells were washed twice with 0.85% NaCl, and 96% ethanol was added for 20 min for the fixation of sedentary cells. After removing ethanol, the plates were air-dried at room temperature. Crystal violet solution (0.1%) was added to each well to stain the bacterial biomass. After 30 min of incubation, the solution was removed, and the wells were washed three times with saline solution. Finally, 33% acetic acid was added to the air-dried wells to completely dissolve crystal violet, and to obtain homogenized solution, the plates were shaken on a rotary shaker. The absorbance was measured at 600 nm by a microplate reader (FLUOstar Omega).

Intracellular ROS detection. The intracellular reactive oxygen species production in bacterial cells, untreated and treated with the preservatives, was measured with the use of the nonfluorescent CM-H_2DCFDA compound according to the manufacturer's instructions and the study of Zawadzka et al.[85]. Briefly, after incubation, each bacterial inoculum was centrifuged at 10,174 × g for 5 min, washed with PBS three times, and resuspended in PBS. Then, the bacterial cells together with different concentrations of the tested compounds (0.489–7.813 mg L^{-1} for MIT and 7.813–125 mg L^{-1} for PCMX) were transferred to a black 96-well plate. H2O2 was used as a positive control at a concentration of 70 µM. Moreover, biotic and abiotic controls were prepared. The plates were incubated in the dark for 15 min at 28 °C. Next, CM-H_2DCFDA solution at a final concentration of 5 µM was added to each well, and the plates were incubated in the dark for 30 min at 37 °C. ROS fluorescence was measured using a FLUOstar Omega reader with an excitation/emission wavelength of 495/520 nm.

Effect of xenobiotics on IAA production. The effect of MIT and PCMX on IAA production by bacterial cultures was determined by the modified method of Maheshwari et al.[86]. The bacterial inoculum after 24 or 48 h of incubation on NB or MHB medium was transferred to fresh NB or MHB broth supplemented with L-tryptophan (0.15%) and the xenobiotics at concentrations of 0.489, 0.977, and 1.954 mg L^{-1} for MIT and 7.813, 31.25, and 62.5 mg L^{-1} for PCMX in a 50-mL Erlenmeyer flask. The biotic and abiotic controls and treated cells were incubated statically at 28 °C for 72 h. The tested samples and controls were measured every 24 h. Following the incubation, 1.5 mL of each sample was centrifuged at 10,174 × g for 10 min, and the clear supernatant was mixed with an equal volume of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 49 mL of 35% $HClO_4$). The mixture was incubated in the dark for 30 min. After that, a quantity of IAA in the sample was measured at λ = 523 nm using the spectrophotometer SPECORD 200 (Analytic Jena, Germany) against a standard graph of pure IAA.

To eliminate false-positive results from the Salkowski assay, LC–MS/MS qualitative analyses for the verification of auxin production were conducted. The samples for qualitative analysis were prepared in the same manner as for the Salkowski method. After incubation, the whole volume of the samples was centrifuged at 2900 × g for 15 min, and the supernatants were acidified to pH 3 with HCl and shaken for 10 min with ethyl acetate (1:1). The collected organic phase was dried with anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at 40 °C. The residues were dissolved in 1 mL ultrapure methanol and then diluted with 2% acetonitrile with the addition of 0.1% formic acid. The detection of IAA was performed on an MS/MS system (4500 QTRAP spectrometer (SCIEX, USA) coupled with a microLC 200 system (Eksigent, USA). The chromatographic separation was carried out on an Eksigent C18 (0.5 mm × 50 mm × 3 mm, 120 Å) column at 50 °C. The mobile phases for the LC–MS/MS analysis consisted of water supplemented with 0.1% formic acid (phase A) and acetonitrile supplemented with 0.1% formic acid (phase B). The gradient profile was as follows: 98% A until 0.2 min, a linear increase to 98% B in 4 min, maintained until 6 min, returned to the initial conditions from 6 to 6.2 min and held until 6.7 min with a constant flow rate of the mobile phases at 35 µL min^{-1}. The injection volume was 10 µL. The MS/MS scanning based on the information-dependent acquisition (IDA) method included multiple reaction monitoring (MRM) pairs and enhanced product ion (EPI) scans in positive ionization mode. IAA was detected based on m/z 176 > 130 (DP: 46, CE: 23) and 176 > 103 (DP: 46, CE: 33) ions. EPI scans worked at m/z 50–200, and mass spectra were collected for the identification of auxin presence. The microESI ion source was used to carry out analyses with set parameters: CUR (25), IS (4500), GS1 (30), GS2 (20), and TEM (400). Data analysis was carried out with Analyst™ software version 1.6.2 (SCIEX, USA).

Evaluation of bacterial membrane permeabilization by SYTOX Green assay. The SYTOX Green assay was used to evaluate the bacterial cell membrane permeability according to the method described by Felczak et al.[87] with modifications. SYTOX Green penetrates the damaged cell membrane and binds to DNA, giving an intense green fluorescence. Bacterial cultures, with and without the addition of xenobiotics, were prepared as in sensitivity testing at concentrations ranging from 0.489 to 7.813 mg L^{-1} (MIT) and 7.813 to 125 mg L^{-1} (PCMX) Adequate controls were also prepared (biotic, abiotic and control of medium). After incubation, bacterial suspensions were stained with 4 µL SYTOX Green dye (50 µM) for 15 min in the dark. Subsequently, the fluorescence of the DNA-bound dye was detected by a FLUOstar OMEGA reader with excitation and emission wavelengths of 485/535 nm. The values are represented as the percentage of the biotic control.

Bacterial phospholipid analysis by HPLC–MS/MS. Phospholipids of the tested bacterial strains were extracted according to a previous method[80] with modifications. Bacterial cultures were incubated in Erlenmeyer flasks (100 mL) on NB or MHB medium supplemented with MIT or PCMX at different concentrations. The final bacterial density was 1×10^6 CFU mL^{-1}. Adequate control wells without the addition of xenobiotics (biotic control) were prepared. After 24 or 48 h of incubation at 28 °C, bacterial biomass was separated by centrifugation at 5723 × g. It was disintegrated with 5 mL methanol and a glass matrix Ø 0.1 mm on a Mixer Mill MM400 (Retsch, Germany). Next, the homogenate was centrifuged (5723 × g, 10 min), and the supernatant was vortexed with 10 mL chloroform and 1 mL 0.85% NaCl for 4 min. The lower organic phase was collected, hydrogenated, and evaporated. The obtained phospholipid extracts were dissolved in 1 mL methanol. The lipid content was determined according to the method described by Zawadzka et al.[80] with the use of an Agilent 1200 HPLC (Agilent, USA) and a 4500 QTRAP mass spectrometer (SCIEX, USA) with an ESI source.

Statistical analysis. The obtained data are expressed as the mean ± SD. The nonparametric Mann–Whitney U test, with values of $^*p < 0.05$ to estimate the statistical significance, was conducted to compare the treated population with the control. The statistical analyses were carried out using TIBCO Statistica™ 13.3 (StatSoft Poland 2017) and Excel, Microsoft 365 Business (Microsoft Corporation, USA).

Data availability

The data presented in this study are available on request from the corresponding author.

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Author contributions

M.N.-L.: conceptualization, methodology, investigation, writing-original draft, writing-review & editing. K.N.: investigation, writing-review & editing. P.B.: methodology, writing-review & editing. K.L.: supervision, writing-review & editing. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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IX.2. Oświadczenia współautorów o udziale w publikacjach

Oświadczenia współautorów o udziale w publikacjach

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*Oświadczam, że mój udział w ww. pracy wynosi **75%** i obejmował: opracowanie koncepcji badań; zaplanowanie i realizację doświadczeń dotyczących oceny zdolności eliminacji badanego ksenobiotyku przez grzyb strzępkowy Phanerochaete chrysosporium, chromatograficznej analizy ilościowej i jakościowej procesów biodegradacji, oceny toksyczności produktów mikrobiologicznego rozkładu badanego związku, określenia stężeń środowiskowych metyloizotiazolinonu, oceny aktywności enzymów cytochromu P450 i lakazy w procesie eliminacji badanego konserwantu; analizę statystyczną uzyskanych danych; opracowanie manuskryptu, w tym opis wstępu, materiałów i metod, wyników, dyskusji i wniosków końcowych; opracowanie graficzne wyników i abstraktu graficznego; edycja tekstu manuskryptu; zebranie danych literaturowych; udział w przygotowaniu odpowiedzi dla recenzentów.*

Nowak-Lange.....

Nowak Marta, Zawadzka Katarzyna, Szemraj Janusz, Góralczyk-Bińkowska Aleksandra, Lisowska Katarzyna (2021). *Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways*. International Journal of Molecular Science, 22(9): 4360, doi: 10.3390/ijms22094360. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ =6.628; pkt. $MEiN_{2021}$ = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **70%** i obejmował: opracowanie koncepcji badań; zaplanowanie i realizację doświadczeń dotyczących oceny zdolności eliminacji badanego ksenobiotyku przez grzyby strzępkowy Cunninghamella elegans i Trametes versicolor, chromatograficznej analizy ilościowej i jakościowej procesów biodegradacji, oceny toksyczności produktów mikrobiologicznego rozkładu badanego związku, oceny aktywności enzymów cytochromu P450 i lakazy w procesie eliminacji testowanego konserwantu; analizę statystyczną uzyskanych danych; opracowanie manuskryptu, w tym opis wstępu, materiałów i metod, wyników, dyskusji i wniosków końcowych; opracowanie graficzne wyników; edycja tekstu manuskryptu; zebranie danych literaturowych; udział w przygotowaniu odpowiedzi dla recenzentów.*

Nowak-Lange.....

Nowak-Lange Marta, Niedziałkowska Katarzyna, Bernat Przemysław, Lisowska Katarzyna. *In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria,* Scientific Reports, 12: 19068, doi: 10.1038/s41598-022-22981-9. (IF_{2021} = 4.996; $IF_{5\text{-letni}}$ = 5.516; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **75%** i obejmował: opracowanie koncepcji badań; zaplanowanie i realizację doświadczeń dotyczących analizy toksyczności środowiskowej metyloizotiazolinonu oraz chloroksylenolu wobec bakterii glebowych, w tym określenia wpływu badanych konserwantów na wzrost, żywotność, tworzenie biofilmu, ilość reaktywnych form tlenu, produkcję kwasu indolo-3-octowego, przepuszczalność błon komórkowych oraz zmiany profilu fosfolipidowego; analizę statystyczną uzyskanych danych; opracowanie manuskryptu, w tym opis wstępu, materiałów i metod, wyników, dyskusji i wniosków końcowych; opracowanie graficzne wyników; edycja tekstu manuskryptu; zebranie danych literaturowych; udział w przygotowaniu odpowiedzi dla recenzentów.*

Nowak-Lange.....

Nowak-Lange Marta, Niedziałkowska Katarzyna, Lisowska Katarzyna. *Cosmetic preservatives — hazardous micropollutants in need of greater attention?* Manuskrypt w recenzji w czasopiśmie International Journal of Molecular Science. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ = 6.628; pkt. MEiN = 140).

Oświadczam, że mój udział w ww. pracy wynosi ***80%****, który obejmował współudział w koncepcji pracy, opracowaniu manuskryptu w tym opis wstępu, wyników, dyskusji i wniosków końcowych; opracowanie graficzne wyników; edycja tekstu manuskryptu; zebranie danych literaturowych; udział w przygotowaniu odpowiedzi dla recenzentów.*

Nowak-Lange.....

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e-mail: katarzyna.lisowska@biol.uni.lodz.pl

Nowak Marta, Zawadzka Katarzyna, **Lisowska Katarzyna** (2020). *Occurrence of methylisothiazolinone in water and soil samples in Poland and its biodegradation by Phanerochaete chrysosporium*, Chemosphere, 254: 126723, doi: 10.1016/j.chemosphere.2020.126723. (IF_{2020} = 7.086; $IF_{5\text{-letni}}$ = 6.956; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

Klisers
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Nowak Marta, Zawadzka Katarzyna, Szemraj Janusz, Góralczyk-Bińkowska Aleksandra, **Lisowska Katarzyna** (2021). *Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways*. International Journal of Molecular Science, 22(9): 4360, doi: 10.3390/ijms22094360. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ = 6.628; pkt. $MEiN_{2021}$ = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

Klisers
.....

Nowak-Lange Marta, Niedziałkowska Katarzyna, Bernat Przemysław, **Lisowska Katarzyna**. *In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria,* Scientific Reports, 12: 19068, doi: 10.1038/s41598-022-22981-9. (IF_{2021} = 4.996; $IF_{5\text{-letni}}$ = 5.516; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

.....

Nowak-Lange Marta, Niedziałkowska Katarzyna, **Lisowska Katarzyna**. *Cosmetic preservatives — hazardous micropollutants in need of greater attention?* Manuskrypt w recenzji w czasopiśmie International Journal of Molecular Science. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ = 6.628; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, który obejmował współudział w opracowaniu koncepcji badań, ocenę postępów pracy, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

.....

Oświadczenia współautorów o udziale w publikacjach

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Nowak Marta, **Zawadzka Katarzyna**, Lisowska Katarzyna (2020). *Occurrence of methylisothiazolinone in water and soil samples in Poland and its biodegradation by Phanerochaete chrysosporium*, Chemosphere, 254: 126723, doi: 10.1016/j.chemosphere.2020.126723. (IF_{2020} = 7.086; $IF_{5\text{-letni}}$ =6.956; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **15%**, i obejmował współudział w: opracowaniu koncepcji badań, jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników, opracowaniu graficznym wyników, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

K. Niedziałkowska .....

Nowak Marta, **Zawadzka Katarzyna**, Szemraj Janusz, Góralczyk-Bińkowska Aleksandra, Lisowska Katarzyna (2021). *Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways*. International Journal of Molecular Science, 22(9): 4360, doi: 10.3390/ijms22094360. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ =6.628; pkt. $MEiN_{2021}$ = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, i obejmował współudział w: opracowaniu koncepcji badań, jakościowej oraz ilościowej analizie chromatograficznej, analizie i interpretacji otrzymanych wyników, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

K. Niedziałkowska .....

Nowak-Lange Marta, **Niedziałkowska Katarzyna**, Bernat Przemysław, Lisowska Katarzyna. *In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria,* Scientific Reports, 12: 19068, doi: 10.1038/s41598-022-22981-9. (IF_{2021} = 4.996; $IF_{5\text{-letni}}$ = 5.516; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, i obejmował współudział w: opracowaniu koncepcji badań, analizie i interpretacji otrzymanych wyników, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Nowak-Lange Marta, **Niedziałkowska Katarzyna**, Lisowska Katarzyna. *Cosmetic preservatives — hazardous micropollutants in need of greater attention?,* Manuskrypt w recenzji w czasopiśmie International Journal of Molecular Science. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ = 6.628; pkt. MEiN = 140).

*Oświadczam, że mój udział w ww. pracy wynosi **10%**, który obejmował współudział w opracowaniu koncepcji pracy, edycji tekstu manuskryptu oraz udział w przygotowaniu odpowiedzi do recenzentów.*

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Nowak-Lange Marta, Niedziałkowska Katarzyna, **Bernat Przemysław**, Lisowska Katarzyna. *In vitro study of the ecotoxicological risk of methylisothiazolinone and chloroxylenol towards soil bacteria*, Scientific Reports, 12: 19068, doi: 10.1038/s41598-022-22981-9. (IF_{2021} = 4.996; $IF_{5\text{-letni}}$ = 5.516; pkt. MEiN = 140

*Oświadczam, że mój udział w ww. pracy wynosi **5%**, który obejmował współudział w analizie profilu fosfolipidowego bakterii glebowych.*

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Oświadczenia współautorów o udziale w publikacjach

Mgr Aleksandra Góralczyk-Bińkowska

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Nowak Marta, Zawadzka Katarzyna, Szemraj Janusz, **Góralczyk-Bińkowska Aleksandra**, Lisowska Katarzyna (2021). *Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways*. International Journal of Molecular Science, 22(9): 4360, doi: 10.3390/ijms22094360. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ =6.628; pkt. MEiN = 140).

Oświadczam, że mój udział w ww. pracy wynosi ***5%****, który obejmował współudział w badaniu aktywności enzymów ligninolitycznych u testowanych drobnoustrojów.*

Aleksandra Góralczyk-Bińkowska

Oświadczenia współautorów o udziale w publikacjach

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Nowak Marta, Zawadzka Katarzyna, **Szemraj Janusz**, Góralczyk-Bińkowska Aleksandra, Lisowska Katarzyna (2021). *Biodegradation of Chloroxylenol by Cunninghamella elegans IM 1785/21GP and Trametes versicolor IM 373: Insight into Ecotoxicity and Metabolic Pathways*. International Journal of Molecular Science, 22(9): 4360, doi: 10.3390/ijms22094360. (IF_{2021} = 6.208; $IF_{5\text{-letni}}$ =6.628; pkt. $MEiN_{2021}$ = 140).

Oświadczam, że mój udział w ww. Pracy wynosi ***5%****, który obejmował analizę ekspresji genów cytochromu P450 oraz reduktazy cytochromu P450 techniką PCR.*

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