WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

Stacjonarne Studia Doktoranckie
Mikrobiologii, Biotechnologii i Biologii Eksperymentalnej

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Rola wybranych antyoksydantów w biodegradacji butylowych związków cyny przez mikroskopowy grzyb strzępkowy *Metarhizium robertsii*

The role of selected antioxidants in the butyltin compounds biodegradation by microscopic filamentous fungus *Metarhizium robertsii*

Praca doktorska

wykonana w Katedrze Mikrobiologii Przemysłowej i Biotechnologii

Instytutu Mikrobiologii, Biotechnologii i Immunologii

pod kierunkiem
dra hab. Przemysława Bernata,
prof. nadzw. UŁ

Łódź, 2019

Podziękowania

Pragnę złożyć serdeczne podziękowania Panu drowi hab. Przemysławowi Bernatowi, prof. nadzw. UŁ za całokształt przekazanej mi wiedzy i doświadczenia w pracy laboratoryjnej, za motywację do krytycznego spojrzenia na problematykę badawczą, a także za kreatywne podejście w poszukiwaniu rozwiązań problemów biologicznych. Pragnę również podziękować za nieocenioną pomoc w trakcie przygotowywania niniejszej pracy doktorskiej oraz za okazaną życzliwość, cierpliwość, wyrozumiałość i zrozumienie dla niektórych moich pomysłów i podejmowanych decyzji.

Chciałabym wyrazić również głęboką wdzięczność Panu prof. Jerzemu Długońskiemu za dostrzeżenie pierwiastka naukowca w mojej osobie, za inspirację do zgłębiania zagadnień naukowych oraz za motywację do opracowania samodzielnych koncepcji badawczych.

Osobne, równie ważne, podziękowania składam mojemu mężowi Dawidowi, na którego pomoc mogłam zawsze liczyć, który wspierał mnie w trudnych chwilach i niejednokrotnie odciążał mnie w obowiązkach domowych oraz rodzicielskich. Dziękuję również moim rodzicom i teściom, którzy wspierali mnie mentalnie i poświęcali czas mojej córeczce Michalinie, abym mogła dopełnić formalności związanych z obroną stopnia naukowego doktora.

Spis treści

Publikacje wchodzące w skład rozprawy doktorskiej

1. **Siewiera P**, Bernat P, Różalska S, Długoński J. 2015. Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*. International Biodeterioration & Biodegradation, 104:258-263.

   IF = 2,429, MNiSW = 30

2. **Siewiera P**, Różalska S, Bernat P. 2017. Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation. Environmental Science and Pollution Research, 24:12118-12127.

   IF = 2,800, MNiSW = 30

3. **Siewiera P**, Różalska S, Bernat P. 2017. Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin. Chemosphere, 185:96-104.

   IF = 4,427, MNiSW = 35

4. **Stolarek P**, Różalska S, Bernat P. 2019. Lipidomic adaptations of the *Metarhizium robertsii* strain in response to butyltin compounds presence. Biochimica et Biophysica Acta - Biomembranes, 1861(1):316-326.

   IF = 3,438, MNiSW = 35

Sumaryczny IF = 13,094

Łączna liczba punktów MNiSW = 130

Wartości IF oraz punktację MNiSW podano zgodnie z rokiem opublikowania.

Finansowanie

1. Grant NCN pt. „Mikrobiologiczna degradacja ksenoestrogenów i estrogenów w obecności metali ciężkich i NaCl”. Nr projektu: 2011/01/B/NZ9/02898. 2014-2015.

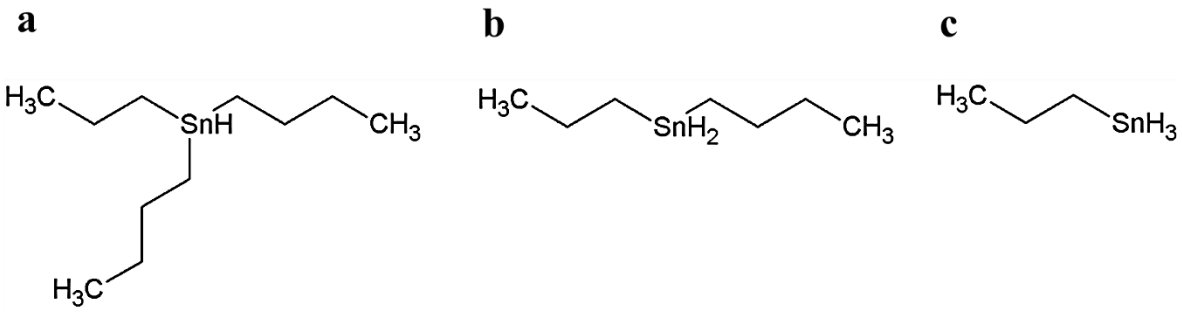
2. Dotacja celowa dla młodych naukowców oraz uczestników studiów doktoranckich pt. „Analiza wpływu antyoksydantów na biodegradację dibutylocyny (DBT) przez wybrane szczepy grzybowe”. Kod projektu: B1611000001201.02. 2016, kierownik.

3. Dotacja celowa dla młodych naukowców oraz uczestników studiów doktoranckich pt. „Analiza jakościowa i ilościowa sfingolipidów grzybni *Metarhizium robertsii* poddanej ekspozycji na dibutylocynę (DBT)”. Kod projektu: B1711000001552.02. 2017, kierownik.

4. Grant NCN pt. „Rola antyoksydantów w procesie biodegradacji butylowych związków cyny przez wybrane szczepy grzybowe, ze szczególnym uwzględnieniem zmian w lipidomie”. Nr projektu: 2015/19/N/NZ9/00459. 2016-2018, kierownik.

I. WPROWADZENIE

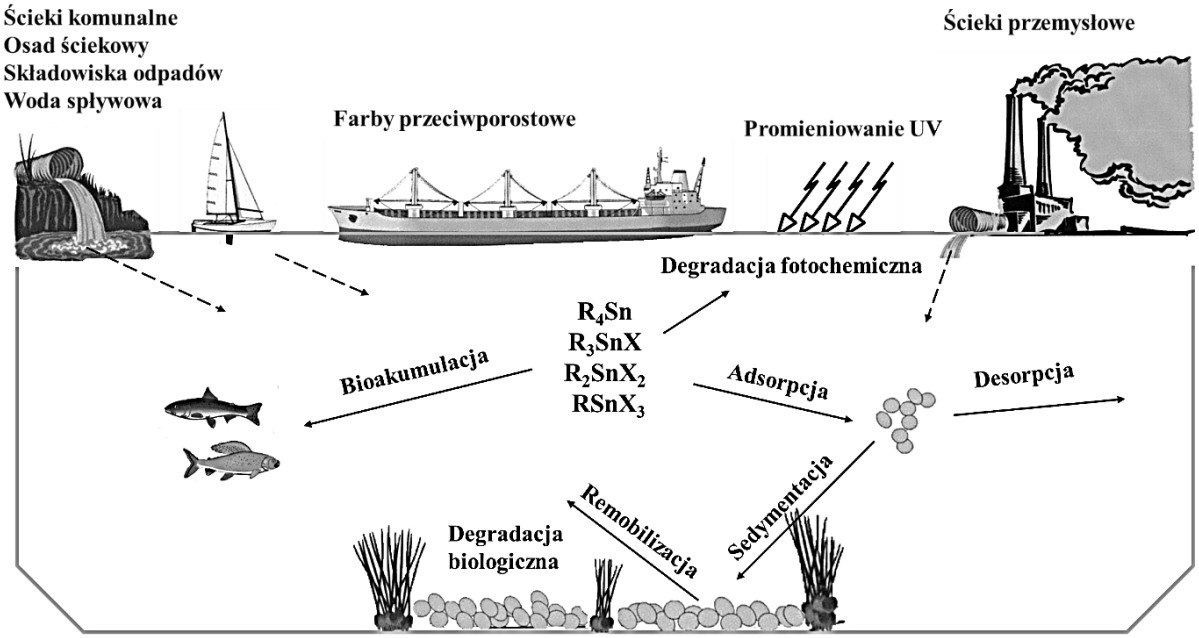
Butylowe związki cyny, stosowane na skalę przemysłową od lat 50. ubiegłego wieku, zbudowane są z jednej lub kilku grup butylowych przyłączonych kowalencyjnie do centralnie położonego atomu cyny (Rys. 1) (Du i in., 2014). Funkcyjność tributylocyny (TBT) przejawia się przede wszystkim w działaniu bójczym, co umożliwiło wykorzystanie związku jako składnika a) farb przeciwporostowych służących do pokrywania kadłubów statków i innych konstrukcji podwodnych, b) środków dezynfekujących stosowanych przez garbarnie, celulozownie i fabryki włókiennicze oraz c) środków konserwujących głównie drewno i inne materiały budowlane. Z kolei aplikacyjność dibutylocyny (DBT) – stabilizatora termicznego oraz katalizatora reakcji estryfikacji – dotyczy głównie przemysłu tworzyw sztucznych, a zwłaszcza produkcji polichlorku winylu, sylikonów i pianek. Natomiast monobutylocynę (MBT) wykorzystuje się podczas obróbki szkła i wytwarzania PCW (Sousa i in., 2014).



Rys. 1. Struktury chemiczne cząsteczek tri- (a), di- (b) i monobutylocyny (c).

W konsekwencji globalnego eksploatowania butylowych związków cyny, substancje te przenikają ze ścieków komunalnych i przemysłowych, z odcieków z wysypisk oraz po bezpośrednim wymyciu z farb przeciwporostowych do ekosystemów wodnych i lądowych (Hoch, 2001). Według danych literaturowych, stężenie butylowych związków cyny nie przekracza 200 ng Sn l^{-1} wód morskich, 17 µg Sn g s.m.$^{-1}$ osadów dennych oraz 100 µg Sn kg s.m.$^{-1}$ gleb (de Carvalho Oliveira i Santelli, 2010; Hoch, 2001; Okoro i in., 2011). W warunkach środowiskowych, substancje te mogą ulegać procesom fotodegradacji, adsorpcji i desorpcji, bioakumulacji oraz biologicznej degradacji (Rys. 2) (Hoch, 2001). Źródłem energii niezbędnej do rozerwania wiązania Sn–C jest promieniowanie UV, stąd też fotoliza cząsteczek butylocyn zachodzi jedynie w warstwach powierzchniowych wód. Z kolei adsorpcja butylowych związków cyny na powierzchni cząsteczek gleby lub osadów dennych zależy od warunków środowiskowych takich jak odczyn pH, zasolenie oraz skład mineralogiczny i chemiczny adsorbenta (Hoch, 2001). Jednakże dane literaturowe donoszą, iż substancje te pozostają

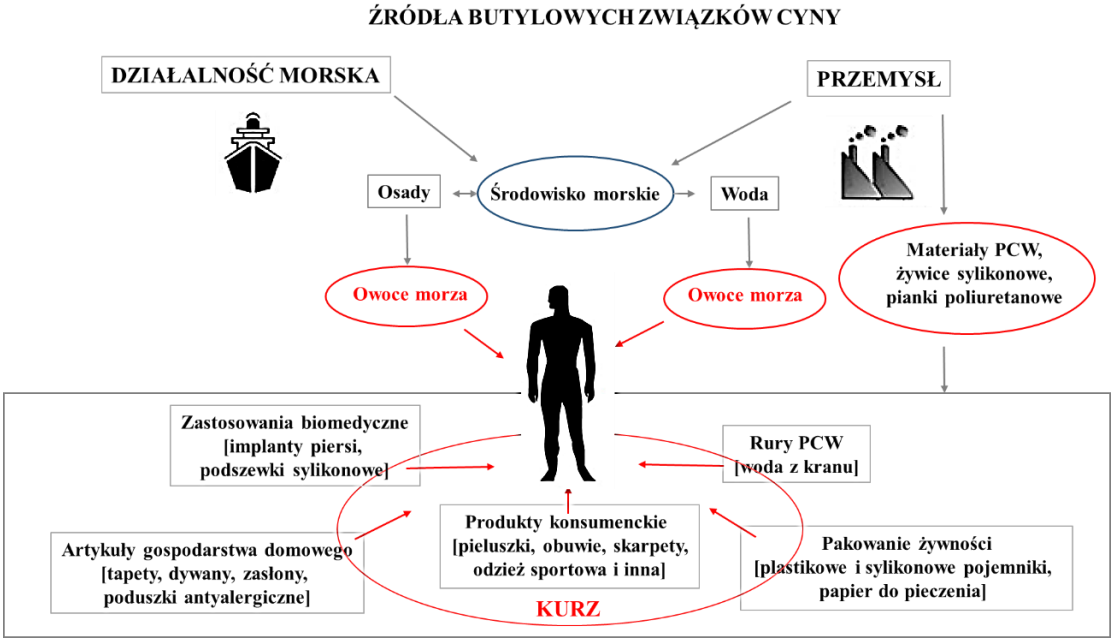
długoterminowymi zanieczyszczeniami środowiska naturalnego (Chiavarini i in., 2014). Z powodu lipofilowego charakteru cząsteczek, butylocyny ulegają również bioakumulacji w tkankach bezkręgowców (takich jak mięczaki i skorupiaki) i kręgowców (przede wszystkim ryb, delfinów, ptaków) zagrażając zdrowiu człowieka, będącego ostatnim ogniwem łańcucha troficznego (Hoch, 2001). Źródła literaturowe wyszczególniają kilkanaście mikroorganizmów wykazujących zdolność do biodegradacji butylowych związków cyny, a wśród nich bakterie z rodzaju *Pseudomonas* i *Streptomyces* (Bernat i Długoński, 2009; Bernat i in., 2014), grzyby strzępkowe należące do gatunków *Cunninghamella elegans* i *Cochliobolus lunatus* (Bernat i Długoński, 2006; Bernat i in., 2013) oraz algi *Chlorella vulgaris* (Tsang i in., 1999).



Rys. 2. Transfer i relokacja butylowych związków cyny oraz procesy, którym ulegają w ekosystemach wodnych (Hoch, 2001).

Ekspozycja człowieka na butylowe związki cyny związana jest z licznymi źródłami antropogenicznymi tych substancji (Rys. 3). Następstwem spożywania skażonej żywności (owoców morza, pokarmów przechowywanych w opakowaniach wykonanych z PCW), wody (transportowanej rurami PCW) i napojów (dystrybuowanych w opakowaniach wyprodukowanych z PCW), podobnie jak skutkiem kontaktu z artykułami gospodarstwa domowego (takimi jak folie i pergaminy do pieczenia, gąbki, odzież, artykuły sanitarne, zabawki dziecięce, tapety, dywany, poduszki) i materiałami medycznymi, czy nawet kurzem domowym (Hoch, 2001; Moser i in., 2009; Sousa i in., 2014) jest mierzalny poziom butylowych

związków cyny w tkankach i wydzielinach ludzkich takich jak krew, wątroba, czy mleko matki (Kannan i Falandysz, 1997; Mino i in., 2008; Rantakokko i in., 2008).



Rys. 3. Główne źródła ekspozycji człowieka na butylowe związki cyny (Sousa i in., 2014).

Wszechstronne narażenie człowieka na obecność butylocyn stwarza niebezpieczeństwo związane z różnorodnymi efektami toksycznymi ich działania. Są to przede wszystkim zaburzenia reprodukcyjne, zmiany neurobehawioralne, zaburzenia immunologiczne, zmiany hepatologiczne, toksyczność sercowo-naczyniowa, różnicowanie tkanki tłuszczowej i otyłość oraz hamowanie osteoklastogenezy (Sousa i in., 2014).

Toksyczność butylowych związków cyny spowodowana jest m. in. interakcją ich cząsteczek ze składnikami błon komórkowych, prowadzącą do zaburzeń prawidłowego funkcjonowania tej bariery ochronnej komórki, stanowiącej zarazem główny cel dla wszystkich substancji toksycznych, a zwłaszcza tych o lipofilowej naturze. Pośrednią przyczyną tego negatywnego oddziaływania jest generowanie reaktywnych form tlenu (RFT), w następstwie czego, komórka poddana jest na działanie stresu oksydacyjnego (Chantong i in., 2014) i peroksydację lipidów błonowych, co wpływa na właściwości fizyko-chemiczne membran oraz na pełnione przez nie funkcje (Niki i in., 2005). Z kolei bezpośrednią przyczyną zaburzeń prawidłowego funkcjonowania błon jest umiejscowienie lipofilowych cząsteczek butylocyn w dwuwarstwie lipidowej, co niesie ze sobą zmiany jej przepuszczalności, które w konsekwencji zakłócają jonowy gradient transbłonowy oraz funkcjonowanie enzymów błonowych (Pagliarani i in., 2013). W sytuacji nadmiernego nagromadzenia butylowych

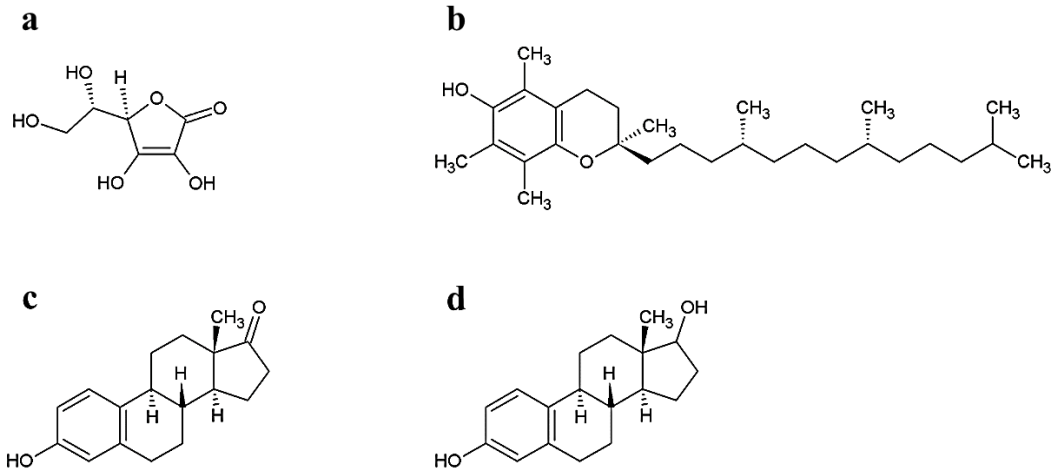
związków cyny w błonie komórkowej, następuje osłabienie oddziaływania między jej składnikami, prowadząc do zniszczenia struktury membranowej (Bojarska-Kujawa i in., 2012).

Powszechnie przyjmuje się, iż toksyczność butylowych związków cyny maleje według przedstawionego ciągu: $R_3SnX > R_2SnX_2 > RSnX_3$, gdzie R oznacza ugrupowanie butylowe, a X dowolne ugrupowanie anionowe (de Carvalho Oliveira i Santelli, 2010). Jednakże Frouin i in. (2008) udowodnili, iż DBT wywiera silniejszy efekt immunotoksyczny u organizmów bezkręgowych i kręgowych niż TBT. Co więcej, istnieją dane literaturowe, które donoszą, iż DBT oddziałuje silniej na funkcje mitochondrialne – niż TBT – hamując aktywność hydrolityczną ATPaz magnezowych i wapniowych (Bouchard i in., 1999; Nesci i in., 2011).

W konsekwencji poszerzenia wiedzy na temat silnej toksyczności i bioakumulacji butylowych związków cyny, uznając potrzebę ochrony środowiska morskiego, Międzynarodowa Organizacja Morska (IMO) opracowała w 2001 roku Konwencję AFS wprowadzającą prohibicję stosowania farb przeciwporostowych opartych na TBT jako substancji czynnej (Sant'Anna i in., 2012; Sousa i in., 2014). Z kolei Unia Europejska uchwaliła Dyrektywę 2002/62/EC i Rozporządzenie 782/2003, na mocy których z dniem 1. stycznia 2008 roku zaczął obowiązywać rozszerzony zakaz korzystania z farb zawierających w swoim składzie tributylocynę obejmujący wszystkie statki wpływające do portów państw członkowskich UE (Sousa i in., 2014). Jednakże produkcja i stosowanie farb na bazie związków cynoorganicznych są nadal możliwe w niektórych krajach rozwijających się, niebędących członkami IMO i UE (Yi i in., 2012). Co więcej, pomimo zakazu stosowania, TBT jest nadal w użyciu na małych łodziach (Toste i in., 2011), a dane literaturowe dowodzą, iż butylowe związki cyny są w dalszym ciągu wykrywane w próbach wód i osadów dennych pobieranych z różnych regionów świata (Chen i in., 2019; de Castro i in., 2012; Filipkowska i in., 2014; Hassan i in., 2019).

Zanieczyszczenie ekosystemów naturalnych butylowymi związkami cyny, w połączeniu z ich zdolnością do bioakumulacji w łańcuchu troficznym i różnorodnymi mechanizmami toksyczności skłoniło naukowców do poszukiwania innowacyjnych rozwiązań służących bioremediacji środowisk skażonych. Na szczególną uwagę zasługują grzyby mikroskopowe, charakteryzujące się tolerancją wobec wysokich stężeń substancji toksycznych, różnorodnością dróg metabolicznych, niską specyficznością enzymów komórkowych oraz wzrostem strzępkowym ułatwiającym dostęp do metabolizowanych substancji (Potin i in., 2006; Samanta i in., 2002).

Z uwagi na fakt potwierdzonych właściwości prooksydacyjnych butylocyn (Chantong i in., 2014; Ishihara i in., 2012), możliwe jest przypuszczenie, iż celowa suplementacja antyoksydantami, chroniąca komórki grzybowe przed stresem oksydacyjnym, może zwiększyć wydajność procesu biodegradacji tych związków. Wśród nieenzymatycznych przeciwutleniaczy, istotną rolę odgrywają witaminy. Witamina C (kwas askorbinowy) – uznawana za zasadniczy antyoksydant fazy wodnej komórki – wygasza reaktywne formy tlenu tworząc stabilizowane rezonansowo, względnie niereaktywne i łatwo poddające się redukcji rodniki askorbinowe (Li i Schellhorn, 2007). Witamina E (α-tokoferol) uważana jest z kolei za elementarny przeciwutleniacz fazy lipofilowej komórki i antyperoksydant lipidów. Według danych literaturowych, α-tokoferol zmiata wolne rodniki najskuteczniej spośród wszystkich tokoferoli. Mechanizm usuwania wtórnych rodników przez α-tokoferole polega na łamaniu 16-węglowego łańcucha antyoksydantu wbudowanego uprzednio w strukturę błonową. W trakcie wymiatania rodników peroksylowych tworzone są formy nierodnikowe i odpowiedni rodnik α-tokoferylowy (Azzi i in., 2007; Sroka i in., 2005). Wśród antyoksydantów wymienia się również związki steroidowe, szczególnie estrogeny naturalne, a spośród nich estron (E1) i 17-βestradiol (E2). Pierścień A wraz z grupą –OH usytuowaną w pozycji 3. jest kluczowym elementem aktywności antyoksydacyjnej estrogenów fenolowych, czyniąc je silnymi przeciwutleniaczami fazy wodnej i mniej skutecznymi w fazie lipidowej. Według niektórych źródeł literaturowych, efektywność zmiatania wolnych rodników przez estrogeny naturalne jest porównywalna, a czasem wyższa niż dla witamin (Ruiz-Larrea i in., 2000). Opisane powyżej substancje o aktywności przeciwutleniającej są naturalnymi, powszechnie występującymi związkami zróżnicowanymi nie tylko pod względem struktury chemicznej (Rys. 4), właściwości i mechanizmów działania, ale również ilości podatnych na jonizację grup hydroksylowych, od których zależy aktywność antyoksydacyjna związku.



Rys. 4. Wzory strukturalne witamin C (a) i E (b) oraz estronu (c) i 17β-estradiolu (d).

Niniejsza praca doktorska dotyczy oddziaływania wybranych substancji o właściwościach antyoksydacyjnych: witamin C i E oraz estrogenów naturalnych - 17β-estradiolu i estronu na proces biodegradacji butylowych związków cyny, z jednoczesnym uwzględnieniem modyfikacji wybranych elementów lipidomu mikroskopowego grzyba strzępkowego *Metarhizium robertsii*. Grzyby należące do gatunku *M. robertsii* występują powszechnie na całym świecie. Ich głównym rezerwuarem jest gleba, a w szczególności ryzosfera roślin (Liao i in., 2013; Sasan i Bidochka, 2012). Grzyb ten zaliczany jest do mikroorganizmów entomopatogennych, czyli infekujących owady (Sasan i Bidochka, 2012), dzięki czemu znalazł zastosowanie w walce z insektami w postaci biopreparatów, zastępując chemiczne środki ochrony roślin (Sasan i Bidochka, 2012). Szczep grzybowy stanowiący model badawczy w niniejszej pracy został wyizolowany z gleby pochodzącej z terenu Łodzi i umieszczony w katalogu kolekcji Katedry Mikrobiologii Przemysłowej i Biotechnologii Uniwersytetu Łódzkiego pod sygnaturą IM 6519. Analizy przeprowadzone przez Różalską i in. (2015) dowiodły, iż grzyb ten wykazuje zdolność do biodegradacji 4-*n*-nonylfenolu. Co więcej, badania wstępne uwidoczniły potencjał szczepu *M. robertsii* IM 6519 do eliminacji butylocyn ze środowiska wzrostu.

II. CELE PRACY

Za główne cele badań przeprowadzanych podczas realizacji pracy doktorskiej przyjęto:

1. Optymalizację procesu biodegradacji dibutylocyny przez mikroskopowy grzyb strzępkowy *Metarhizium robertsii* IM 6519.
2. Analizę wpływu wybranych substancji o właściwościach antyoksydacyjnych na rozkład butylowych związków cyny przez szczep IM 6519.
3. Badanie toksycznego oddziaływania di- i tributylocyny (w/bez obecności witaminy C) na wybrane składniki lipidomu grzybowego szczepu *M. robertsii* IM 6519.

III. REALIZACJA POSZCZEGÓLNYCH CELÓW PRACY

III. 1. Opis optymalizacji procesu biodegradacji dibutylocyny przez mikroskopowy grzyb strzępkowy *Metarhizium robertsii* IM 6519

Głównym źródłem dibutylocyny w ekosystemach wodnych i lądowych jest uwalnianie związku z materiałów PCW oraz degradacja środowiskowej tributylocyny za pośrednictwem aktywności mikrobiologicznej i/lub reakcji fotochemicznych (Hoch, 2001). Dostępne dane literaturowe wskazują, iż DBT wykazuje działanie cyto-, immuno-, hepato-, terato-, neuro- i genotoksyczne, a także charakteryzuje się właściwościami prooksydacyjnymi (Chantong i in., 2014; Farr i in., 2001; Ferreira i in., 2013; Gumy i in., 2008; Jenkins i in., 2004; Ueno i in., 2003), stąd też wnioskuje się, iż obecność tego związku w bezpośrednim otoczeniu stwarza zagrożenie dla wszystkich organizmów. Niestety, konwencjonalne metody oczyszczania środowisk skażonych obarczone są wysokimi kosztami oraz stosunkowo niską skutecznością, dlatego też dostrzega się potencjał rozkładu zanieczyszczeń z udziałem mikroorganizmów, który może posłużyć redukcji środowiskowych poziomów dibutylocyny.

Podczas realizacji pierwszego celu niniejszej rozprawy doktorskiej przeprowadzono optymalizację biodegradacji DBT przez mikroskopowy grzyb strzępkowy *Metarhizium robertsii*. Weryfikacja podłóż hodowlanych zróżnicowanych pod kątem składników odżywczych umożliwiła dokonanie doboru najlepszego źródła węgla (tj. glukozy) i azotu (tj. ekstraktu drożdżowego) pożywki wzrostowej. Kolejno, eliminowano poszczególne źródła makroelementów (magnezu, wapnia i potasu) oraz modyfikowano a) odczyn pH pożywki (w zakresie 6-8), b) temperaturę inkubacji (w zakresie 25-30 °C) i c) natlenienie hodowli (porównanie kolb laboratoryjnych oraz bioreaktora) kontrolując wpływ zmienianych warunków hodowli na tempo biodegradacji DBT. Ze względu na fakt, iż deficyt Mg, Ca i K, podobnie jak odczyn pH i temperatura hodowli (w badanych zakresach) nie oddziaływały istotnie na efektywność biodegradacji badanego związku [dane nieopublikowane], do dalszych etapów badań wybrano podłoże wzrostowe o następującym składzie: K_2HPO_4 (4,36 g), KH_2PO_4 (1,7 g), $MgSO_4 \cdot 7H_2O$ (0,2 g), $MnSO_4$ (0,05 g), $FeSO_4 \cdot 7H_2O$ (0,01 g), $CaCl_2 \cdot 2H_2O$ (0,03 g), glukoza (40 g), ekstrakt drożdżowy (10 g), woda destylowana (do 1 l) i odczynie pH równym 6,8. Inkubację prowadzono w optymalnej temperaturze do hodowli grzybów strzępkowych - 28 °C, przy minimalnym nasyceniu podłoża hodowlanego tlenem wynoszącym 20%. Właściwe warunki wzrostowe zostały zapewnione poprzez użycie bioreaktora Labfors 5 o obj. roboczej 3,6 l. Hodowle grzybowe prowadzono w objętości 1 l, z kontrolowanym

natlenianiem (przepływ powietrza 1 l min^{-1}) i mieszaniem (200–250 rpm) oraz nieregulowanym odczynem pH pożywki.

Efektem zapewnienia optymalnych warunków inkubacji badanego grzyba strzępkowego, ze szczególnym naciskiem na intensywne natlenianie pożywki, było istotne przyspieszenie zarówno wzrostu jak i tempa biodegradacji butylowych związków cyny. Wartość maksymalnego, specyficznego współczynnika wzrostu drobnoustroju (μ_{max}) wzrosła z 0,061 do 0,086 h^{-1}, a proces biodegradacji DBT został przyspieszony 10-krotnie (w porównaniu z wynikami otrzymanymi dla hodowli prowadzonych w kolbach laboratoryjnych) doprowadzając do całkowitego usunięcia di- i monobutylocyny ze środowiska wzrostu, czego nie udało się osiągnąć podczas badań prowadzonych w kolbach. Co więcej, dotychczas nie opublikowano innego modelu biologicznego zdolnego do szybkiego i całkowitego przekształcania butylowych związków cyny do cyny nieorganicznej, poza szczepem *M. robertsii* IM 6519 inkubowanym w warunkach intensywnego natlenienia. Zaobserwowana zależność pomiędzy krzywą wzrostu drobnoustroju, a ubytkiem DBT skłoniła do przeprowadzenia badań nad asymilacją glukozy z pożywki hodowlanej. Analizy wykonane z użyciem wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas HPLC-MS/MS umożliwiły wyznaczenie trendu ubytku glukozy z podłoża wzrostowego, obrazując jednoczesne wykorzystanie źródła węgla i energii oraz DBT przez komórki grzybowe, wskazując na kometaboliczną naturę procesu rozkładu ksenobiotyku. Z kolei, obserwacje mikroskopowe grzybni inkubowanej w warunkach zróżnicowanego natlenienia ukazały różnice morfologiczne wpływające bezpośrednio na wydajność procesu biodegradacji DBT. W warunkach intensywnego napowietrzania, grzybnia przyjmowała postać rozproszoną z długimi strzępkami (określaną typu „*hairy*"), podczas gdy niedostateczna ilość doprowadzonego tlenu do hodowli w kolbach skutkowała formowaniem kulistych, gęsto upakowanych peletek grzybowych z pojedynczymi, krótkimi strzępkami w regionie peryferyjnym (morfologia typ „*smooth*") oraz z najprawdopodobniej martwym wnętrzem. Opisując modyfikacje morfologiczne szczepu *M. robertsii* IM 6519 wyznaczano stosunek pola powierzchni rdzenia peletki do pola powierzchni całej peletki. Zaobserwowano, iż otrzymane wartości były istotnie niższe dla grzybni inkubowanej w bioreaktorze niż w kolbach, potwierdzając hipotezę, iż zmniejszenie powierzchni rdzenia peletki na rzecz aktywnych metabolicznie strzępek znajdujących się w regionie peryferyjnym zwiększa wydajność biodegradacji DBT, najprawdopodobniej ze względu na lepszą wymianę masy pomiędzy wnętrzem peletki, a środowiskiem zewnętrznym.

Stwierdzenia cząstkowe dotyczące optymalizacji procesu biodegradacji dibutylocyny przez mikroskopowy grzyb strzępkowy *Metarhizium robertsii* IM 6519

1. Szczep grzybowy *Metarhizium robertsii* IM 6519 wykazuje zdolność do całkowitej eliminacji dibutylocyny ze środowiska wzrostu.

2. Intensywne napowietrzanie hodowli wgłębnej badanego szczepu prowadzi do zmian morfologicznych grzybni tj. powstawania rozproszonych form z długimi strzępkami w zamian gęsto upakowanych peletek.

3. Ułatwiona wymiana substancji odżywczych oraz tlenu i dwutlenku węgla między biomasą, a środowiskiem wzrostu skutkuje wzmożonym wzrostem grzybowym i poprawą wydajności biodegradacji DBT i MBT.

4. Jednoczesne wykorzystanie glukozy i dibutylocyny wskazuje na kometaboliczny charakter eliminacji ksenobiotyku.

5. Wykrycie hydroksylowanej monobutylocyny sugeruje zaangażowanie monooksygenaz cytochromu P450 w proces biodegradacji DBT.

III. 2. Opis analizy wpływu wybranych związków o właściwościach antyoksydacyjnych na rozkład butylowych związków cyny przez szczep IM 6519

Wśród antyoksydantów, czyli donorów elektronów neutralizujących niezwykle reaktywne i niestabilne cząsteczki wolnych rodników, wyszczególnia się różnorodne związki takie jak witaminy, karotenoidy, związki fenolowe i wiele innych (Sroka i in., 2005). Znaczenie suplementacji środowiska wzrostu drobnoustrojów niskocząsteczkowymi związkami przeciwutleniającymi dla skutecznej eliminacji substancji o charakterze lipofilowym lub właściwościach prooksydacyjnych zostało dostrzeżone w światowej literaturze (Ponce i in., 2011; Słaba i in., 2013), jednakże wiedza na temat podłoża molekularnego procesu jest nadal ograniczona.

Realizację drugiego celu niniejszej pracy doktorskiej dotyczącego oddziaływania wybranych substancji o właściwościach antyoksydacyjnych na biodegradację butylowych związków cyny przez szczep *M. robertsii* IM 6519 rozpoczęto od weryfikacji wpływu witamin C i E na kinetykę rozkładu DBT, która pozwoliła na stwierdzenie, iż suplementacja przeciwutleniaczami odgrywa większą rolę w usuwaniu monobutylocyny, jako głównego produktu ubocznego powstającego w trakcie rozkładu DBT, niż samego związku macierzystego. Eliminacja MBT w obecności witaminy C następowała 3-krotnie szybciej niż w hodowli bez dodatku przeciwutleniacza. Podczas gdy, dodatek witaminy E wpływał negatywnie na tempo rozkładu MBT, spowolniając go o 40% w trakcie pierwszych 7 godz. prowadzenia hodowli. Biorąc pod uwagę fakt zaangażowania enzymów cytochromu P450 w ω-hydroksylację łańcucha węglowodorowego witaminy E (Sontag i Parker, 2002), przyczyną negatywnego wpływu na tempo biodegradacji MBT jest przypuszczalnie konkurencja tych cząsteczek o miejsce aktywne cytochromu P450. Wykonane analizy ilościowe dialdehydu malonowego (MDA), jako jednego z najczęściej wymienianych markerów peroksydacji lipidów, potwierdziły potencjał antyoksydacyjny witaminy C i ochronę błon grzybowych przed stresem oksydacyjnym. Poziom MDA w grzybni poddanej ekspozycji na DBT (151 μM l^{-1}) zmalał o 45% przy jednoczesnej obecności witaminy C i nie uległ istotnym zmianom po wprowadzeniu witaminy E. Prawdopodobną przyczyną nieskuteczności witaminy E w badanym układzie jest a) zbyt niskie stężenie antyoksydantu, którego aktywność według danych literaturowych jest skuteczna w stanach fizjologicznych (nie patologicznych), a czasem wymaga podania dodatkowych przeciwutleniaczy (Azzi, 2007) lub b) brak różnorodności

wielonienasyconych kwasów tłuszczowych, których uszkodzeniom zapobiega mechanizm przerwania łańcucha węglowodorowego antyoksydantu (Raederstorff i in., 2015).

Uzyskane wyniki pozwoliły stwierdzić, iż witamina C jest skuteczniejszą substancją (z punktu widzenia prowadzonych analiz) niż witamina E, co może być powiązane z przeważającą ilością grup hydroksylowych (jako donorów atomu wodoru) w strukturze kwasu askorbinowego (4 grupy –OH) w porównaniu z α-tokoferolem (1 grupa –OH). Co więcej, zjawisko to sugeruje iż, reaktywne formy tlenu indukowane obecnością butylowego związku cyny powstają w fazie wodnej, a nie lipofilowej. Kluczowym elementem realizacji niniejszego etapu pracy doktorskiej była identyfikacja pochodnych powstających w trakcie procesu biodegradacji DBT. Wnikliwa analiza widm masowych uzyskanych z użyciem techniki HPLC-MS/MS umożliwiła wskazanie produktu pośredniego pomiędzy DBT, a MBT, którym był związek o wzorze ogólnym $OHBuSnH_2$ (MBTOH), zwracający uwagę w kierunku monooksygenaz grzybowego cytochromu P450 katalizujących reakcje hydroksylacji ksenobiotyków.

Wyniki badań prowadzonych nad wpływem natlenienia oraz obecności witamin C i E na kinetykę rozkładu butylowych związków cyny przez szczep *M. robertsii* IM 6519 oraz rezultaty eksperymentów mających na celu wyjaśnienie zaobserwowanych zjawisk tj. opis zmian morfologicznych, znaczenie tempa asymilacji glukozy oraz analizy a) ilościowe MDA i b) jakościowe metabolitów dibutylocyny opublikowano w pracy oryginalnej „Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation” (Environmental Science and Pollution Research, IF = 2,741, 30 pkt MNiSW).

Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation

[Environmental Science and Pollution Research (2017), 24:12118-12127]

Environ Sci Pollut Res (2017) 24:12118–12127
DOI 10.1007/s11356-017-8764-4

RESEARCH ARTICLE

Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation

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Received: 17 November 2016 / Accepted: 7 March 2017 / Published online: 27 March 2017

Abstract Dibutyltin (DBT) is an environmental pollutant characterized by immunotoxic, neurotoxic, and pro-oxidant properties. In this study, an attempt was made to enhance DBT elimination by the *Metarhizium robertsii* strain. We observed enhanced fungal growth in the bioreactor ($pO_2 \geq 20\%$) compared to flask cultures (μ_{max} increased from 0.061 to 0.086 h^{-1}). Moreover, under aerated conditions, *M. robertsii* mycelium with "hairy" morphology biodegraded DBT (20 mg l^{-1}) 10-fold faster in the bioreactor than in the flask cultures. Monobutyltin (MBT) and a hydroxylated derivative of MBT ($OHBuSnH_2$) were detected as by-products of dibutyltin debutylation. Simultaneous usage of glucose and butyltins indicates the comatabolic nature of monobutyltin and dibutyltin removal. In order to protect fungal cells from oxidative stress caused by DBT presence, vitamin C (20 mg l^{-1}) was applied. Supplementation with ascorbic acid (AA) resulted in a 3-fold acceleration of MBT removal during the first 7 h of incubation. Using the HPLC-MS/MS technique, a quantitative analysis of malondialdehyde (MDA), a marker of oxidative stress, was performed. In the AA presence, a decrease in the MDA amount (about 45%) was observed compared to the case with fungal cells exposed to DBT alone.

Responsible editor: Diane Purchase

Electronic supplementary material The online version of this article (doi:10.1007/s11356-017-8764-4) contains supplementary material, which is available to authorized users.

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Keywords *Metarhizium robertsii* · Dibutyltin utilization · Intense aeration · Hyphae morphology · Antioxidants · Oxidative stress alleviation · Malondialdehyde · Liquid chromatography

Introduction

Dibutyltin (DBT) is a useful heat stabilizer of polyvinyl chloride, a curing agent for silicone rubbers, and a catalyst for esterification reactions. Because of its widespread use, the compound is found in the environment and in dietary sources (Moser et al. 2009). This organotin is mainly introduced into sediments and water by leaching from PVC materials. Additionally, dibutyltin is formed as a major degradation product of environmental tributyltin (TBT)—the most toxic of all organotin compounds. Due to its hydrophilicity, DBT rapidly enters into water and is accumulated in tissues of mussels and other marine invertebrates (Nesci et al. 2011). The concentrations of DBT in mussels from the Northern Adriatic Sea ranges from 15 to 2660 ng Sn g^{-1} (Nemanič et al. 2009). The levels of DBT in mussels collected from the Polish coast (Baltic Sea) are between 0.5 and 24 ng Sn g^{-1}. On the other hand, dibutyltin found in the liver of European flounder in the Gdansk Bay represents 72–86% of total butyltin content (Albalat et al. 2002). The presence of DBT in human blood (4.7–36.7 ng Sn ml^{-1}) and liver samples (0.4–12.8 ng Sn g^{-1}) has been proved (Whalen et al. 1999; Nielsen and Strand 2002). Liver enzymes are not effective in DBT elimination (Albalat et al. 2002).

In contrast to tributyltin, the toxic effects of dibutyltin are less well known and there is little information available. A reduction of the toxicity of metabolites compared to the initial compound is assumed. Unfortunately, DBT is more immunotoxic to invertebrates and vertebrates than TBT

(Frouin et al. 2008). Moreover, DBT has a stronger effect on mitochondrial functions than TBT, as it inhibits hydrolytic Mg-ATPase and Ca-ATPase activities in mussels (Bouchard et al. 1999; Nesci et al. 2011). A potential neurotoxic effect of DBT, which may lead to apoptotic death of the hippocampus and neocortex cells in rats, has also been reported (Jenkins et al. 2004). DBT is an inducer of oxidative stress and an amplifier of pro-inflammatory cytokine expression in microglia cells (Chantong et al. 2014).

In this study, an attempt was undertaken to improve the process of dibutyltin biodegradation using a microscopic fungus *Metarhizium robertsii.* The selected strain can eliminate TBT with high levels of efficiency by protecting the fungal cells from oxidative stress through the application of 17β-estradiol (Siewiera et al. 2015). In the present research, ascorbic acid (AA, vitamin C) and α-tocopherol (vitamin E) were chosen as primary antioxidants of the aqueous and lipophilic phases, respectively (Li and Schellhorn 2007). The effectiveness of vitamins in free radical scavenging was verified by quantitative analysis of malondialdehyde (MDA), a lipid peroxidation product. In order to speed up the process of DBT degradation, an additional oxygen supply ($pO_2 \geq 20\%$) was prepared. Moreover, there was an attempt at the identification of metabolic intermediates formed during organotin dealkylation.

Materials and methods

Chemicals

Dibutyltin dichloride, ascorbic acid, α-tocopherol, methyl magnesium bromide, tropolone, anhydrous sodium sulfate, and 1,1,3,3-tetraethoxypropane were purchased from Sigma-Aldrich Chemical Co. (Germany). Stock solutions of $DBTCl_2$, α-tocopherol (vitamin E), and ascorbic acid (vitamin C), each at a concentration of 10 mg ml^{-1}, were prepared in ethanol, dimethyl sulfoxide, and distilled water, respectively. The solvents for organotin extraction such as methanol, hexane, and ethyl acetate were purchased from POCH S.A. (Poland). Other high purity organic solvents used during gas and liquid chromatography analyses originated from J.T. Baker Chemical Co. (the Netherlands).

Microorganism and growth conditions

The ascomycete insect pathogenic fungus *M. robertsii* IM 6519 from the Department of Industrial Microbiology and Biotechnology (University of Lodz, Poland) was the subject of the study. The ability of the microorganism to degrade organotins was confirmed in an earlier paper (Siewiera et al. 2015).

Fourteen-day-old fungal cultures on ZT slants were used to inoculate synthetic medium (Lobos et al. 1992) in 100-ml Erlenmeyer flasks. The medium was modified and consisted of (grams per liter) K_2HPO_4 (4.36), KH_2PO_4 (1.7), $MgSO_4 \cdot 7H_2O$ (0.2), $MnSO_4$ (0.05), $FeSO_4 \cdot 7H_2O$ (0.01), $CaCl_2 \cdot 2H_2O$ (0.03), glucose (40), yeast extract (10), and distilled water (up to 1 l), pH 6.8. The cultivation was carried out at 28 °C with shaking at 160 rpm for 24 h. The precultures were transferred to fresh medium (1:1 ratio) and incubated for another 24 h. In 100-ml flasks, the synthetic medium with DBT (20 mg l^{-1}) or without the organotin (the control cultures) was inoculated with 20% of a homogeneous preculture. Incubation was conducted for 120 h in the above–mentioned conditions. Samples for analyses were collected after 0, 24, 48, 72, 96, and 120-h cultivations.

Batch cultivations

Batch cultivations of the *M. robertsii* strain were conducted in a 3.6-l bioreactor (Labfors 5; Iris 6 software; Infors AG, Switzerland) with a culture volume of 1 l. The fungal preculture, obtained as described above, was additionally transferred to the fresh medium (1:2 ratio) and incubated for a further 24 h. Finally, the homogeneous preculture was introduced into 800 ml of the synthetic medium with DBT (20 mg l^{-1}), either alone or in a mixture with one of the vitamins (C or E, both 20 mg l^{-1}) or without the tested compounds (the control culture). The applied concentrations of the vitamins did not affect *M. robertsii* growth. The fungal cultures were incubated for 72 h with controlled aeration (air flow 1 l min^{-1}), stirring (200–250 rpm), temperature (28 °C), and level of dissolved oxygen ($pO_2 \geq 20\%$). The quantities of O_2 in the introduced and exhaust gasses were measured with a gas analyzer (Infors AG, Switzerland). The pH of the medium was not regulated during the cultivation. In order to determine the fungal growth and DBT utilization, the samples were collected regularly: after 0, 3, 7, 12, 24, 48, and 72 h.

Fungal biomass estimation

Fungal mycelia were separated from culture media by filtration through Whatman#1 filter paper and drying at 105 °C to reach a constant weight. The maximum specific growth rate (μ_{max}) was calculated in accordance with the formula $\mu_{max} = [(\ln X_2 - \ln X_1) / (t_2 - t_1)]$, where X_2 is the biomass concentration at time t_2 and analogously for X_1. Based on the growth curves, logarithmic growth phases of the *M. robertsii* strain were indicated. Mycelium amounts required for the calculations were taken from 0 and 24-h incubation and from 3 and 7-h cultivation for flask and batch cultures, respectively.

Glucose content analysis

Glucose amounts in the supernatant were determined using an Agilent 1200 HPLC coupled with a QTRAP 3200 mass

spectrometer (AB Sciex), according to the Bernat et al. (2013) procedure.

Sample preparation and organotin determination

Acidified (pH 2) fungal biomass was suspended in methanol and homogenized with glass beads by ball milling (Retsch MM 400, Germany). After disruption, the samples were prepared according to the procedure by Bernat et al. (2013).

The analysis of butyltin intermediates

The cultures (20 ml) were transferred into Falcon tubes and centrifuged for 10 min at 10,000×*g*. The mycelium was suspended in methanol and homogenized using a mixer mill with glass beads for 5 min at 30 m s^{-1}. The extraction of the homogenate and supernatant mixture with ethyl acetate (1:1 *v*/*v*) was carried out twice. The organic layers were dehydrated with the use of anhydrous sodium sulfate and evaporated to dryness. The precipitate was dissolved in methanol.

Separation of butyltins was performed with the Agilent Technologies 1200 HPLC system equipped with a Phenomenex Aqua C18 125A column (50 mm × 2.0 mm × 5 μm) and maintained at 37 °C. The mobile phase consisted of water (A) and methanol (B), both supplemented with 2 mM ammonium formate and 0.2% formic acid. The run time was 9 min, and the solvent gradient was initiated at 60% B. After 1 min, the amount of B was increased to 100% over the following 2 min, and this was maintained for two additional minutes before returning to the initial solvent composition over the next 2 min, and this then being maintained for 2 min. The flow rate was 0.5 ml min^{-1} with an autosampler temperature of 10 °C and an injection volume of 10 μl, respectively.

To identify DBT derivatives in fungal samples by LC-MS/MS, an information-dependent acquisition (IDA) method was developed consisting of a precursor ion scan (PI) and an enhanced product ion (EPI) scan mode. IDA experiments were performed on a hybrid Q-Trap 3200 mass spectrometer (QTRAP; AB Sciex) connected to the HPLC system. The ion source conditions were set as follows: curtain gas (CUR) = 25, collision gas (CAD) = high, ionspray voltage (IS) = 5500, temperature (TEM) = 500, ion source gas 1 (GS1) = 40, and ion source gas 2 (GS2) = 50. Nitrogen was used as a nebulizer and an auxiliary gas. For the PI-EPI analysis, a PI scan of *m*/*z* 179 ($BuSnH_2^+$) was run in positive mode at a scan range from *m*/*z* 200 to *m*/*z* 650. The EPI scan was run in positive mode at a scan range for daughter ions from *m*/*z* 100 to *m*/*z* 700. Declustering potential (DP), entrance potential (EP), and collision energy (CE) were set to 25, 10, and 28, respectively.

Characterization of morphological modifications

In order to determine the morphology of the *M. robertsii* hyphae from the exponential growth phase, image analysis was used. Fungal pellet morphology was performed for 30 pellets from each culture using the software package Axiovision 4.4 (Carl Zeiss, Germany). According to the Casas López et al. (2005) method, a central compact core region and a peripheral "hairy" region of the fungal pellets were separated. Subsequently, values of the pellet and pellet core projected area (mm^2) were estimated.

Quantitative determination of malondialdehyde

Samples were prepared according to the procedure by Wei et al. (2010) with some modifications. After 7 h of batch cultivation, 10 ml of the culture was collected and filtrated. The mycelium was washed with distilled water and transferred into Eppendorf tubes containing 1 ml of cooled water and glass beads. The homogenization process with the use of FastPrep 24 (MP Biomedicals, USA) was performed three times for 20 s at 4 m s^{-1} with 2-min breaks for cooling samples on ice. Subsequently, samples were centrifuged without beads for 10 min at 4000×*g* at 4 °C. The upper layers were transferred into inserts of dark glass vials for LC-MS/MS analysis. According to the Csallany et al. (1984) procedure, the MDA standard for quantitative determinations was obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane.

Measurement of MDA was performed using an Agilent 1200 HPLC (Santa Clara CA, USA) system and a 3200 QTRAP mass spectrometer (AB Sciex, Framingham, MA, USA) with an ESI source. For reversed-phase chromatographic analysis, 10 μl of the sample was injected into a Phenomenex Aqua C18 125A column (50 mm × 2.0 mm × 5 μm). The mobile phase consisted of water (A) and methanol (B); 5 mM ammonium formate was also used in all solvents as an additive. The solvent gradient was initiated at 20% B; after 0.5 min, this was increased to 100% B over 1 min and maintained at 100% B for four additional minutes before returning to the initial solvent composition over 2 min. The run time was 7 min. The column temperature was maintained at 37 °C, and the flow rate was 600 μl min^{-1}. The instrumental settings were as follows: spray voltage −4500 V, curtain gas (CUR) 25, nebulizer gas (GS1) 55, turbo gas (GS2) 60, and ion source temperature of 500 °C. Data analysis was performed with Analyst™ v1.5.2 software (AB Sciex, Framingham, MA, USA). The monitored multiple reaction monitoring (MRM) pair for MDA was *m*/*z* 71–42.

Statistical analysis

The experiments were carried out with triplicate samples. The Student's *t* test and Spearman's correlation were performed

using Excel 2007 (Microsoft Corporation, USA). An average standard deviation (±SD) was calculated. Values were considered significant when $p \leq 0.05$.

Results and discussion

Growth kinetics and DBT biotransformation by the *M. robertsii* strain incubated in flasks

Based on the results of preliminary studies (data not shown), synthetic medium with an addition of yeast extract as a source of both organic nitrogen and various vitamins (Lee and Little 2015) was chosen for the *M. robertsii* strain cultivation. The growth kinetics and glucose assimilation by the fungal cells are shown in Fig. 1a. After 120-h incubation, the biomass amount was almost 16 g l^{-1}, while the glucose uptake was complete after 72 h of cultivation. In the presence of DBT (20 mg l^{-1}), an increase in the fungal dry weight of about 22% was observed (Fig. 1b). During the first 24 h of the experiment, glucose assimilation by those fungal cells exposed to the organotins was lower than in control cultures. At the same time, the most rapid decrease in DBT level was observed (Fig. 1b). No significant differences were noted in the substrate assimilation after 72 h of cultivation between the cultures, either with or without DBT. Due to the simultaneous utilization of glucose and the organotins, the cometabolic

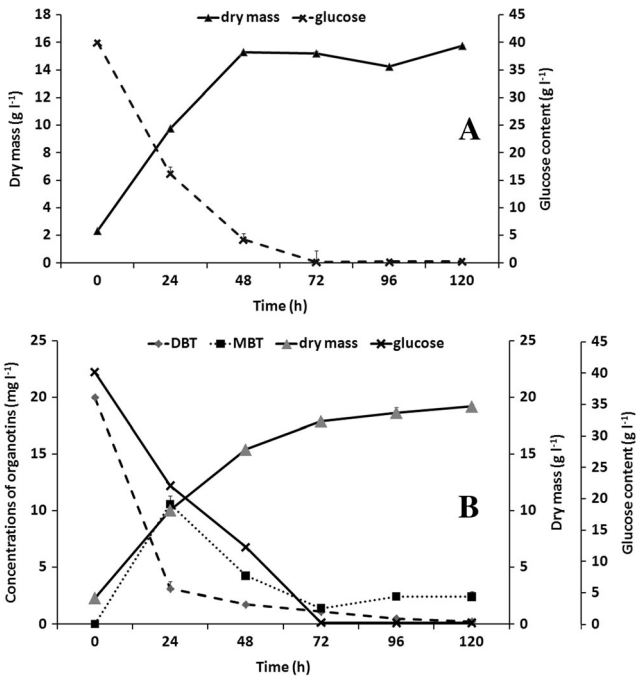


Fig. 1 Biomass synthesis and glucose assimilation by the *M. robertsii* strain during flask cultivation without (**a**) and with an addition of DBT (**b**) for 120 h in synthetic medium. Additionally, in **b**, the curves for DBT and MBT biodegradation by the examined fungus incubated in the above conditions are shown

character of the compound removal was indicated. The same phenomenon has previously been described for DBT biodegradation by *Cochliobolus lunatus* and TBT elimination by both *Cunninghamella elegans* and *M. robertsii* (Bernat and Długoński 2006; Bernat et al. 2013; Siewiera et al. 2015).

The ability of the *M. robertsii* strain to eliminate not only dibutyltin but also its derivative compound—monobutyltin (MBT)—was confirmed. The efficiency of DBT removal after 5-day incubation on modified synthetic medium was estimated as 98%. On the other hand, a fifth of the MBT remained in the culture (Fig. 1b).

Fungal growth and organotin utilization by the *M. robertsii* strain cultivated under intensive aeration conditions

Although hyphae are not as sensitive as tissues (Bilodeau et al. 2005), mechanical agitation in a bioreactor undoubtedly causes stress resulting in morphological modifications in microorganism cells (Boswell et al. 2003; Chamsartra et al. 2005). However, the benefits of bioprocesses carried out on a bioreactor scale, such as culture homogeneity and facilitated transfers of nutrients, respiration gasses, and metabolic products (Garcia-Ochoa and Gomez 2009), seem to be more important than damage caused by mechanical stress. One positive impact of intensive oxygenation on fungal growth and organotin biodegradation has previously been discovered (Bernat and Długoński 2006). Therefore, in the next stage of the study, an experiment was performed with the aid of a bioreactor. After 72 h of batch cultivation, the fungal biomass from the control culture was lower (about 30% in comparison with the flask cultures) (Fig. 2a). Maximum specific growth rates (μ_{max}) of the *M. robertsii* cells were also determined. The parameters for the fungus cultivated without DBT both in flask and batch cultures were 0.060 and 0.078 h^{-1}, respectively. The acceleration of fungal growth in the bioreactor was caused by the efficient system supply of O_2. Oxygen plays a key role in aerobic processes, especially in microorganism growth and metabolite production (Garcia-Ochoa and Gomez 2009). In our studies, the minimal level of dissolved oxygen was 20%. In the presence of DBT, pO_2 = 20% was achieved after 17 h of incubation, 5 h later than in the fungal culture without the organotin. The changes in the productivity of the fungal biomass exposed to DBT were not significant (Fig. 2b), despite an increase in the μ_{max} value up to 0.086 h^{-1}. A reverse dependency was observed in flask cultures supplemented with DBT. The maximum specific growth rates of the *M. robertsii* cells remained constant, despite the increase in the biomass amount compared to the control culture.

The rate of butyltin biotransformation by the fungal strain cultivated under intensive aeration conditions is presented in Fig. 2b. Although the final effectiveness of DBT elimination is comparable to the rate of the compound removal in flask

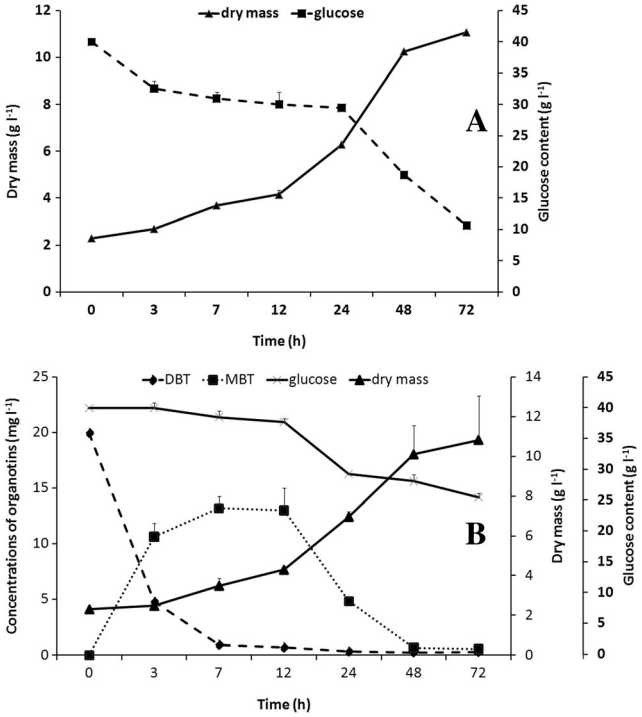


Fig. 2 Biomass synthesis and glucose assimilation by the *M. robertsii* strain during batch cultivation without (**a**) and with an addition of DBT (**b**) for 72 h in synthetic medium. Additionally, in **b**, the curves for DBT and MBT biodegradation by the examined fungus incubated in the above conditions are shown

cultures, a supply of oxygen resulted in a 10-fold acceleration of dibutyltin removal. A similar effect was described by Bernat and Długoński (2006) for TBT utilization by an additional oxygen supply to a growing *C. elegans* strain. The influence of the level of culture oxygenation on the progress of DBT debutylation by the *M. robertsii* is crucial. Moreover, complete metabolism of MBT by the fungus was possible only in the bioreactor culture. The positive effect of batch conditions on fungal growth and the organotin utilization by the microorganism is connected with a facilitated exchange of nutrients and respiratory gasses between the biomass and growth medium (Garcia-Ochoa and Gomez 2009).

In contrast to TBT, a compound biodegraded by bacteria (e.g., *Moraxella osloensis* (Yáñez et al. 2015), *Pseudomonas* sp. (Bernat et al. 2014), and *Enterobacter cloacae* (Sakultantimetha et al. 2011)), fungi (e.g., *C. elegans* (Bernat and Długoński 2006), *Cunninghamella echinulata* (Soboń et al. 2016), and *Coniophora puteana* (White et al. 1999)), the alga *Chlorella vulgaris* (Tsang et al. 1999), and crab *Thalamita crenata* (Chen et al. 2016), DBT and MBT have been described as being degraded by only a few microbial strains. Among the fungi, only *C. lunatus* has been mentioned as a strain efficiently degrading dibutyltin and monobutyltin. During incubation on Sabouraud medium, 92% of the initial DBT concentration (10 mg l^{-1}) and approximately 70% of formed MBT were removed from the fungal culture after 24 and 168 h of cultivation, respectively (Bernat et al. 2013). On the other hand, the yield of DBT (20 mg l^{-1}) transformation by *Streptomyces* sp. was equal to 90% after 1-day cultivation on synthetic medium with a 2-fold higher amount of MBT in comparison to the *C. lunatus* strain. Moreover, within 7 days, almost 90% of produced MBT was removed from the bacterial culture (Bernat and Długoński 2009). The most significant advantage of our results, compared to those obtained in other published studies, is the reduction of the time required for the bioremediation process. During the first 24 h of cultivation, differences were slight, because all strains achieved a high (90%) efficiency of DBT elimination. However, MBT was still detected as a major by-product (until day 7), in both *Streptomyces* sp. (Bernat and Długoński 2009) and *C. lunatus* (Bernat et al. 2013) cultures, while as early as after 2 days of the *M. robertsii* cultivation, both butyltins were completely eliminated.

Unfortunately, no literature data concerning the comparison of dibutyltin elimination efficiency in flask and batch conditions are available. According to Moscoso et al. (2012), benzo[a]anthracene (BaA), one of the polycyclic aromatic hydrocarbons, is biodegraded by *Staphylococcus warneri* and *Bacillus pumilus* strains more efficiently in batch experiments than in flask conditions. After 2-day cultivation of the bacterial consortium on minimal medium with BaA (100 μM), 8 and 75% of initial compound concentration were removed in flask and bioreactor, respectively (Moscoso et al. 2012). On the other hand, in further studies on BaA biotransformation in the same conditions by the bacterial strain *Pseudomonas stutzeri*, a reverse dependency was observed. The efficiency of BaA utilization was equal to 94% in flasks and 81% in the bioreactor after 7 days of bacterial incubation (Moscoso et al. 2015). The published results indicate the importance of the metabolic abilities of microorganisms.

The influence of vitamins C and E on DBT removal by the examined fungus

In our previous paper (Siewiera et al. 2015), an increase in the efficiency (about 14%) of TBT elimination by the *M. robertsii* strain cultivated in the presence of 17β-estradiol was confirmed. DBT, as well as TBT, promotes production of reactive oxygen species (ROS) (Chantong et al. 2014), which can inflict direct damage on cell components. In order to increase fungal tolerance to oxidative stress induced by the presence of DBT, vitamins C and E were applied. The influence of ascorbic acid (20 mg l^{-1}) or α-tocopherol (20 mg l^{-1}) on fungal biomass synthesis and organotin biodegradation is demonstrated in Fig. 3. In the presence of vitamins, the assimilation of glucose was about 50% higher for vitamin C (Fig. 3a) and about 68% higher for vitamin E (Fig. 3b) compared to culture with DBT alone. Moreover, biomass production increased about 34 and 58%, respectively. After supplementation of

the growth medium with the tested vitamins, the values of μ_{max} were decreased compared to the control cultures and remained at the same level (~0.063 h^{-1}). In the presence of antioxidants, faster oxygen uptake from growth medium by fungal cells was observed in comparison to the culture with DBT alone. pO_2 = 20% was achieved after 14 and 9 h for vitamins C and E, respectively.

Vitamin supplementation played a greater role in MBT than in DBT elimination. The addition of ascorbic acid to the fungal culture led to a 3-fold acceleration of the rate of monobutyltin decomposition. The importance of vitamin C in heavy metal removal by the filamentous fungi was noted. According to Słaba et al. (2013), the addition of 1 mM ascorbic acid enhanced the uptake of lead and copper into the cell walls of *Paecilomyces marquandi*. In this study, in contrast to vitamin C, the negative impact of α-tocopherol on the MBT elimination was observed (about 40% during the first 7 h of incubation). The involvement of cytochrome P450 enzymes in vitamin E metabolism has been proved (Sontag and Parker 2002). Thus potentially, α-tocopherol could be a competitive inhibitor of CYP450, decreasing the rate of organotin debutylation. However, the involvement of the enzymatic complex in butyltin biodegradation by the *M. robertsii* strain has not yet been examined. On the other hand, the importance of vitamin E was confirmed in the biodegradation of polychlorobiphenyls as environmental pollutants. According to Ponce et al. (2011), α-tocopherol (1.6 μM) improved degradation of biphenyl and 4-chlorobiphenyl (4-CB) by the bacterial strain *Burkholderia xenovorans* cultivated in an aqueous solution. In the presence of the antioxidant, an increase in the rate of biphenyl degradation (40 μM) from 0.8 ± 0.1 to 1.32 ± 0.2 μM min^{-1} was observed. However, the efficiency of 4-CB (1 mM) elimination after 24-h incubation reached 40% in the absence of α-tocopherol and 100% in the presence of the antioxidant (Ponce et al. 2011).

In our experiments, butyltins were identified separately in the fungal biomass and the supernatant (data not shown). On this basis, the high affinity of DBT with the biomass was discovered. During the first hours of the study, as much as 69% of the initial concentration of the compound was attached to the *M. robertsii* mycelium. On the other hand, the location of the monobutyl derivative was dependent on the oxygen supply and incubation time. In the fungal cultures conducted in the bioreactor, continuous MBT excretion to the growth medium was detected. However, in the flask cultivation of the fungus, MBT was mostly transported to the substrate only for 24 h. Afterwards, accumulation of the monobutyl compound was observed in the hyphae.

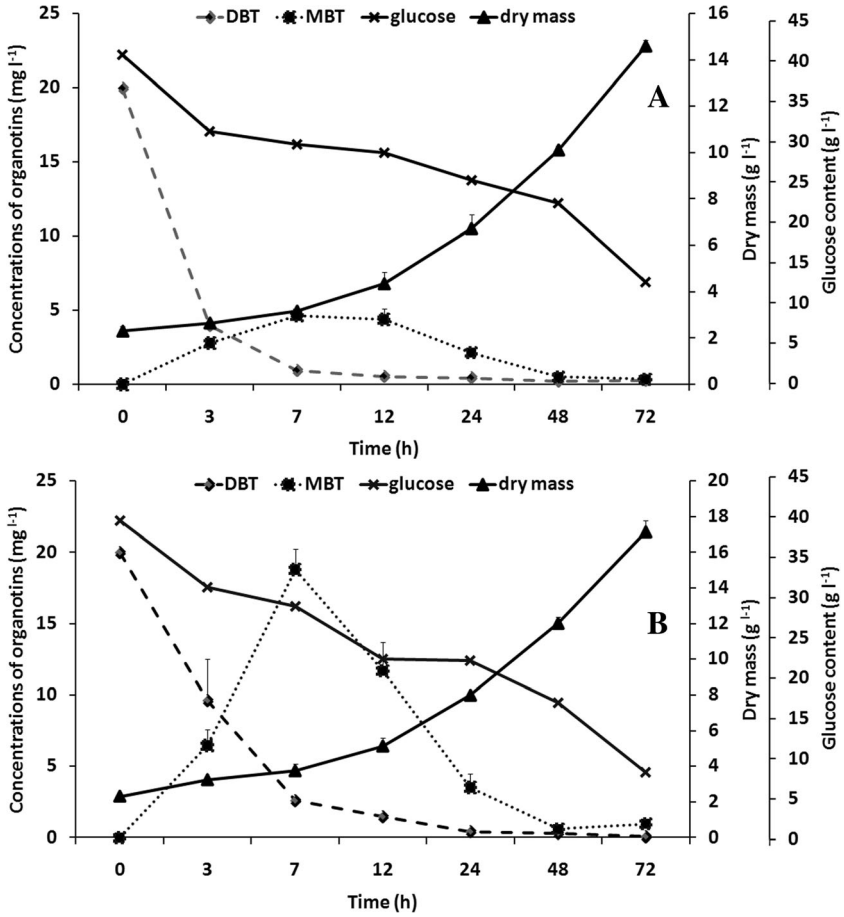


Fig. 3 Growth curve, glucose assimilation, and butyltin utilization by the examined fungus incubated for 72 h in a bioreactor on synthetic medium supplemented with vitamin C (**a**) or vitamin E (**b**)

Table 1 Kinetic parameters of *M. robertsii* growth and butyltin removal by the fungus cultivated on flask and bioreactor scale

Scale	Culture	Biomass parameters			Butyltin degradation parameters				
		X_{max} (g l^{-1})	μ_{max} (h^{-1})	R^2	Max DBT removal (%)	R^2	Max MBT concentration (mg l^{-1})	Max MBT removal (%)	R^2
Flasks	Control	15.75 ± 0.22	0.060	0.997	–	–	–	–	–
	DBT	19.20 ± 0.15	0.061	1.000	98.80 ± 0.15	0.997	10.57 ± 0.77	77.20 ± 2.10	0.987
	DBT + vitamin C	21.18 ± 0.87	0.064	0.999	94.65 ± 0.16	0.996	16.00 ± 0.17	67.25 ± 2.65	0.995
	DBT + vitamin E	22.00 ± 0.98	0.069	0.999	96.75 ± 0.21	0.986	11.07 ± 0.50	69.56 ± 2.17	0.989
Bioreactor	Control	11.07 ± 0.59	0.078	0.988	–	–	–	–	–
	DBT	10.83 ± 2.30	0.086	0.995	98.60 ± 0.10	0.999	13.20 ± 1.08	95.90 ± 0.90	0.984
	DBT + vitamin C	14.58 ± 0.28	0.063	1.000	98.60 ± 0.05	0.998	4.64 ± 0.20	92.46 ± 0.22	0.993
	DBT + vitamin E	17.15 ± 0.64	0.063	0.999	99.65 ± 0.20	0.996	18.78 ± 1.43	94.94 ± 0.05	0.877

R^2 coefficients refer to polynomial regression ($n = 4$)

A summary of the most important kinetic parameters of the *M. robertsii* growth and its efficiency in butyltin biodegradation is presented in Table 1, i.e., maximum values of biomass (X_{max}), specific growth rate (μ_{max}), DBT removal (%), MBT concentrations, and MBT removal (%). The regression coefficients (R^2) were higher than 0.98 in all cases, except for MBT removal (%) in the batch culture of the *M. robertsii* supplemented with DBT and vitamin E, where R^2 was equal to 0.877. The values of R^2 suggested at least good or very good fitting of the model, which confirmed its suitability for use in biodegradation processes.

Intensive aeration and the presence of vitamin C and glucose in medium ensured optimal conditions for fungal growth and butyltin biodegradation. The use of the nutrients is not cost-effective. However, the application of agricultural wastes as a rich source of carbon and energy for microorganisms (Singh and Nain 2014) is the first step in the reduction of costs with a simultaneous enhancement of biodegradation efficiency. Cometabolism could be a novel way of facilitating the removal of not only butyltins but also other pollutants.

A qualitative analysis of butyltin metabolites

The extracts of the *M. robertsii* cultures from the exponential growth phase were chosen for the studies because of the rapid decrease in the DBT level at that time. The metabolites formed during the organotin biotransformation were qualitatively analyzed using gas and liquid chromatography coupled with mass spectrometry.

MBT, the primary by-product of DBT biotransformation by the *M. robertsii* cells, was determined with GC-MS/MS. HPLC-MS/MS chromatograms revealed the presence of DBT and MBT and the formation of its metabolite at retention time 4.15 (Fig. 4). The retention times for DBT and MBT were 4.0 and 4.6, respectively. The spectrum of the analyte showed ions 197 and 179, and the appearance of the $[SnH_3]^+$ ion at *m*/*z* 123

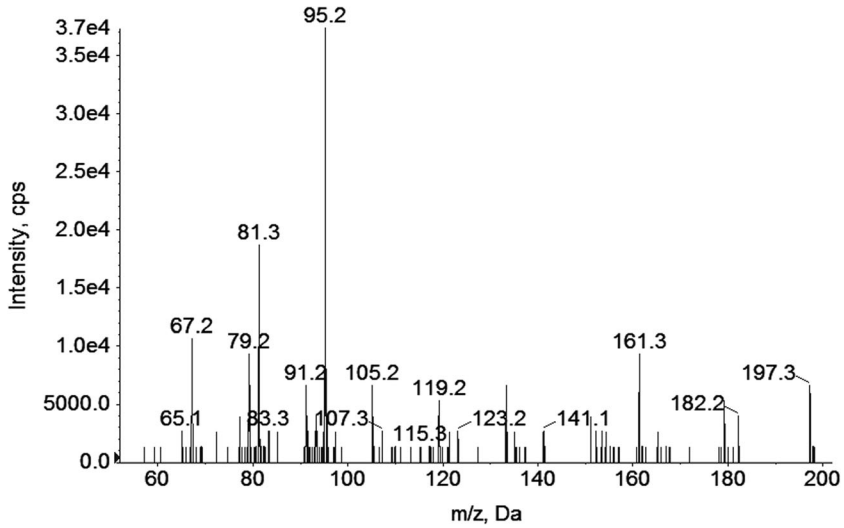


Fig. 4 Mass spectrum showing the main ion group of $OHBuSnH_2$ acquired on the first day of the *M. robertsii* incubation in the presence of DBT

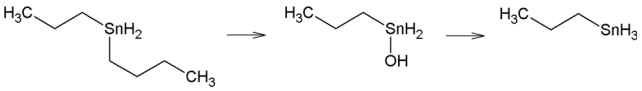


Fig. 5 A proposed pathway of DBT biodegradation by the *M. robertsii* strain

and all of these exhibited a typical tin isotopic pattern. The difference in masses at 18 Da between 197 and 179 $[BuSnH_2]^+$ indicated that the obtained compound could be the hydroxylated derivative of MBT—$OHBuSnH_2$. The hydroxylated derivative of DBT was determined in samples from the first to the third day of the *M. robertsii* incubation. Therefore, considering the obtained results, it seems that the presence of $OHBuSnH_2$ was probably associated with DBT debutylation by the fungal strain (Fig. 5). A similar mechanism was described by Suzuki et al. (1992) and Matsuda et al. (1993) for TBT or DBT, which were metabolized either in vitro or in vivo by rat or fish liver microsome enzyme systems to MBT, DBT, and/or hydroxylated products at the third and fourth positions of DBT or TBT.

Fungal cell morphology

Based on macroscopic observations during the experiments, the morphological modifications of the *M. robertsii* pellets (from the exponential growth phase) cultivated under various levels of oxygen supply were noted (Supplementary Fig. 1). Primarily, the shape and size of fungal morphological forms were examined. In the conditions of intensified oxygenation, the fungus grew as a non-spherical, dispersed form with long hyphae, while the fungus cultivated in flasks formed spherical, densely packed pellets with single hyphae outside the peripheral region. According to Papagianni's (2004) hypothesis, hyphae aggregation and growth as pellets occur as a result of insufficient oxygen levels in the growth medium.

In order to characterize the *M. robertsii* cell morphology, the ratio between the pellet core and the projected area of the whole pellet was calculated (Table 2). A high ratio implied that the pellet had a "smooth" morphology while lower values of the ratio suggested a hairy morphology (Różalska et al. 2014). Densely packed fungal pellets with a smooth morphology were noted in the flask cultures, unlike the pellets from batch cultivation which had a large, actively growing hairy zone. Due to the exposure to DBT and supplementation with vitamins C or E, an increase in the hairy zone was observed. Moreover, there was a positive correlation ($r = 0.803$) between the degree of hyphae compaction and the efficiency of DBT biodegradation. Papagianni (2004) also reported that hyphae surrounding the pellet core are characterized by high activity. Taking into account the results obtained for the *M. robertsii* during butyltin and 4-n-nonylphenol biotransformation (Różalska et al. 2014), hyphae compaction seems to be a common feature of the xenobiotic metabolism by fungi from the genus *Metarhizium*.

Analysis of lipid peroxidation products

Dibutyltin contributes indirectly to lipid oxidation by inducing reactive oxygen species (Chantong et al. 2014). MDA is the most mutagenic among secondary products formed during the process of lipid peroxidation (Ayala et al. 2014). In this study, quantitative analyses of MDA, one of the most popular and reliable markers of oxidative stress, were conducted with the use of liquid chromatography coupled with mass spectrometry. Extracts of the *M. robertsii* batch cultures exposed to DBT (with or without the antioxidants) from the exponential growth phase were examined. The highest level of MDA, i.e., 151 μM l^{-1}, was determined for those fungal cells supplemented with the organotin alone. In the presence of ascorbic acid and α-tocopherol, a decrease in the MDA amount was observed, about 45 and 2%, respectively. The same effect was characterized by Lu et al. (2007), who investigated pancreatic damage in rats induced by the presence of dibutyltin dichloride. The action of AA, determined as soothing, was supported by the measurement of reduced MDA levels after the antioxidant treatment (Lu et al. 2007). In order to detect MDA in the organs of rabbits exposed to stannous chloride (the ROS inducer), a reaction with thiobarbituric acid was applied. Researchers have reported that treatment with ascorbic acid causes a decrease in the TBARS levels in all tested organs (El-Demerdash et al. 2005). These findings are consistent with our results, despite the use of different test organisms.

Undoubtedly, the reduction of MDA quantity in fungal membranes damaged by DBT was a result of the efficient process of free radical scavenging by ascorbic acid. Consequently, restriction of oxidative stress was the main

Table 2 The ratio between the pellet core and the projected area of the whole pellet of the *M. robertsii* strain cultivated in flasks and in a bioreactor on synthetic medium in the absence of DBT, in the presence of DBT alone, or in a mixture with one of the vitamins

	Control	DBT	DBT + vitamin C	DBT + vitamin E
Cultures in flasks	0.732 ± 0.076	0.950 ± 0.029	n.t.	n.t.
Cultures in bioreactor	0.430 ± 0.020	0.562 ± 0.006	0.629 ± 0.048	0.536 ± 0.090

n.t. not tested

reason for the improved yield of the process of DBT biodegradation by the examined fungus.

Conclusions

This report is the first to show that the *M. robertsii* can degrade both DBT and MBT with high levels of efficiency. An additional supply of oxygen led to a hairy morphology for the *M. robertsii* hyphae instead of densely interwoven pellets. A facilitated exchange of nutrients and respiratory gasses between the biomass and growth medium contributed to intensive fungal growth and finally improved butyltin biodegradation. Due to simultaneous utilization of glucose and the organotins, the cometabolic character of the described process is suggested. Moreover, supplementation of the growth medium with ascorbic acid protects fungal cells exposed to DBT from oxidative stress.

Acknowledgements This study was supported by the National Science Centre, Poland (Project No. 2015/19/N/NZ9/00459).

References

Albalat A, Potrykus J, Pempkowiak J, Porte C (2002) Assessment of organotin pollution along the Polish coast (Baltic Sea) by using mussels and fish as sentinel organisms. Chemosphere 47:165–171

Ayala A, Muñoz MF, Argüelles A (2014) Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative Med Cell Longev. doi:10.1155/2014/360438

Bernat P, Długoński J (2006) Acceleration of tributyltin chloride (TBT) degradation in liquid cultures of the filamentous fungus *Cunninghamella elegans*. Chemosphere 62(1):3–8

Bernat P, Długoński J (2009) Isolation of *Streptomyces* sp. strain capable of butyltin compounds degradation with high efficiency. J Hazard Mater 171:660–664

Bernat P, Szewczyk R, Krupiński M, Długoński J (2013) Butyltins degradation by *Cunninghamella elegans* and *Cochliobolus lunatus* co-culture. J Hazard Mater 246-247:277–282

Bernat P, Siewiera P, Soboń A, Długoński J (2014) Phospholipids and protein adaptation of *Pseudomonas* sp. to the xenoestrogen tributyltin chloride (TBT). World J Microbiol Biotechnol 30:2343–2350

Bilodeau K, Couet F, Boccafoschi F, Mantovani D (2005) Design of a perfusion bioreactor specific to the regeneration of vascular tissues under mechanical stresses. Artif Organs 29:906–912

Boswell CD, Nienow AW, Gill NK, Kocharunchitt S, Hewitt CJ (2003) The impact of fluid mechanical stress on *Saccharomyces cerevisiae* cells during continuous cultivation in an agitated, aerated bioreactor; its implication for mixing in the brewing process and aerobic fermentations. Food Bioprod Process 81:23–32

Bouchard N, Pelletier É, Fournier M (1999) Effects of butyltin compounds on phagocytic activity of hemocytes from three marine bivalves. Environ Toxicol Chem 18(3):519–522

Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Rodríguez Porcel EM, Chisti Y (2005) Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. J Biotechnol 116(1):61–77

Chamsartra S, Hewitt CJ, Nienow AW (2005) The impact of fluid mechanical stress on *Corynebacterium glutamicum* during continuous cultivation in an agitated bioreactor. Biotechnol Lett 27:693–700

Chantong B, Kratschmar DV, Lister A, Odermatt A (2014) Dibutyltin promotes oxidative stress and increases inflammatory mediators in BV-2 microglia cells. Toxicol Lett 230(2):177–187

Chen CW, Chen CF, Ju YR, Dong CD (2016) Assessment of the bioaccumulation and biodegradation of butyltin compounds by *Thalamita crenata* in Kaohsiung Harbor, Taiwan. Int Biodeterior Biodegradation 113:97–104

Csallany AS, Guan MD, Manwaring JD, Addis PB (1984) Free malonaldehyde determination in tissues by high-performance liquid chromatography. Anal Biochem 142:277–283

El-Demerdash FM, Yousef MI, Zoheir MA (2005) Stannous chloride induces alterations in enzyme activities, lipid peroxidation and histopathology in male rabbit: antioxidant role of vitamin C. Food Chem Toxicol 43(12):1743–1752

Frouin H, Lebeuf M, Saint-Louis R, Hammill M, Pelletier E, Fournier M (2008) Toxic effects of tributyltin and its metabolites on harbour seal (*Phocavitulina*) immune cells in vitro. Aquat Toxicol 90(3):243–251

Garcia-Ochoa F, Gomez E (2009) Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnol Adv 27:153–176

Jenkins SM, Ehman K, Barone S Jr (2004) Structure–activity comparison of organotin species: dibutyltin is a developmental neurotoxicant in vitro and in vivo. Behav Brain Res 151:1–12

Lee JS, Little BJ (2015) Technical note: electrochemical and chemical complications resulting from yeast extract addition to stimulate microbial growth. Corrosion 71(12):1434–1440

Li Y, Schellhorn HE (2007) New developments and novel therapeutic perspectives for vitamin C. J Nutr 137(10):2171–2184

Lobos JH, Leib TK, Su TM (1992) Biodegradation of bisphenol A and other bisphenols by a gram-negative aerobic bacterium. Appl Environ Microbiol 58:1823–1831

Lu XL, Song YH, Fu YB, Si JM, Qian KD (2007) Ascorbic acid alleviates pancreatic damage induced by dibutyltin dichloride (DBTC) in rats. Yonsei Med J 48(6):1028–1034

Matsuda R, Suzuki T, Saito Y (1993) Metabolism of tri-n-butyltin chloride in male rats. J Agric Food Chem 41:489–495

Moscoso F, Teijiz I, Deive FJ, Sanromán MA (2012) Efficient PAHs biodegradation by a bacterial consortium at flask and bioreactor scale. Bioresour Technol 119:270–276

Moscoso F, Deive FJ, Longo MA, Sanromán MA (2015) Insights into polyaromatic hydrocarbon biodegradation by *Pseudomonas stutzeri* CECT 930: operation at bioreactor scale and metabolic pathways. Int J Environ Sci Technol 12:1243–1252

Moser VC, McGee JK, Ehman KD (2009) Concentration and persistence of tin in rat brain and blood following dibutyltin exposure during development. J Toxicol Environ Health A 72(1):47–52

Nemanič TM, Milačič R, Ščančar J (2009) A survey of organotin compounds in the Northern Adriatic Sea. Water Air Soil Pollut 196:211–224

Nesci S, Ventrella V, Trombetti F, Pirini M, Borgatti AR, Pagliarani A (2011) Tributyltin (TBT) and dibutyltin (DBT) differently inhibit the mitochondria Mg-ATPase activity in mussel digestive gland. Toxicol in Vitro 25:117–124

Nielsen JB, Strand J (2002) Butyltin compounds in human liver. Environ Res 88:129–133

Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 22(3):189–259

Ponce BL, Latorre VK, González M, Seeger M (2011) Antioxidant compounds improved PCB-degradation by *Burkholderia xenovorans* strain LB400. Enzym Microb Technol 49(6–7):509–516

Różalska S, Glińska S, Długoński J (2014) *Metarhizium robertsii* morphological flexibility during nonylphenol removal. Int Biodeterior Biodegradation 95:285–293

Sakultantimetha A, Keenan HE, Beattie TK, Bangkedphol S, Cavoura O (2011) Bioremediation of tributyltin contaminated sediment: degradation enhancement and improvement of bioavailability to promote treatment processes. Chemosphere 83:680–686

Siewiera P, Bernat P, Różalska S, Długoński J (2015) Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*. Int Biodeterior Biodegradation 104:258–263

Singh S, Nain L (2014) Microorganisms in the conversion of agricultural wastes to compost. Proc Indian Natn Sci Acad 80:473–481

Słaba M, Gajewska E, Bernat P, Fornalska M, Długoński J (2013) Adaptive alterations in the fatty acids composition under induced oxidative stress in heavy metal-tolerant filamentous fungus *Paecilomyces marquandii* cultured in ascorbic acid presence. Environ Sci Pollut R 20:3423–3434

Soboń A, Szewczyk R, Długoński J (2016) Tributyltin (TBT) biodegradation induces oxidative stress of *Cunninghamella echinulata*. Int Biodeterior Biodegradation 107:92–101

Sontag TJ, Parker RS (2002) Cytochrome P450 ω-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. J Biol Chem 277(28):25290–25296

Suzuki T, Matsuda R, Saito Y (1992) Molecular species of tri-n-butyltin compounds in marine products. J Agric Food Chem 40(8):1437–1443

Tsang CK, Lau PS, Tam NFY, Wong YS (1999) Biodegradation capacity of tributyltin by two *Chlorella* species. Environ Pollut 105:289–297

Wei R, Li G, Seymour AB (2010) High-throughput and multiplexed LC/MS/MRM method for targeted metabolomics. Anal Chem 82:5527–5533

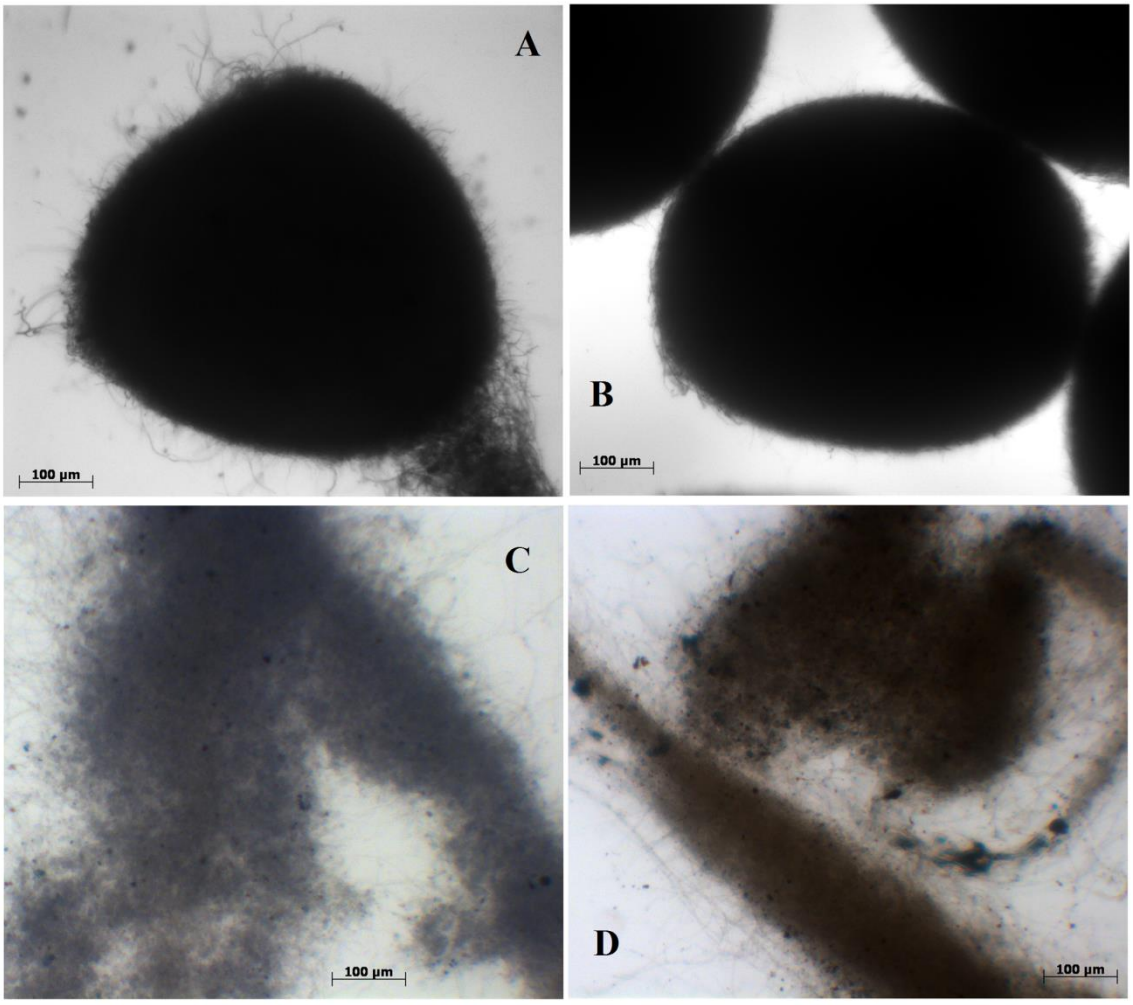
Whalen MM, Loganathan BG, Kannan K (1999) Immunotoxicity of environmentally relevant concentrations of butyltins on human natural killer cells in vitro. Environ Res 81:108–116

White JS, Tobin JM, Cooney JJ (1999) Organotin compounds and their interactions with microorganisms. Can J Microbiol 45:541–554

Yáñez J, Riffo P, Santander P, Mansilla HD, Mondaca MA, Campos V, Amarasiriwardena D (2015) Biodegradation of tributyltin (TBT) by extremophile bacteria from Atacama Desert and speciation of tin by-products. Bull Environ Contam Toxicol 95:126–130

Supplementary

Fig.1. Confocal micrographs of mycelium structures from flask (A, B) and bioreactor cultures (C, D) conducted on synthetic medium supplemented with DBT (B, D) or without the compound (A, C). Microscope magnification was 10x. The scale bar represents 100 µm.



Realizację drugiego celu pracy doktorskiej kontynuowano poddając analizie wpływ dwóch estrogenów naturalnych – estronu i 17β-estradiolu – na efektywność biodegradacji DBT przez grzyb strzępkowy *M. roberstii*. W trakcie pierwszych 24 godz. inkubacji, początkowe stężenie DBT (20 mg l^{-1}) zostało zredukowane do 3,1 mg l^{-1}, z jednoczesnym wytworzeniem MBT na poziomie 10,5 mg l^{-1}. W obecności E1/E2, pozostałości DBT były niemalże 2-krotnie wyższe niż w hodowlach bez dodatku estrogenów naturalnych. Negatywne oddziaływanie zarówno estronu jak i 17β-estradiolu na efektywność usuwania DBT przez szczep grzybowy IM 6519 zostało wyjaśnione podczas wykonywania eksperymentów z udziałem proadifenu (SKF, 1 mM) w roli niespecyficznego inhibitora cytochromu P450 (CYP450). Zaplanowane analizy miały głównie na celu zweryfikowanie udziału grzybowych monooksygenaz w procesie biodegradacji butylowych związków cyny. W obecności SKF, eliminacja DBT nie uległa całkowitemu zahamowaniu, jednakże pozostałości związku macierzystego były 4-krotnie wyższe niż w hodowlach bez dodatku inhibitora CYP450. Ponadto, brak obecności hydroksylowanej monobutylocyny w próbach zawierających jednocześnie DBT i SKF potwierdziły ostatecznie udział kompleksu enzymatycznego CYP450 w procesie biotransformacji DBT. Z kolei wyższe stężenia zarówno E1 jak i E2 odnotowane w hodowlach z dodatkiem SKF zweryfikowały pozytywnie udział CYP450 w rozkładzie tych związków wskazując na inhibicję kompetycyjną jako przyczynę negatywnego oddziaływania estrogenów naturalnych na efektywność usuwania DBT. Istnieją doniesienia literaturowe na temat zaangażowania enzymów ludzkiego cytochromu P450 w metabolizm estrogenów naturalnych (Tsuchiya i in., 2005), jednakże brakuje danych odnoszących się do monooksygenaz grzybowych.

Pomimo udowodnionej nadprodukcji reaktywnych form tlenu (RFT) w obecności DBT (Chantong i in., 2014; Gennari i in., 2000), prace na temat rodzajów RFT i ich relacji ilościowych nadal nie są dostępne. Podjęto ten problem podczas realizacji niniejszej pracy. Przeprowadzono detekcję *in situ* anionorodnika ponadtlenkowego ($O_2^{\bullet-}$), nadtlenku wodoru (H_2O_2) i tlenku azotu ($NO^{\bullet}$) w grzybni *M. robertsii* poddanej ekspozycji na działanie dibutylocyny i oszacowano ich poziomy na odpowiednio 66%, 4% i 28% po 24-godzinnej inkubacji. Co więcej, w trakcie dodatkowych analiz udowodniono, iż to nie DBT, lecz MBT indukuje powstawanie RFT i reaktywnych form azotu (RFA). Jak dotąd, tylko Ferreira i in. (2013) wskazali na MBT jako silniejszego niż TBT induktora RFT w komórkach neuroblastomy.

Aplikacja zarówno estronu jak i 17β-estradiolu ograniczyła stres oksydacyjny i nitrozowy poprzez skuteczne usuwanie RFT i RFA w grzybni szczepu IM 6519, których poziom zmniejszył się od 1,5 do 21 razy w zależności od użytego estrogenu naturalnego. Badania wykonane nad obroną enzymatyczną szczepu IM 6519 przed reaktywnymi formami tlenu indukowanymi obecnością MBT wykluczyły udział katalazy i dysmutazy ponadtlenkowej w usuwaniu H_2O_2 i $O_2^{\bullet-}$, podkreślając znaczenie E1 i E2 w łagodzeniu skutków stresu oksydacyjnego, a jednocześnie wskazując na fazę wodną jako obszar powstawania badanych RFT.

Efekty wypracowane podczas analizy znaczenia estronu i 17β-estradiolu dla wydajności biodegradacji DBT przez szczep grzybowy IM 6519 opublikowano w kolejnej pracy autorskiej „Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin" (Chemosphere, IF = 4,208, 35 pkt MNiSW).

Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin

[Chemosphere (2017), 185:96-104]

Chemosphere 185 (2017) 96–104

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin

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HIGHLIGHTS

- Cytochrome P450 enzymes participate in the biotransformation of DBT and estrogens.
- MBT (not DBT) induces the formation of $O_2^{\bullet -}$, H_2O_2, and $NO^{\bullet}$ in *M. robertsii* mycelium.
- SOD and CAT of the fungal cells are not involved in ROS detoxification.
- E1 and E2 are efficient scavengers of nitrogen and oxygen reactive species.

ARTICLE INFO

Article history:
Received 11 April 2017
Received in revised form
20 June 2017
Accepted 29 June 2017
Available online 30 June 2017

Handling Editor: A. Gies

Keywords:
Metarhizium robertsii
Monobutyltin
Natural estrogens
Oxygen and nitrogen reactive species
Antioxidant enzymes
Cytochrome P450 monooxygenases

ABSTRACT

Dibutyltin (DBT) is a global pollutant characterized by pro-oxidative properties. The fungal strain *Metarhizium robertsii* can eliminate high levels of DBT efficiently. In this study, induction of oxidative stress as well as its alleviation through the application of natural estrogens during the elimination of DBT by *M. robertsii* were evaluated. During the first 24 h of incubation, the initial concentration of DBT (20 mg l^{-1}) was reduced to 3.1 mg l^{-1}, with simultaneous formation of a major byproduct – monobutyltin (MBT). In the presence of estrone (E1) or 17β-estradiol (E2), the amounts of dibutyltin residues in the fungal cultures were found to be approximately 2-fold higher compared to cultures without estrogens, which was associated with the simultaneous utilization of the compounds by cytochrome P450 enzymes. On the other hand, MBT levels were approximately 2.5 times lower in the fungal cultures with the addition of one of the estrogens. MBT (not DBT) promotes the generation of $O_2^{\bullet -}$, H_2O_2, and $NO^{\bullet}$ at levels 65.89 ± 18.08, 4.04 ± 3.62, and 27.92 ± 1.95, respectively. Superoxide dismutase and catalase activities did not show any response of the *M. robertsii* strain against the overproduction of superoxide anion and hydrogen peroxide. Application of E1 as well as E2 ensured non-enzymatic defense against nitrosative and oxidative stress through scavenging of nitrogen and oxygen reactive species, and limited their levels from 1.5-fold to 21-fold, depending on the used estrogen.

1. Introduction

Dibutyltin (DBT) is one of the organometallic chemicals used in the industry, primarily as a thermal stabilizer of polyvinyl chloride materials (Okoro et al., 2011). Because widespread applications, DBT has become a global pollutant that has been detected at levels from nanogram to microgram of tin per liter of seawater or per gram of dry weight of sediments in Europe (Scrimshaw et al., 2005; Díez et al., 2005, 2006), Asia and Oceania (Bhosle et al., 2006; Harino et al., 2007), and North America (Landmeyer et al., 2004).

A major byproduct of DBT biotransformation is monobutyltin (MBT), which also constitutes contamination of environmental matrices (Okoro et al., 2011). It can remain in the environment for a longer period than the parent compound because of its stronger sorption to solid particles, especially in decreased pH and salinity (Dai et al., 2003). According to the report published by World Health Organization (Dobson et al., 2006), only limited data on toxicity and possible health effects of MBT are available.

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http://dx.doi.org/10.1016/j.chemosphere.2017.06.130
0045-6535/

Both DBT and MBT tend to bioaccumulate through the food chain, particularly in mussels (Harino et al., 2007), gastropods (Gomez-Ariza et al., 2006), fishes, oysters (Bhosle et al., 2004), and crabs (Chen et al., 2016). The consumption of contaminated seafoods has resulted in measurable levels of the butyltins in human blood and liver samples (Whalen et al., 1999; Nielsen and Strand, 2002).

Due to various mechanisms of toxicity such as cyto-, immuno-, hepato-, terato-, and genotoxic activity of DBT (Farr et al., 2001; Ueno et al., 2003; Gumy et al., 2008; Ferreira et al., 2013) and unrecognized effects of MBT activity, the presence of these compounds in the environment carries the risk for all organisms. The biodegradation process could be used for the reduction of environmental levels of butyltins. Moreover, application of antioxidants seems to be the proper way to increase the fungal ability for elimination of organotins (Siewiera et al., 2015, 2017). Although overproduction of reactive oxygen species (ROS) in the presence of DBT has been reported (Gennari et al., 2000; Chantong et al., 2014), the knowledge about the types of intracellular ROS induced by the compound is still limited. The data that have been published so far are concerned only about the measurement of total amounts of ROS (Gennari et al., 2000; Ferreira et al., 2013; Chantong et al., 2014), while no information about individual chemical species is available.

Reactive oxygen species are characterized by different formation rate and different reactivity and hence they have various targets of action. Moreover, ROS not only can attack biological molecules, but they can also interact with other radicals. In order to prevent the ROS-mediated damage, more data about free radicals species that are overproduced in the cells are required (Li et al., 2009). On the other hand, oxidative stress is related with the imbalance between the excessive formation of ROS and limited antioxidant defense, and so the information about the antioxidant response needs to be provided.

This paper describes the study on the induction of oxidative stress as well as its alleviation through the application of antioxidants during the elimination of dibutyltin using the fungal strain *Metarhizium robertsii*. Two natural estrogens, i.e., 17β-estradiol (E2) and estrone (E1), a major E2 metabolite with a higher radical-scavenging activity than the parent compound (Ruiz-Larrea et al., 2000), were used as antioxidant compounds. The tested compounds were analyzed quantitatively using gas and liquid chromatography techniques. Involvement of cytochrome P450 (CYP450) monooxygenases in biotransformation of both natural estrogens and butyltins by the *M. robertsii* strain was verified through the application of a nonspecific inhibitor of CYP450. Investigations on ROS induction were done through the *in situ* detection of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) as the products of the sequential reduction of molecular oxygen. Considering the literature data for DBT effect on nitric oxide synthase (Chantong et al., 2014), the level of nitric oxide (NO•) was also estimated in our study. The antioxidant defense of the fungal strain was studied by measuring the activities of catalase (CAT) and superoxide dismutase (SOD), the major enzymes removing H_2O_2 and $O_2^{\bullet -}$, respectively (Carillon et al., 2013; Sooch et al., 2014).

2. Materials and methods

2.1. Chemicals

Dibutyltin dichloride, monobutyltin trichloride, 17β-estradiol, estrone, proadifen, methyl magnesium bromide, tropolone, nitrotetrazolium blue chloride (NBT), sodium azide (NaN_3), 3,3′-diaminobenzidine-tetrahydrochloride (DAB), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), bovine serum albumin (BSA), Bradford reagent, ethylenediaminetetraacetic acid (EDTA), polyvinylpyrrolidone (PVP), ascorbic acid, methionine, and riboflavin were purchased from Sigma–Aldrich (Germany). The other chemicals used for extraction were obtained from J.T. Baker Chemical Co. (the Netherlands) while ultrapure solvents required for chromatography analyzes were purchased from POCH S.A. (Poland). Stock solutions of $DBTCl_2$ and 17β-estradiol (E2), both at 10 mg ml^{-1}, were prepared in ethanol. Estrone (E1) was dissolved in dimethyl sulfoxide to the final concentration of 5 mg ml^{-1}.

2.2. Microorganism and growth conditions

In this study, the filamentous fungus *Metarhizium robertsii* was used. The ability of the IM 6519 strain to remove tri-, di-, and monobutyltin had been reported by Siewiera et al. (2015, 2017).

Fourteen-day-old fungal cultures on ZT agar slants (Paraszkiewicz et al., 2007) were used to inoculate 20 ml of synthetic medium (Lobos et al., 1992). The growth medium was modified by the addition of glucose and yeast extract and it finally consisted of (per liter): 4.36 g K_2HPO_4, 1.7 g KH_2PO_4, 0.2 g $MgSO_4 \times 7H_2O$, 0.05 g $MnSO_4$, 0.01 g $FeSO_4 \times 7H_2O$, 0.03 g $CaCl_2 \times 2H_2O$, 40 g glucose, 10 g yeast extract and distilled water (up to 1 L), pH 6.8. The preculture was placed on a rotary shaker (160 rpm) at 28 °C, for 24 h, and later transferred into a fresh medium (in the ratio 1:1) and incubated for another day. A volume of 4 ml of the homogenous preculture was introduced into 16 ml of medium containing only DBT (20 mg l^{-1}), to the mixture of DBT with one of natural estrogens (E1 at 5 mg l^{-1} or E2 at 10 mg l^{-1}), and to a medium without any of the compounds as a control culture. Additionally, some cultures were supplemented with proadifen (SKF, 1 mM), a nonselective CYP450 inhibitor. The cultures were incubated for 72 h in conditions described above.

The concentrations of DBT, E1, E2, and SKF, which did not inhibit the growth of *M. robertsii,* were applied in the study. The effect of the higher level of DBT (20 mg l^{-1}) on the fungal cells might be different from that observed in environmental matrices. However, further research on the mechanisms of fungal resistance to the butyltins could be unsuccessful because the DBT concentrations would not be high enough to stimulate a noticeable response of the cells. Samples, which undergo bioremediation, might contain concentrated pollution.

2.3. Fungal biomass estimation

The *M. robertsii* mycelia from 24- and 72-h cultivation were separated from culture media by filtering with a vacuum pump and then dried at 105 °C, until the weight was constant.

2.4. Extraction and quantification of butyltins

The extraction of butyltin compounds from the fungal cultures was carried out according to the method proposed by Siewiera et al. (2015). The amounts of methylated di- and monobutyltin from the extracts were determined quantitatively using a gas chromatography mass spectrometry (GC-MS) (Hewlett-Packard Model 6890 equipped with an HP 5973 Mass Detector).

The cultures containing thermally killed fungal biomass (2 times for 20 min, at 121 °C) were extracted independently according to the above procedure to exclude the impact of abiotic factors, such as UV radiation and adsorption of the compounds to a glass surface, on the efficiency of DBT biodegradation.

2.5. Qualitative analysis of DBT derivatives

In order to determine butyltin intermediates in the fungal

cultures cultivated with the addition of the CYP450 inhibitor, the samples were prepared and then analyzed chromatographically according to the earlier described procedure (Siewiera et al., 2017).

2.6. Determination of E1 and E2 contents

Estrone and 17β-estradiol were extracted using a QuEChERS (*Quick, Easy, Cheap, Effective, Rugged, Safe*) method. Next, the quantitative analyzes of the estrogens were performed by liquid chromatography mass spectrometry (LC-MS, Agilent 1200 coupled with tandem mass spectrometer AB Sciex QTRAP 4500). The detailed procedure has been described in the paper of Siewiera et al. (2015).

2.7. In situ detection of superoxide anion

An assay of $O_2^{\bullet -}$ accumulation in the *M. robertsii* hyphae was carried out according to a modified method described previously by Trujillo et al. (2004). The cultures were filtered using a vacuum pump. Next, the biomass (100 mg) was transferred into an Eppendorf tube containing a solution of 0.1% NBT and 10 mM NaN_3 in sodium phosphate buffer (PBS) of pH 7.8. The mixtures were incubated for 30 min in the dark, at room temperature. The samples were centrifuged (5000 × *g*, 5 min) and then the fungal pellets were washed twice with PBS. Finally, the biomass was suspended in 1 ml of the buffer and examined immediately under a confocal laser scanning microscope Pascal 5 (Zeiss).

2.8. In situ detection of hydrogen peroxide

An accumulation of H_2O_2 in the fungus hyphae was detected according to the modified method of Orozco-Cárdenas and Ryan (1999). Firstly, 100 mg of wet biomass was placed in an Eppendorf tube containing 1 ml of DAB solution (1 mg ml^{-1}) in PBS of pH 3.8. Next, the samples were incubated for 15 h in the light, at room temperature. The mycelium was washed twice with PBS and then suspended in the buffer. At the end, the fungal pellets were transferred to the microscope slides and were photographed with an Axiovert 200 M inverted microscope equipped with an HRC digital camera (Zeiss).

2.9. In situ detection of nitric oxide

The NO• overproduction in *M. robertsii* mycelium was detected via H_2DCFDA, according to a slightly modified method by Liu et al. (2010). Firstly, 1 ml of the fungal culture was transferred into an Eppendorf tube and centrifuged at 5000 × *g* for 5 min. Next, the supernatant was removed and the pellets were suspended in 1 ml of PBS (pH 7.4) containing 50 μM H_2DCFDA (dissolved in DMSO). The incubation of samples was carried out for 30 min in the dark, at room temperature. Finally, the mycelium was washed twice with PBS and placed on a microscope slide. The images were taken using a confocal laser scanning microscope (LSM510 Meta, Zeiss) combined with an Axiovert 200 M (Zeiss, Germany) inverted fluorescence microscope equipped with a Plan-Apochromat objective (63 × /1.25 oil). The mycelia were excited with a 488 argon laser line and the emission was recorded using a 530 nm bandpass filter. The same laser line was used for Nomarski DIC. Constant exposure time was applied for all experiments. The detection of green fluorescence was attributed to the presence of NO•.

2.10. Evaluation of superoxide anion, hydrogen peroxide, and nitric oxide quantities

In situ ROS and RNS generation was visualized by the formation of precipitates (blue – $O_2^{\bullet -}$ or brown – H_2O_2) or by the occurrence of green fluorescence (in NO• presence). Accumulation of $O_2^{\bullet -}$, H_2O_2, and NO• was calculated as a ratio of the fluorescent/colored mycelial area (mm^2) to the area of all mycelia (mm^2), for at least 20 independent images.

2.11. Preparation of enzyme extracts

Firstly, a wet biomass (250 mg) was placed into a cooled mortar and then it was homogenized in 2.5 ml of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 1% PVP, and 1 mM ascorbic acid. The homogenate was centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant was transferred into a fresh Eppendorf tube placed on the ice and it was subsequently used for enzyme assays.

2.12. Assays of CAT and SOD activities

Measurement of CAT activity through H_2O_2 conversion was carried out according to the modified method of Dhindsa et al. (1981). The reaction mixture consisted of 1.8 ml of 50 mM PBS (pH 7.0), 100 μl of the enzyme extract, and 150 μl of 1% H_2O_2. An absorbance decrease over time (4 min) was observed spectrophotometrically at 240 nm. Enzyme activity was expressed in U mg^{-1}.

The activity of SOD was monitored by the inhibition of NBT reduction under UV radiation, according to the method of Beauchamp and Fridovich (1971) with few modifications. Firstly, 50 mM PBS (pH 7.8) containing 13 mM methionine, 75 μM NBT and 1 mM EDTA was prepared. The reactive mixture consisted of 2.9 ml of the buffer, 50 μl of the enzyme extract, and 2 μM of riboflavin. Additionally, a blank sample was made using the buffer instead of the enzyme extract. All samples were transferred to a glass Petri dish and then incubated under UV lamps for 30 min. Finally, the absorbances were measured at λ = 560 nm. Enzyme activity was expressed in U mg^{-1}.

2.13. Measurement of protein content

The protein content in the enzyme extracts was determined by the Bradford (1976) method. A mixture of the extract (50 μl) and the Bradford reagent (1 ml) was incubated for 15 min at room temperature and then the absorbance of samples was measured (λ = 595 nm). The standard curve was prepared in the same way, using bovine serum albumin instead of the supernatant. Next, the curve equation was calculated and it was applied for the estimation of the amount of protein.

2.14. Statistical analyzes

The experiments were carried out in triplicate. The one-way analysis of variance (ANOVA) and Spearman's correlation were performed using Excel 2016 (Microsoft Corporation, USA). Sample variability was given as a standard deviation (±SD). Differences at $p < 0.05$ were considered as significant.

3. Results and discussion

3.1. Growth of M. robertsii strain exposed to DBT alone or to its mixture with E1/E2

The influence of DBT (with or without the estrogens) on the *M. robertsii* growth was observed as late as after 72 h of cultivation

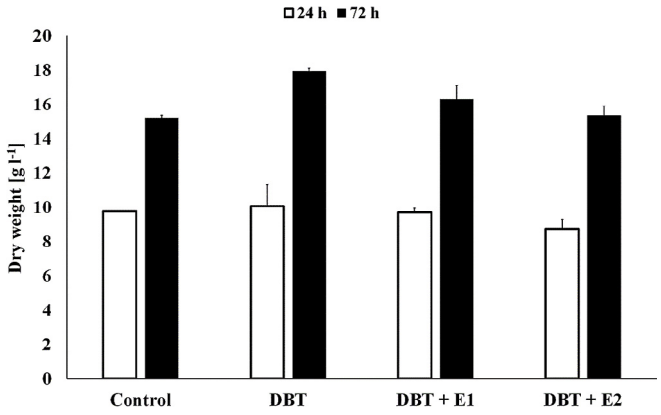

Fig. 1. Dry weight of the *M. robertsii* strain after 24- and 72-h incubation on modified synthetic medium with: DBT (20 mg l^{-1}), DBT and E1 (5 mg l^{-1}) or E2 (10 mg l^{-1}), as well as without these compounds.

(Fig. 1). Dry weight of the tested strain exposed to DBT alone has increased by 18% compared to the biotic control. The phenomena can be connected with the butyltin utilization, followed by the inclusion of the obtained carbon and hydrogen into the biomass, or with the intensified uptake of glucose from the growth medium. The amount of biomass exposed to the mixture of DBT and E1 was higher by 7%, contrary to the quantity of the mycelium incubated in DBT with E2, which remained at a level similar to that of the control culture. But, in the previous study on tributyltin (TBT) biodegradation by the *M. robertsii* strain, the addition of E2 resulted in a high increase of the 5-day biomass (Siewiera et al., 2015). The divergences between the obtained results might have resulted from a stronger toxic effect of TBT, than DBT, on the tested strain, which resulted in the requirement of greater antioxidant protection for TBT-treated cells. Moreover, it could have been related with differences in both growth media composition and incubation time, which was adapted to show the dissimilarity between cultures conducted with and without the addition of the antioxidant. However, due to various conditions of the cultures and different mechanisms of DBT and TBT toxicity, present and earlier results should not be compared.

3.2. Influence of the natural estrogens on DBT removal and involvement of the fungal CYP450 in this process

The residues of di- and monobutyltin, a major byproduct of DBT debutylation, are shown in Fig. 2. During the first 24 h of incubation, the initial concentration of DBT (20 mg l^{-1}) was reduced to 3.1 mg l^{-1}, achieving 85% efficiency of DBT elimination. Bernat et al. (2013) have reported that *Cochliobolus lunatus* strain removed 92% of the initial DBT concentration (10 mg l^{-1}) during the first 24 h of incubation on Sabouraud medium. Because of DBT dealkylation, a byproduct with one butyl group was formed. In the *C. lunatus* culture, the MBT concentration was greater than 6 mg l^{-1} (Bernat et al., 2013), while in our studies DBT was converted to 10.5 mg l^{-1} of MBT. The total amount of di- and monobutyltin was not equal to 20 mg l^{-1} due to a partial conversion of MBT to inorganic tin.

Although the intention of this paper was improvement of DBT elimination, dibutyltin levels were found to be approximately 2-fold higher while monobutyltin amounts were approximately 2.5 times lower in the fungal culture in the presence of estrogens (Fig. 2), compared to the cultures with DBT alone. The probable cause of this phenomenon was competitive inhibition, which means that DBT competed with E1 or E2 in binding to the active site of the enzyme involved in biotransformation of these compounds. A similar dependence was observed during the biodegradation of volatile aromatic compounds by the fungus *Cladophialophora* sp. The process was slower when the fungal culture was exposed to a mixture of toluene and xylene because of simultaneous conversion of the compounds conducted at the side chain by the same monooxygenase enzyme (Prenafeta-Boldú et al., 2002). On the other hand, supplementation of the *M. robertsii* fungus with E2 resulted in improved TBT removal (about 14%), compared to the cultures without the estrogen (Siewiera et al., 2015). Literature data indicate the significance of antioxidants application for the elimination of other ROS inducers such as polychlorobiphenyls, chloroacetanilides, and heavy metals by microorganisms (Ponce et al., 2011; Słaba et al., 2013, 2015).

Monooxygenases of CYP450, which play an important role in

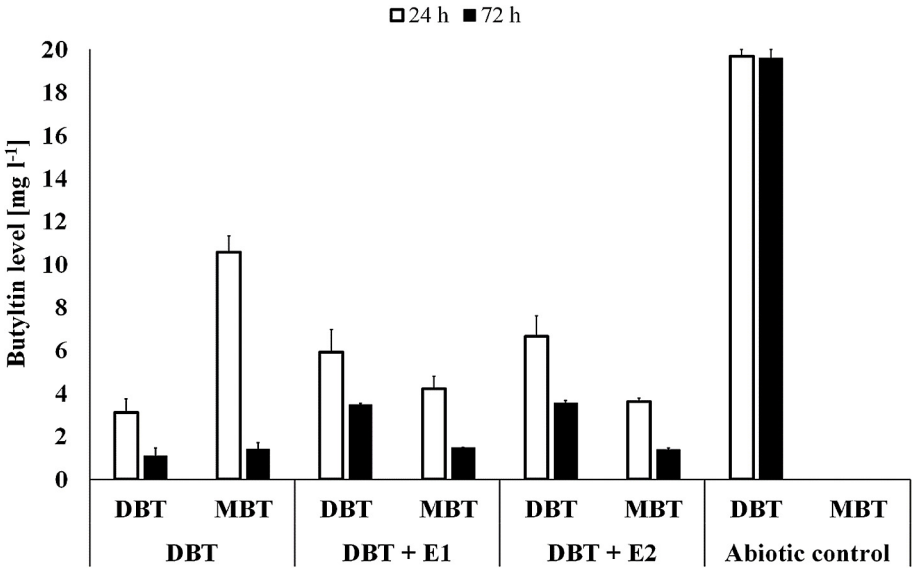

Fig. 2. The DBT and MBT residues in the fungal culture after 24 and 72 h of cultivation in the absence or in the presence of natural estrogens.

bioremediation processes (Kumar, 2010), are commonly found in the fungal kingdom (Jawallapersand et al., 2014). Moreover, Lin et al. (2011) showed that CYP450 enzymes of *M. robertsii* species are required for utilization of hydrocarbons.

In our study, the involvement of CYP450 monooxygenases in the elimination of butyltins was verified indirectly using a proadifen (SKF), a nonselective inhibitor of this enzymatic complex. In the cultures with SKF, DBT biodegradation was not inhibited completely but DBT level was 4-fold higher compared to the cultures without inhibitor (Fig. 3). Moreover, the hydroxylated metabolite of MBT ($OHBuSnH_2$), described previously in our paper (Siewiera et al., 2017), was not detected in the cultures with SKF, which confirmed the involvement of CYP450 monooxygenases in DBT bioconversion by the *M. robertsii* strain.

The research team of Bernat and Długoński (2002, 2009) also proved that microbial CYP450 is involved in the dealkylation of organotin compounds. Therefore, hydroxylation-mediated metabolism of the butyltins seems to be a universal pathway of the compound removal by bacteria and filamentous fungi.

3.3. The quantitative analyzes of the natural estrogens in the fungal cultures conducted with or without the inhibitor of CYP450

Due to the ability of the *M. robertsii* strain to biotransform estrogens, the steroids were analyzed quantitatively using a LC-MS/MS. During the first 24 h of incubation, E2 was utilized by the fungal cells almost 2-fold faster than E1. The concentration of 17β-estradiol was reduced from 10 mg l^{-1} to 2.52 mg l^{-1}, while the estrone level was decreased from 5 mg l^{-1} to 2.84 mg l^{-1} (Table 1).

In the presence of proadifen, the amounts of E1 and E2 were higher by 67% and 34%, respectively, compared to cultures without SKF. The results indicate that the CYP450 of the *M. robertsii* strain may participate in the biotransformation of E1 and E2. However, dehydrogenation of ring D at C-17 position of 17β-estradiol molecule was weakly inhibited, which led to the partial conversion of E2 to E1. Mediation of fungal laccases in the degradation of estrogens has been described by Lloret et al. (2010) and Tran et al. (2010). Unfortunately, no data about the involvement of cytochrome monooxygenases in the biotransformation of natural estrogens by filamentous fungi are available, contrary to the mechanism of transformation of the compounds by human CYP450 (Tsuchiya et al., 2005; Zhu and Lee, 2005).

Table 1
The quantities of natural estrogens in 1-day-old cultures of the *M. robertsii* strain cultivated in the presence of DBT (with or without the CYP450 inhibitor).

Culture	Proadifen [1 mM] presence	Estrogen level [mg l^{-1}]
DBT + E1 [5 mg l^{-1}]	–	2.84 ± 0.28
	+	4.73 ± 0.33
DBT + E2 [10 mg l^{-1}]	–	2.52 ± 0.23
	+	3.39 ± 0.35

3.4. Overproduction of $O_2^{\bullet-}$, H_2O_2, and NO•

The quantities of superoxide anion, hydrogen peroxide, and nitric oxide in the 3-h and 24-h *M. robertsii* mycelia exposed to DBT and/or the estrogens are presented in Table 2. In contrast to the estrogens, whose presence in the fungal culture had no effect on ROS and RNS production, the addition of DBT caused a significant increase in the levels of all detected reactive molecules since the first hours of incubation. However, the reactive molecules were present at the highest levels in the 1-day-old mycelium. A superoxide anion, the first product of sequential univalent reduction of the molecular oxygen, was overproduced at the level of 65.89 ± 18.08 initiating the ROS cascade. The level of hydrogen peroxide, formed through the reduction of $O_2^{\bullet-}$, was estimated as 4.04 ± 3.62.

Similarly, Chantong et al. (2014) described the influence of DBT (200 nM) on the overproduction of both the intracellular and the mitochondrial ROS in BV-2 microglia cells. Moreover, a quantitative real-time PCR revealed a significant increase in the mRNA expression of nitric oxide synthase (NOS) of BV-2 cells treated with the organotin (Chantong et al., 2014). In our studies, we found that NOS of the *M. robertsii* strain could also be activated in the presence of DBT, because the level of NO• was significantly higher (than in the control cultures) and amounted to 27.92 ± 1.95. On the other hand, Ohashi et al. (2004) indicate an opposite (inhibitory) effect of DBT on the activeness of rat neuronal nitric oxide synthase.

After 1-day incubation with the natural estrogens, the levels of $O_2^{\bullet-}$, H_2O_2, and NO• in the DBT-treated mycelium were lower even

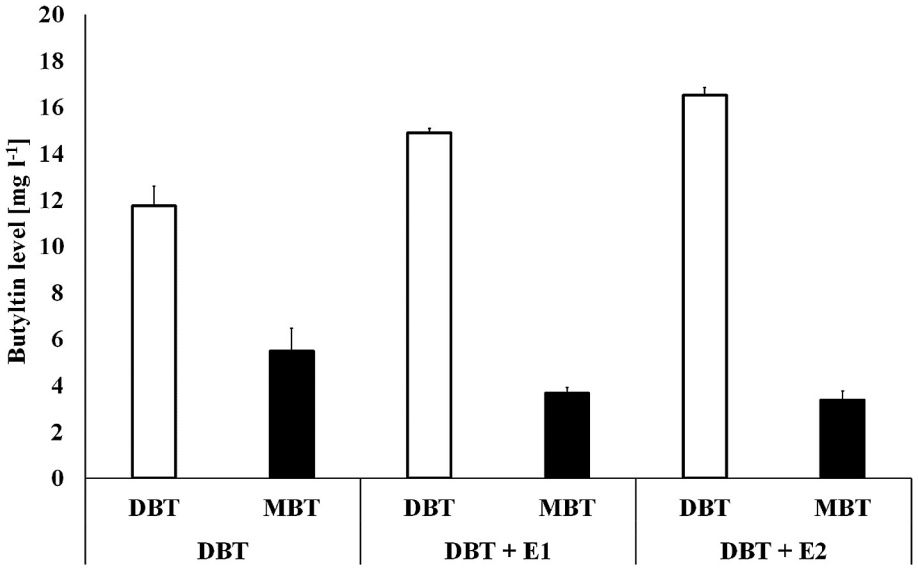

Fig. 3. The concentrations of the butyltin compounds in the culture of the examined fungus, after 24-h cultivation on growth medium supplemented with the inhibitor of CYP450.

Table 2
The ratio between the area of colored/fluorescent hyphae and total hyphal area of the *M. robertsii* strain cultivated in the presence of DBT, and/or the antioxidant, or without these compounds.

Culture	$O_2^{\bullet-}$ (blue colored)		H_2O_2 (brown colored)		NO•(green fluorescent)	
	3 h	24 h	3 h	24 h	3 h	24 h
Control	0.27 ± 0.05	0.36 ± 0.30	0.14 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.89
E1	1.53 ± 1.46	0.33 ± 0.18	0.20 ± 0.09	0.00 ± 0.00	0.03 ± 0.00	0.00 ± 0.00
E2	0.56 ± 0.19	5.27 ± 2.99	0.24 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
DBT	17.95 ± 1.37	65.89 ± 18.08	0.66 ± 0.34	4.04 ± 3.62	6.51 ± 4.17	27.92 ± 1.95
DBT + E1	0.70 ± 0.34	4.61 ± 1.73	0.24 ± 0.14	0.39 ± 0.12	0.00 ± 0.00	7.23 ± 0.72
DBT + E2	0.83 ± 0.08	17.68 ± 3.54	0.31 ± 0.11	0.19 ± 0.01	2.76 ± 0.98	17.61 ± 5.61

14 times, 10 times, and almost 4 times in the presence of E1, as well as 3.5-fold, 21-fold, and 1.5-fold in the presence of E2, compared to the mycelium exposed to DBT alone. The protective effect of E2 on TBT-induced neuronal damage and enhanced permeability of fungal membranes has been described previously (Ishihara et al., 2015; Siewiera et al., 2015). 17β-estradiol is known due to its protective properties for mitochondria (Borrás et al., 2010), i.e., places where intracellular ROS are produced (Dan Dunn et al., 2015). E2 exerts a positive effect on the activity of membrane-linked ATPases (Kumar et al., 2011), the enzymes whose modulation by DBT (Srivastava, 1990; Nesci et al., 2011, 2013) leads to the inhibition of mitochondrial respiration (Ueno et al., 2003) and finally results in ROS generation. Furthermore, the 17β-estradiol structure contains two free phenolic OH groups, which have been considered the quintessential feature in conferring protection against oxidative stress (Prokai et al., 2013); therefore, E2 should be a stronger antioxidant than E1 (the compound with only one hydroxyl group) (E1). Despite all these useful properties of E2, the addition of E1 more efficiently limited the overproduction of both ROS and RNS in the *M. robertsii* hyphae exposed to DBT. According to Ruiz-Larrea et al. (2000), E1 has a higher radical-scavenging activity than E2. On the other hand, the obtained results could be associated with a longer presence of E1 in the fungal culture, contrary to E2, which was rapidly utilized by the fungal cells.

The levels of $O_2^{\bullet-}$, H_2O_2, and NO• detected in the 1-day-old mycelium were negatively correlated with DBT quantities and positively related to MBT concentrations remained in these cultures. The correlation was strong, because the coefficients were higher than 0.745 in all cases. The reactive molecules were additionally detected in the *M. robertsii* mycelium exposed to MBT at concentration of about 10 mg l^{-1}. The levels of $O_2^{\bullet-}$, H_2O_2, and NO• amounted to 45.35 ± 9.18, 2.03 ± 0.57, and 16.25 ± 3.74, respectively.

The biological activity of organotin compounds is determined by the number of organic groups linked to the tin atom, and it decreases as follows: $R_3SnX > R_2SnX_2 > RSnX_3$ (de Carvalho Oliveira and Santelli, 2010). Our results draw attention to an unexpected threat associated with the presence of MBT, the compound that appeared to be the least toxic among all organotins. Contrary to the numerous studies carried out on TBT-mediated oxidative stress (Ishihara et al., 2012; Bernat et al., 2014; Mitra et al., 2014; Soboń et al., 2016), only Ferreira et al. (2013) have indicated MBT as the compound causing stronger ROS induction in neuroblastoma cells than TBT. There are studies indicating that MBT is more toxic to methanogenic and sulfate-reducing bacteria than TBT (Belay et al., 1990; Boopathy and Daniels, 1991; Lascourrèges et al., 2000). Moreover, MBT causes more intense potassium efflux from marine yeast than DBT (Cooney et al., 1989; Laurence et al., 1989). Furthermore, Doolittle and Cooney (1992) reported that MBT flocculated particles of bacteriophage T4, inactivating it more effectively than TBT or DBT. Unfortunately, further studies on toxic effects of MBT have not been carried out.

3.5. CAT and SOD activities

Oxygen can be toxic to every aerobic organism, including the *M. robertsii* strain, which requires a large supply of the O_2 for efficient elimination of butyltins (Siewiera et al., 2017). Moreover, MBT promotes generation of both the nitrogen and oxygen reactive species. Nevertheless, after the addition of DBT (with or without the estrogens), the levels of CAT (Fig. 4a) and SOD (Fig. 4b) were not significantly different, in comparison to the results obtained for the control mycelia.

In the research of toxic effects of organotins on natural populations of bats (*Myotis daubentonii*), the inhibition of SOD activity was determined (Lilley et al., 2013). In our studies, ROS cascade was blocked ($O_2^{\bullet-}$ was not converted into H_2O_2) but SOD levels in the hyphae, which were exposed to the butyltin, were not changed. It was assumed that the reason for the above could be caused by little reactivity of superoxide anion with non-radical species in aqueous solutions (Li et al., 2009). Similar to the superoxide dismutase activity in the *M. robertsii* mycelium exposed to DBT alone or to its mixture with one of the natural estrogens, the catalase activity also remained at the same level. The amount of hydrogen peroxide was low and it could be essential for the signaling pathways (Pourova et al., 2010). On the other hand, other enzymes such as fungal peroxidases (Hofrichter et al., 2010) could be involved in the H_2O_2 removal.

Detoxification of NO• has been a more complicated issue because of the occurrence of various fungal-denitrifying systems such as CYP450 nitric oxide reductase, or detoxification via oxidation by flavohemoglobin YHb, or action of regulatory protein Yap1 (Lewinska and Bartosz, 2006; Lushchak et al., 2010; Shoun et al., 2012). Due to the lack of information about antioxidant defense of the *M. robertsii* strain against NO• formation, it was difficult to establish the nitrosative stress. However, the fungal cells are certainly exposed to oxidative stress, because of ROS overproduction and defense enzymatic limitation. In published reports concerning biodegradation of other xenobiotics such as chloroacetanilide herbicides, quinclorac, bensulfuron-methyl, and atrazine, SOD as well as CAT activities showed active response of microorganisms against oxidative stress (Lin et al., 2009; Lü et al., 2009; Martins et al., 2011; Zhang et al., 2012; Słaba et al., 2015). In our studies, SOD and CAT activities were limited, which was most likely associated with non-enzymatic defense of the fungal cells initiated by the antioxidants application. Moreover, the unchanged level of the *M. robertsii* antioxidant enzymes clearly indicates the action of the natural estrogens in scavenging of $O_2^{\bullet-}$, H_2O_2, and NO• induced by the presence of MBT.

4. Conclusions

The presented study shows that CYP450 of the *M. robertsii* strain is involved in DBT biodegradation. Moreover, generation of $O_2^{\bullet-}$, H_2O_2, and NO• is not induced by the parent compound, but by the

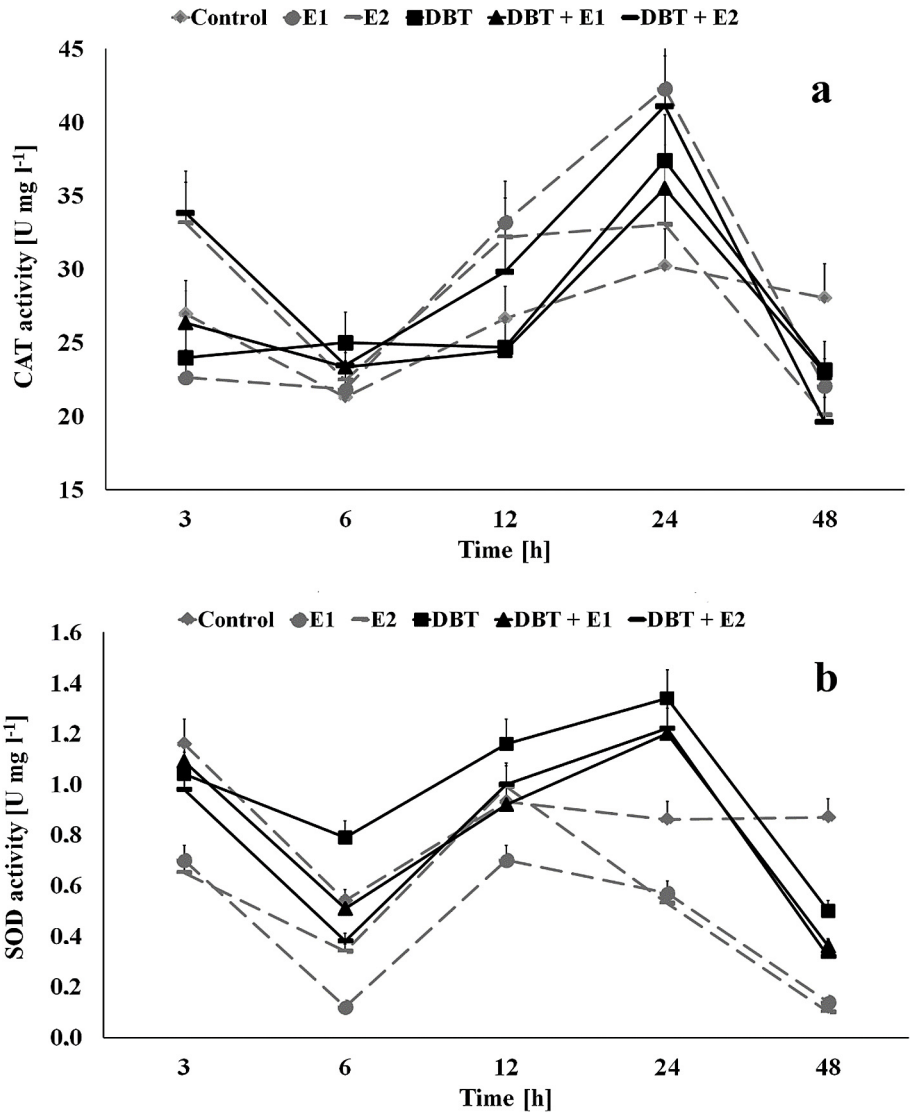

Fig. 4. Activity of catalase (a) and superoxide dismutase (b) in the *M. robertsii* mycelium, incubated in the presence of DBT, and/or antioxidant, or without these compounds.

presence of MBT – a byproduct of DBT dealkylation, which is considered as the least toxic of all organotins. Superoxide dismutase as well as catalase of the fungal cells do not participate in ROS detoxification. Estrone and 17β-estradiol are effective scavengers of nitrogen and oxygen reactive species, protecting *M. robertsii* cells against both nitrosative and oxidative stress.

The obtained results could initiate further research focusing on the molecular background of MBT action on microorganisms, including the consequences of ROS and RNS overproduction. Expanding the knowledge in the area of biodegradation of butyltins by filamentous fungi in the presence of antioxidants may in future lead to the creation of new, effective bioremediation systems.

Acknowledgements

This study was supported by the National Science Centre, Poland (Project No. 2015/19/N/NZ9/00459).

References

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and assay applicable to acrylamide gels. Anal. Biochem. 44 (1), 276–287.

Belay, N., Rajagopal, B.S., Daniels, L., 1990. Effects of alkyltin compounds on hydrogen-oxidizing anaerobic bacteria. Curr. Microbiol. 20 (5), 329–334.

Bernat, P., Długoński, J., 2002. Degradation of tributyltin by the filamentous fungus *Cunninghamella elegans*, with involvement of cytochrome P-450. Biotechnol. Lett. 24 (23), 1971–1974.

Bernat, P., Długoński, J., 2009. Isolation of *Streptomyces* sp. strain capable of butyltin compounds degradation with high efficiency. J. Hazard. Mater. 171 (1–3), 660–664.

Bernat, P., Gajewska, E., Szewczyk, R., Słaba, M., Długoński, J., 2014. Tributyltin (TBT) induces oxidative stress and modifies lipid profile in the filamentous fungus *Cunnighamella elegans*. Environ. Sci. Pollut. Res. Int. 21 (6), 4228–4235.

Bernat, P., Szewczyk, R., Krupiński, M., Długoński, J., 2013. Butyltins degradation by *Cunninghamella elegans* and *Cochliobolus lunatus* co-culture. J. Hazard. Mater. 246–247, 277–282.

Bhosle, N.B., Garg, A., Harji, R., Jadhav, S., Swant, S.S., Krishnamurthy, V., Anil, C., 2006. Butyltins in the sediments of Kochi and Mumbai harbours, west coast of India. Environ. Int. 32 (2), 253–258.

Bhosle, N.B., Garg, A., Jadhav, S., Harjee, R., Sawant, S.S., Venkat, K., Anil, A.C., 2004. Butyltins in water, biofilm, animals and sediments of the west coast of India. Chemosphere 57 (8), 897–907.

Boopathy, R., Daniels, L., 1991. Pattern of organotin inhibition of methanogenic bacteria. Appl. Environ. Microbiol. 57 (4), 1189–1193.

Borrás, C., Gambini, J., López-Grueso, R., Pallardó, F.V., Viña, J., 2010. Direct antioxidant and protective effect of estradiol on isolated mitochondria. Biochim. Biophys. Acta 1802 (1), 205–211.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254.

Carillon, J., Rouanet, J.M., Cristol, J.P., Brion, R., 2013. Superoxide dismutase administration, a potential therapy against oxidative stress related diseases: several routes of supplementation and proposal of an original mechanism of action. Pharm. Res. 30 (11), 2718–2728.
Chantong, B., Kratschmar, D.V., Lister, A., Odermatt, A., 2014. Dibutyltin promotes oxidative stress and increases inflammatory mediators in BV-2 microglia cells. Toxicol. Lett. 230 (2), 177–187.
Chen, C.W., Chen, C.F., Ju, Y.R., Dong, C.D., 2016. Assessment of the bioaccumulation and biodegradation of butyltin compounds by *Thalamita crenata* in Kaohsiung Harbor, Taiwan. Int. Biodeterior. Biodegrad. 113, 97–104.
Cooney, J.J., deRome, L., Laurence, O., Gadd, G.M., 1989. Effects of organotin and organolead compounds on yeasts. J. Ind. Microbiol. 4 (4), 279–288.
Dai, S.G., Sun, H.W., Wang, Y.Q., Chen, W.P., Li, N., 2003. Biogeochemistry of butyltin compounds in estuarine environments of the Haiha River, China. ACS Symp. Ser. 835, 370–387.
Dan Dunn, J., Alvarez, L.A., Zhang, X., Soldati, T., 2015. Reactive oxygen species and mitochondria: a nexus of cellular homeostasis. Redox Biol. 6, 472–485.
de Carvalho Oliveira, R., Santelli, R.E., 2010. Occurrence and chemical speciation analysis of organotin compounds in the environment: a review. Talanta 82 (1), 19–24.
Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A., 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decrease levels of superoxide dismutase and catalase. J. Exp. Bot. 32 (1), 93–101.
Díez, S., Jover, E., Albaigés, J., Bayona, J.M., 2006. Occurrence and degradation of butyltins and wastewater marker compounds in sediments from Barcelona harbor. Spain. Environ. Int. 32 (7), 858–865.
Díez, S., Lacorte, S., Viana, P., Barceló, D., Bayona, J.M., 2005. Survey of organotin compounds in rivers and coastal environments in Portugal 1999-2000. Environ. Pollut. 136 (3), 525–536.
Dobson, S., Howe, P.D., Floyd, P., 2006. Mono- and Disubstituted Methyltin, Butyltin, and Octyltin Compounds. http://www.who.int/iris/handle/10665/43549.
Doolittle, M.M., Cooney, J.J., 1992. Inactivation of bacteriophage T4 by organic and inorganic tin compounds. J. Ind. Microbiol. 10 (3), 221–228.
Farr, C.H., Reinisch, K., Holson, J.F., Neubert, D., 2001. Potential teratogenicity of di-n-butyltin dichloride and other dibutyltin compounds. Teratog. Carcinog. Mutagen 21 (6), 405–415.
Ferreira, M., Blanco, L., Garrido, A., Vieites, J.M., Cabado, A.G., 2013. In vitro approaches to evaluate toxicity induced by organotin compounds tributyltin (TBT), dibutyltin (DBT), and monobutyltin (MBT) in neuroblastoma cells. J. Agric. Food Chem. 61 (17), 4195–4203.
Gennari, A., Viviani, B., Galli, C.L., Marinovich, M., Pieters, R., Corsini, E., 2000. Organotins induce apoptosis by disturbance of $[Ca^{2+}]_i$ and mitochondrial activity, causing oxidative stress and activation of caspases in rat thymocytes. Toxicol. Appl. Pharmacol. 169 (2), 185–190.
Gomez-Ariza, J.L., Santos, M.M., Morales, E., Giraldez, I., Sanchez-Rodas, D., Vieira, N., Kemp, J.F., Boon, J.P., Ten-Hallers-Tjabbes, C.C., 2006. Organotin contamination in the atlantic ocean of the Iberian Peninsula in relation to shipping. Chemosphere 64 (7), 1100–1108.
Gumy, C., Chandsawangbhuwana, C., Dzyakanchuk, A.A., Kratschmar, D.V., Baker, M.E., Odermatt, A., 2008. Dibutyltin disrupts glucocorticoid receptor function and impairs glucocorticoid-induced suppression of cytokine production. PLoS One 3 (10), e3545.
Harino, H., Yamamoto, Y., Eguchi, S., Kawai, S., Kurokawa, Y., Arai, T., Ohji, M., Okamura, H., Miyazaki, N., 2007. Concentrations of antifouling biocides in sediment and mussel samples collected from Otsuchi Bay, Japan. Arch. Environ. Contam. Toxicol. 52 (2), 179–188.
Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T., 2010. New and classic families of secreted fungal heme peroxidases. Appl. Microbiol. Biotechnol. 87 (3), 871–897.
Ishihara, Y., Kawami, T., Ishida, A., Yamazaki, T., 2012. Tributyltin induces oxidative stress and neuronal injury by inhibiting glutathione S-transferase in rat organotypic hippocampal slice cultures. Neurochem. Int. 60 (8), 782–790.
Ishihara, Y., Takemoto, T., Ishida, A., Yamazaki, T., 2015. Protective actions of 17-estradiol and progesterone on oxidative neuronal injury induced by organometallic compounds. Oxid. Med. Cell Longev. http://dx.doi.org/10.1155/2015/343706.
Jawallapersand, P., Mashele, S.S., Kovačič, L., Stojan, J., Komel, R., Pakala, S.B., Kraševec, N., Syed, K., 2014. Cytochrome P450 monooxygenase CYP53 family in fungi: comparative structural and evolutionary analysis and its role as a common alternative anti-fungal drug target. PLoS One 9 (9), e107209.
Kumar, P., Kale, R.K., Baquer, N.Z., 2011. Estradiol modulates membrane-linked ATPases, antioxidant enzymes, membrane fluidity, lipid peroxidation, and lipofuscin in aged rat liver. J. Aging Res. http://dx.doi.org/10.4061/2011/580245.
Kumar, S., 2010. Engineering cytochrome P450 biocatalysts for biotechnology, medicine and bioremediation. Expert Opin. Drug Metab. Toxicol. 6 (2), 115–131.
Landmeyer, J.E., Tanner, T.L., Watt, B.E., 2004. Biotransformation of tributyltin to tin in freshwater river-bed sediments contaminated by an organotin release. Environ. Sci. Technol. 38 (15), 4106–4112.
Lascourrèges, J.F., Caumette, P., Donard, O.F.X., 2000. Toxicity of butyltin, phenyltin and inorganic tin compounds to sulfate-reducing bacteria isolated from anoxic marine sediments. Appl. Organometal. Chem. 14, 98–107.
Laurence, O.S., Cooney, J.J., Gadd, G.M., 1989. Toxicity of organotins towards the marine yeast *Debaryomyces hansenii*. Microb. Ecol. 17 (3), 275–285.
Lewinska, A., Bartosz, G., 2006. Yeast flavohemoglobin protects against nitrosative stress and controls ferric reductase activity. Redox Rep. 11 (5), 231–239.
Li, Q., Harvey, L.M., McNeil, B., 2009. Oxidative stress in industrial fungi. Crit. Rev. Biotechnol. 29 (3), 199–213.
Lilley, T.M., Ruokolainen, L., Meierjohann, A., Kanerva, M., Stauffer, J., Laine, V.N., Atosuo, J., Lilius, E.-M., Nikinmaa, M., 2013. Resistance to oxidative damage but not immunosuppression by organic tin compounds in natural populations of Daubenton's bats (*Myotis daubentonii*). Comp. Biochem. Physiol. C 157 (3), 298–305.
Lin, L., Fang, W., Liao, X., Wang, F., Wei, D., St Leger, R.J., 2011. The MrCYP52 cytochrome P450 monoxygenase gene of *Metarhizium robertsii* is important for utilizing insect epicuticular hydrocarbons. PLoS One 6 (12), e28984.
Lin, X., Xu, X., Yang, C., Zhao, Y., Feng, Z., Dong, Y., 2009. Activities of antioxidant enzymes in three bacteria exposed to bensulfuron-methyl. Ecotoxicol. Environ. Saf. 72 (7), 1899–1904.
Liu, P., Luo, L., Guo, J., Liu, H., Wang, B., Deng, B., Long, C.A., Cheng, Y., 2010. Farnesol induces apoptosis and oxidative stress in the fungal pathogen *Penicillium expansum*. Mycologia 102 (2), 311–318.
Lloret, L., Eibes, G., Lú-Chau, T.A., Moreira, M.T., Feijoo, G., Lema, J.M., 2010. Laccase-catalyzed degradation of anti-inflammatories and estrogens. Biochem. Eng. J. 51 (3), 124–131.
Lobos, J.H., Leib, T.K., Su, T.M., 1992. Biodegradation of bisphenol A and other bisphenols by a gram-negative aerobic bacterium. Appl. Environ. Microbiol. 58 (6), 1823–1831.
Lü, Z., Sang, L., Li, Z., Min, H., 2009. Catalase and superoxide dismutase activities in a *Stenotrophomonas maltophilia* WZ2 resistant to herbicide pollution. Ecotoxicol. Environ. Saf. 72 (1), 136–143.
Lushchak, O.V., Inoue, Y., Lushchak, V.I., 2010. Regulatory protein Yap1 is involved in response of yeast *Saccharomyces cerevisiae* to nitrosative stress. Biochemistry 75 (5), 629–635.
Martins, P.F., Carvalho, G., Gratão, P.L., Dourado, M.N., Pileggi, M., Araújo, W.L., Azevedo, R.A., 2011. Effects of the herbicides acetochlor and metolachlor on antioxidant enzymes in soil bacteria. Process Biochem. 46 (5), 1186–1195.
Mitra, S., Srivastava, A., Khanna, S., Khandelwal, S., 2014. Consequences of tributyltin chloride induced stress in Leydig cells: an ex-vivo approach. Environ. Toxicol. Pharmacol. 37 (2), 850–860.
Nesci, S., Ventrella, V., Pagliarani, A., 2013. Modulation of the F_1F_O-ATPase function by butyltin compounds. Appl. Organomet. Chem. 27 (4), 199–205.
Nesci, S., Ventrella, V., Trombetti, F., Pirini, M., Borgatti, A.R., Pagliarani, A., 2011. Tributyltin (TBT) and dibutyltin (DBT) differently inhibit the mitochondria Mg-ATPase activity in mussel digestive gland. Toxicol In Vitro 25 (1), 117–124.
Nielsen, J.B., Strand, J., 2002. Butyltin compounds in human liver. Environ. Res. 88 (2), 129–133.
Ohashi, K., Kominami, S., Yamazaki, T., Ohta, S., Kitamura, S., 2004. Inhibitory effect of organotin compounds on rat neuronal nitric oxide synthase through interaction with calmodulin. Biochem. Biophys. Res. Commun. 324 (1), 178–185.
Okoro, H.K., Fatoki, O.S., Adekola, F.A., Ximba, B.J., Snyman, R.G., 2011. Sources, environmental levels and toxicity of organotin in marine environment-a review. Asian J. Chem. 23 (2), 473–482.
Orozco-Cárdenas, M., Ryan, C.A., 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and system in via the octadecanoid pathway. Proc. Natl. Acad. Sci. U. S. A. 96 (11), 6553–6557.
Paraszkiewicz, K., Frycie, A., Słaba, M., Długoński, J., 2007. Enhancement of emulsifier production by *Curvularia lunata* in cadmium, zinc and lead presence. BioMetals 20 (5), 797–805.
Ponce, B.L., Latorre, V.K., González, M., Seeger, M., 2011. Antioxidant compounds improved PCB-degradation by *Burkholderia xenovorans* strain LB400. Enzyme Microb. Technol. 49 (6–7), 509–516.
Pourova, J., Kottova, M., Voprsalova, M., Pour, M., 2010. Reactive oxygen and nitrogen species in normal physiological processes. Acta Physiol. 198 (1), 15–35.
Prenafeta-Boldú, F.X., Vervoort, J., Grotenhuis, J.T.C., Van Groenestijn, J.W., 2002. Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbons by the fungus *Cladophialophora* sp. strain T1. Appl. Environ. Microbiol. 68 (6), 2660–2665.
Prokai, L., Rivera-Portalatin, N.M., Prokai-Tatrai, K., 2013. Quantitative structure-activity relationships predicting the antioxidant potency of 17β-estradiol-related polycyclic phenols to inhibit lipid peroxidation. Int. J. Mol. Sci. 14 (1), 1443–1454.
Ruiz-Larrea, M.B., Martín, C., Martínez, R., Navarro, R., Lacort, M., Miller, N.J., 2000. Antioxidant activities of estrogens against aqueous and lipophilic radicals; differences between phenol and catechol estrogens. Chem. Phys. Lipids 105 (2), 179–188.
Scrimshaw, M.D., Wahlen, R., Catterick, T., Lester, J.N., 2005. Butyltin compounds in a sediment core from the old Tilbury basin, London, UK. Mar. Pollut. Bull. 50 (12), 1500–1507.
Shoun, H., Fushinobu, S., Jiang, L., Kim, S.W., Wakagi, T., 2012. Fungal denitrification and nitric oxide reductase cytochrome P450nor. Philos. Trans. R. Soc. Lond B Biol. Sci. 367 (1593), 1186–1194.
Siewiera, P., Bernat, P., Różalska, S., Długoński, J., 2015. Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*. Int. Biodeterior. Biodegrad. 104, 258–263.
Siewiera, P., Różalska, S., Bernat, P., 2017. Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under intensive aeration conditions and ascorbic acid supplementation. Environ. Sci. Pollut. Res. http://dx.doi.org/10.1007/s11356-017-8764-4.
Słaba, M., Gajewska, E., Bernat, P., Fornalska, M., Długoński, J., 2013. Adaptive alterations in the fatty acids composition under induced oxidative stress in heavy

metal-tolerant filamentous fungus *Paecilomyces marquandii* cultured in ascorbic acid presence. Environ. Sci. Pollut. Res. 20 (5), 3423–3434.
Słaba, M., Różalska, S., Bernat, P., Szewczyk, R., Piątek, M.A., Długoński, J., 2015. Efficient alachlor degradation by the filamentous fungus *Paecilomyces marquandii* with simultaneous oxidative stress reduction. Bioresour. Technol. 197, 404–409.
Soboń, A., Szewczyk, R., Długoński, J., 2016. Tributyltin (TBT) biodegradation induces oxidative stress of *Cunninghamella echinulata*. Int. Biodeterior. Biodegrad. 107, 92–101.
Sooch, B.S., Kauldhar, B.S., Puri, M., 2014. Recent insights into microbial catalases: isolation, production and purification. Biotechnol. Adv. 32 (8), 1429–1447.
Srivastava, S.C., 1990. In vivo effects of di-n-butyltin dichloride on some enzymes and lipids of rat liver plasma membrane. Toxicol. Lett. 52 (3), 287–291.
Tran, N.H., Urase, T., Kusakabe, O., 2010. Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. J. Water Environ. Technol. 8 (2), 125–140.
Trujillo, M., Kogel, K.H., Hückelhoven, R., 2004. Superoxide and hydrogen peroxide play different roles in the nonhost interaction of barley and wheat with inappropriate *formae speciales* of *Blumeria graminis*. Mol. Plant Microbe Interact. 17 (3), 304–312.
Tsuchiya, Y., Nakajima, M., Yokoi, T., 2005. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. Cancer Lett. 227 (2), 115–124.
Ueno, S., Kashimoto, T., Susa, N., Shiota, Y., Okuda, M., Mutoh, K., Hoshi, F., Watanabe, K., Tsuda, S., Kawazoe, S., Suzuki, T., Sugiyama, M., 2003. Effects of butyltin compounds on mitochondrial respiration and its relation to hepatotoxicity in mice and Guinea pigs. Toxicol. Sci. 75 (1), 201–207.
Whalen, M.M., Loganathan, B.G., Kannan, K., 1999. Immunotoxicity of environmentally relevant concentrations of butyltins on human natural killer cells in vitro. Environ. Res. 81 (2), 108–116.
Zhang, Y., Meng, D., Wang, Z., Guo, H., Wang, Y., Wang, X., Dong, X., 2012. Oxidative stress response in atrazine-degrading bacteria exposed to atrazine. J. Hazard. Mater. 229–230, 434–438.
Zhu, B.T., Lee, A.J., 2005. NADPH-dependent metabolism of 17β-estradiol and estrone to polar and nonpolar metabolites by human tissues and cytochrome P450 isoforms. Steroids 70 (4), 225–244.

Realizację drugiego celu pracy doktorskiej podtrzymywano badając oddziaływanie 17β-estradiolu na wydajność biodegradacji TBT przez grzyb mikroskopowy *M. roberstii.* Po 5 dniach prowadzenia hodowli z dodatkiem TBT (2,5 mg l^{-1}) odnotowano 50% zahamowanie wzrostu szczepu IM 6519. Pomimo wysokiej toksyczności związku w stosunku do wybranego modelu badawczego, widma masowe ekstraktów hodowlanych (uzyskanych techniką chromatografii gazowej sprzężonej ze spektrometrią mas – GC-MS) ukazały obecność DBT i MBT jako głównych metabolitów biodegradacji TBT. Dodatek E2 (10 mg l^{-1}) spowodował nie tylko większy przyrost biomasy grzybowej (o 20%) ale przede wszystkim poprawę wydajności eliminacji TBT (o 14%), w porównaniu z wynikami osiągniętymi dla hodowli niezawierającej estrogenu naturalnego. Użycie wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas umożliwiło przeprowadzenie analizy ilościowej 17β-estradiolu, która wskazywała na szybki ubytek związku przeciwutleniającego z wytworzeniem następujących metabolitów: estronu i estriolu oraz koniugatów E2 i E1 z siarczanami i glukoronidami.

Poddając analizie wpływ badanych związków (wprowadzanych do hodowli oddzielnie lub w postaci mieszaniny) na skład fosfolipidów (PLs) błonowych szczepu *M. robertsii* IM 6519, zidentyfikowano główne klasy lipidów, którymi były fosfatydylocholiny (PC), fosfatydyloetanoloaminy (PE), fosfatydyloglicerole (PG), fosfatydyloseryny (PS) oraz fosfatydyloinozytole (PI). PC i PE przeważały ilościowo nad pozostałymi klasami reprezentując kolejno ponad 66% i 17% całkowitej zawartości lipidów. Grzybnia eksponowana na działanie TBT charakteryzowała się zmniejszoną ilością PC i odwrotnie zwiększoną zawartością PE, co wskazywało na usztywnienie błon komórkowych w obszarze główek fosfolipidowych związane z upakowaniem mniej rozległych obszarowo etanoloamin niż cholin. Podstawowymi kwasami tłuszczowymi budującymi cząsteczki PLs były kwasy: palmitynowy i stearynowy wśród nasyconych oraz oleinowy i linolowy wśród nienasyconych. Analiza głównych składowych (PCA) wykonana dla 14 dominujących z 62 oznaczonych rodzajów PLs wykazała, iż profil fosfolipidowy grzybni inkubowanej w obecności mieszaniny TBT i E2 miał charakter pośredni między profilami zmodyfikowanymi przez badane substancje wprowadzane samodzielnie. Wyniki analizy PCA sugerowały, iż dodatek 17β-estradiolu do hodowli ograniczał zmiany w składzie fosfolipidów błon grzybowych zmodyfikowanych obecnością TBT, umożliwiając dążenie membran do osiągnięcia stanu fizjologicznego.

Analizy mikroskopowe przeprowadzone z użyciem jodku propidu ukazały 3500-krotny wzrost intensywności fluorescencji barwnika jądrowego w strzępkach szczepu *M. robertsii*

IM 6519 odzwierciedlając zwiększoną przepuszczalność błon komórek poddanych ekspozycji na działanie TBT. W obecności 17β-estradiolu nastąpił 3-krotny spadek intensywności fluorescencji fluoroforu, równoznaczny z ograniczeniem liczby komórek uszkodzonych przez TBT, co potwierdziło antyoksydacyjne właściwości ochronne badanego estrogenu naturalnego.

Wyniki badań prowadzonych nad wpływem 17β-estradiolu na efektywność biodegradacji TBT przez grzyb strzępkowy *M. roberstii* opublikowano w pracy oryginalnej „Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*" (International Biodeterioration & Biodegradation, IF = 2,429, 30 pkt MNiSW), stanowiącej pierwsze opublikowane doniesienie na temat możliwości wykorzystania estrogenu naturalnego w celu zwiększenia wydajności biodegradacji lipofilowego, silnie toksycznego związku o właściwościach prooksydacyjnych.

Stwierdzenia cząstkowe dotyczące analizy wpływu wybranych substancji o właściwościach antyoksydacyjnych na rozkład butylowych związków cyny przez szczep IM 6519

1. Suplementacja pożywki wzrostowej witaminą C skutkuje 3-krotnym przyspieszeniem tempa biodegradacji MBT przez szczep grzybowy *M. robertsii* IM 6519.
2. Wyniki analizy ilościowej dialdehydu malonowego – powszechnego markera peroksydacji lipidów – wskazują na skuteczność witaminy C w ochronie grzybowych błon komórkowych przed stresem oksydacyjnym.
3. Induktorem reaktywnych form tlenu i azotu w badanej grzybni jest monobutylocyna – substancja uznawana za najmniej toksyczną spośród butylowych związków cyny.
4. Enzymy antyoksydacyjne tj. katalaza i dysmutaza ponadtlenkowa nie biorą udziału w usuwaniu nadtlenku wodoru i anionorodnika ponadtlenkowego.
5. Estron i 17β-estradiol skutecznie usuwają reaktywne formy tlenu.
6. Przyczyną negatywnego oddziaływania estrogenów naturalnych na proces biodegradacji DBT jest występowanie zjawiska inhibicji kompetycyjnej monooksygenaz cytochromu P450.
7. 17β-estradiol ogranicza zmiany w składzie fosfolipidów i zwiększa integralność błon komórkowych szczepu IM 6519 eksponowanego na działanie TBT, w konsekwencji zwiększając efektywność eliminacji badanego związku.

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[International Biodeterioration & Biodegradation (2015), 104:258-263]

International Biodeterioration & Biodegradation 104 (2015) 258–263

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*

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ARTICLE INFO

Article history:
Received 13 May 2015
Received in revised form
18 June 2015
Accepted 18 June 2015
Available online xxx

Keywords:
Organotins
Tributyltin
Phospholipids
Degradation
Estradiol

ABSTRACT

Tributyltin chloride (TBT) is a toxic and persistent organic pollutant that is extensively employed in a variety of industrial products. TBT, an endocrine disrupting chemical, disturbs lipids homeostasis in living cells and is responsible for generating reactive oxygen species (ROS), which affect cell viability. The aim of this study was to test whether estradiol (E2), which exhibits antioxidant activity, could increase the TBT degradation efficiency by the filamentous fungus *Metarhizium robertsii*. After five days of incubation in Sabouraud growth medium, 27% of TBT (added at the initial concentration 2.5 mg l^{-1}) was converted to dibutyltin (DBT) and monobutyltin (MBT). The data obtained for fungal cultures incubated with TBT and E2 simultaneously demonstrated that, in the presence of estradiol, the TBT-induced growth inhibition was reduced and 44% of TBT was metabolised. Moreover, the determination of phospholipids and the assessment of propidium iodide influx showed a strong disturbance in the membrane integrity and the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio for TBT-stressed mycelia, whereas fungal cells treated with the mixture of TBT and E2 presented a lower level of membrane disintegration. It is suggested that E2 protects fungal cells against TBT toxicity and improves biocide degradation.

1. Introduction

Tributyltin (TBT) is one of the most toxic organotin compounds. Various mechanisms of TBT action have been identified. Among them, oxidative stress, associated with the overproduction of reactive oxygen species (ROS), has been shown to cause damage to lipids, proteins and nucleic acids (Ishihara et al., 2012). Moreover, interference in the membrane structure by lipophilic TBT molecules leads to changes in the membrane permeability and the functioning of the transmembrane enzymes (Pagliarani et al., 2013).

Several studies suggest that biotic degradation is a major pathway for the removal of TBT from the environment (Bernat et al., 2013). Nevertheless, only a few fungal strains have been described as TBT degrading microorganisms. Among the fungi, *Coniophora puteana, Trametes versicolor*, *Chaetomium globosum* and *Cunninghamella elegans* have been mentioned (Bernat et al., 2014).

Because the increase in the ROS levels affects the fungal growth and decreases the fungal survival (Culakova et al., 2012), it has been postulated that oxidative stress could reduce TBT degradation by microorganisms. Therefore, the aim of this study was to increase the efficiency of TBT degradation of a *Metarhizium robertsii* strain by adding an antioxidant compound that could increase its tolerance to oxidative stress.

Estradiol (E2) was chosen as an antioxidant. Literature data indicate that the treatment of living cells with E2 decreases free radical formation and reduces lipid peroxidation levels, which further improves the membrane fluidity (Kumar et al., 2011). Moreover, according to Ishihara et al. (2014), E2 diminishes TBT-induced neurotoxicity. E2 is also the main estrogen compound released into the environment, which is excreted by all humans and animals (Lucas and Jones, 2006). Therefore, E2 and TBT are widespread pollutants that could occur simultaneously in many aquatic and terrestrial environments (Dubascoux et al., 2008).

The impact of E2 on the TBT degradation process was examined. Moreover, because TBT caused modifications in the membrane structure, changes in the phospholipid profile and membrane

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http://dx.doi.org/10.1016/j.ibiod.2015.06.014
0964-8305/

permeability of fungal cells simultaneously exposed to E2 and TBT were described.

2. Materials and methods

2.1. Chemicals

Tributyltin chloride, other organotins and estradiol were purchased from Sigma–Aldrich. The other chemicals were purchased from Supelco, J. T. Baker, Fluka and POCh (Gliwice, Poland). All of the chemicals were high-purity grade reagents. Stock solutions of TBT and E2 at 5 mg ml^{-1} were prepared in ethanol. The phospholipid standards: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Microorganism and growth conditions

A fungal strain *M. robertsii* IM 6519, obtained from the collection of the Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, Poland, was applied in this study. This entomopathogenic strain is capable of degrading 4-n-nonylphenol (Różalska et al., 2015b).

Fourteen-day spores of an *M. robertsii* strain from cultures on ZT agar slants (Paraszkiewicz et al., 2007) were used to inoculate 20 ml Sabouraud medium (Difco, pH 5,8) in 100-ml Erlenmeyer flasks. The cultivation was carried out for 48 h at 28 °C on a rotary shaker (160 rpm). Subsequently, a homogenous preculture (20%) was introduced into the Sabouraud medium with TBT (2.5 mg l^{-1}) and/or E2 (10 mg l^{-1}) or without the compounds in the control cultures. The cultures were incubated for 5 days at 28 °C on a rotary shaker (160 rpm).

2.3. Organotins analysis

The analysis of organotin compounds was carried out according to the procedure by Bernat and Długoński (2009). The cultures after 5 days of incubation were acidified (pH 2), then transferred into Falcon tubes and centrifuged for 10 min at 8000 ×*g*. The fungal biomass suspended in methanol with glass beads was homogenized twice for 3 min at 30 m/s. A homogenate was mixed with supernatant. An extraction process was carried out twice with 20 ml mixture of 0.05% tropolone in hexane. The organic layers were dehydrated using anhydrous Na_2SO_4 and evaporated to dryness. A precipitate was dissolved in 2 ml of hexane. Next, a derivatization reaction of organotins was performed. After adding 0.5 ml of a Grignard reagent (3 M methyl magnesium bromide in anhydrous diethyl ether), the reaction was carried out for 20 min in darkness at room temperature. The derivatization process was interrupted by the slow addition of cooled, 20% aqueous NH_4Cl (2 ml). After centrifugation (2000 ×*g*) for 10 min, 250 μl of the upper layer was transferred into the chromatographic vials for a GC/MS analysis.

A methylated organotin compounds were determined using a gas chromatograph (Hewlett–Packard Model 6890 equipped with HP 5973 Mass Detector). The temperature of the column (HP 5 MS methyl polysiloxane, 30 m × 0.25 mm × 0.25 μm) reached 60 °C and was maintained for 3 min. Next, the temperature was increased to 280 °C at a rate of 20 °C min^{-1} and maintained for 3 min. The flow rate of helium as a carrier gas was set at 1.2 ml min^{-1}. The temperature of the injection port was 250 °C. The injection volume was 2 μl. The organotin compounds were separated and identified based on their retention times and typical ions. Quantitative ions 165 for MBT, 151 for DBT and 193 for TBT were performed.

2.4. E2 analysis

The analysis of E2 and its metabolites was carried out using the QuEChERS method (Anastassiades et al., 2003). Five-day fungal cultures were transferred into 50-ml tubes. Then, 10 ml of acetonitrile with glass beads was added. Using a ball mill, biomass homogenization was carried out twice for 10 min. A mixture of salt (2 g $MgSO_4$, 0.5 g NaCl, 0.5 g $C_6H_5Na_3O_7 \times 2H_2O$, 0.25 g $C_6H_6Na_2O_7 \times 1.5H_2O$) was added to the homogenate and vortexed for 2 min. Then, the sample was centrifuged for 5 min (4 °C at 2000 ×*g*). Finally, 2 ml of the top layer was collected for the chromatographic analysis.

Estrogens were determined using liquid chromatography-mass spectrometry (LC-MS/MS) with an Agilent 1200 coupled with mass detector QTRAP 3200 (AB Sciex, USA) (Różalska et al., 2015a). The injection volume was 5 μl. The separation was carried out in a capillary Zorbax Eclipse XDB-C18 column (50 mm × 4.6 mm, 1.8-μm particle size; Agilent, USA) at 35 °C. The total flow rate of the mobile phase (solvent mixtures A and B) was 0.6 ml min^{-1}. The gradient was started at 80% B and 20% A; at 2 min it was linearly increased to 100% B and held for 4 min; then, in 2 min it was linearly decreased to 20% B. The mobile phase consisted of 5 mM ammonium formate in water (A) and 5 mM ammonium formate in methanol (B). The mass spectrometric detection was conducted on a 3200 QTRAP LC-MS/MS system from ABSciex (USA) equipped with a turbo ion spray source. The detection of estrogens was performed with the use of an MS/MS detector working in the multiple reaction monitoring (MRM) negative ionization mode. Additionally, a mass spectrometry of E2 potential metabolites was performed using electrospray ionisation (ESI) in a negative ion mode. Information-dependent acquisition (IDA) methods: EMS (enhanced MS) and PI (precursor ion)/EPI (enhanced product ion), were used to identify possible E2 metabolites. Both Q1 and Q3 were operated at low and unit mass resolution, respectively. The spectra were obtained over a range from m/z 50 to m/z 500.

2.5. Phospholipids (PLs) extraction and analysis

Phospholipids of *M. robertsii* from the stationary phase of growth (120 h) were collected. To perform the extraction of lipids, the Folch method (Folch et al., 1957) was applied with some modifications. The fungal biomass was filtered and transferred to a 50-ml tube with a 10 ml of a chloroform/methanol mixture (2:1 v/v). The samples were homogenized with glass beads into 50-ml tubes using a ball mill (Retsch, Germany). The homogenization process was conducted twice for 10 min each time. To separate the methanol and chloroform layers, 2 ml of 0.85% NaCl was added to the homogenate. The mixture was vortexed for 2 min. Then, the sample was centrifuged for 5 min (2000 ×*g*). The bottom layer was collected, dehydrated by the addition of anhydrous sodium sulphate and finally evaporated. Extracts dissolved in 2 ml of methanol were analysed according to the procedure of Bernat et al. (2014) using the LC-MS/MS technique. The chromatographic conditions had been described previously by Bernat et al. (2014).

Using MarkerView™ Software, principal component analysis (PCA), indicating variations in phospholipids contents, was performed. Additionally, the Double Bonds Index (DBI) describing fatty acid unsaturation was calculated in accordance with the formula: $DBI = [\sum(N \times \%\ \text{lipid molecular species})]/100$, where N is a number of double bonds in each lipid molecular species and % refers to the % of a complex lipid class (Su et al., 2009).

2.6. Test of the cell membrane permeability

The test was performed according to the modified procedure of Słaba et al. (2013). First, 1 ml each of the control and the tested samples was transferred into Eppendorf tubes and centrifuged for 2 min at 6000 ×g. After removing the supernatant, the mycelium was suspended in 1 ml PBS (Phosphate Buffered Saline) and centrifuged under the same conditions. Next, the previous step of the procedure was repeated, 1 μl of propidium iodide (PrI, 2 μM) was added and the mixture was incubated for 5 min in the dark. Subsequently, the sample was centrifuged in the above-described conditions and the final precipitate was suspended in 0.5 ml PBS. Microscopic samples were prepared and examined on a confocal microscope (Pascal II) at the wavelength $\lambda = 543$ nm. The total area of fungal hyphae and areas emitting red fluorescence were calculated. Using these data, the percentage of hyphae exhibiting fluorescence and the percentage of damaged/dead cells were simultaneously calculated.

2.7. Statistical analysis

Experiments were carried out in triplicate. Statistical analyses were performed using Excel 2007 (Microsoft Corporation, USA) and MarkerView™ Software 1.2.1 (AbSciex, USA).

3. Results

3.1. The growth of the M. robertsii strain in the presence of TBT and/or E2

Fungal biomass growth after exposure to TBT (2.5 mg l^{-1}), E2 (10 mg l^{-1}) or a mixture of these two compounds was investigated. After 5 days of incubation in Sabouraud medium, the dry fungal weight of the control biomass was estimated to be 16.7 g l^{-1} (Fig. 1). In the samples with E2, a slight decrease in the fungal growth (approximately 8%) was observed. A strong modification (50%) occurred in the cultures with the addition of TBT, whereas 31% growth inhibition was noted in the samples containing both chemicals.

3.2. E2 influence on TBT degradation and TBT impact on E2 transformation by M. robertsii

The effect of E2 on the TBT degradation process and the influence of TBT on the E2 transformation were examined. After 5 days of incubating the fungus with the organotin, the level of tributyltin decreased by almost 30% (Fig. 2). The GC/MS chromatogram of *M. robertsii* culture extracts incubated with TBT revealed that, despite the inhibition of the mycelial growth, TBT was converted to less toxic derivatives, which were dibutyltin (DBT) and monobutyltin (MBT). The TBT elimination was associated with the fungal growth, which suggests a cometabolic nature of TBT degradation. On the other hand, during the study on the efficiency of tributyltin elimination by *C. elegans* the level of over 70% TBT conversion to derivatives had been noted under the same culture conditions (Bernat and Długoński, 2007).

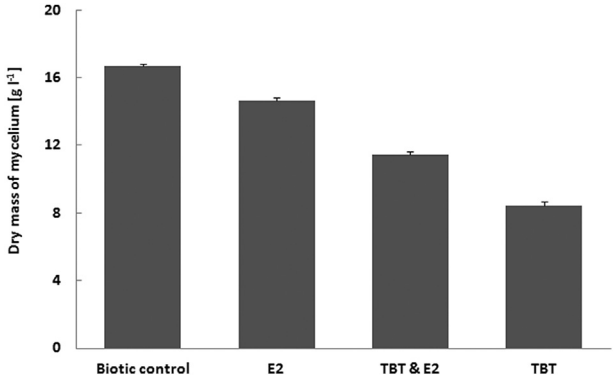

Fig. 1. Dry mass of *M. robertsii* mycelium after 5-day incubation on Sabouraud medium with TBT (2.5 mg l^{-1}) and/or E2 (10 mg l^{-1}) or without the examined compounds.

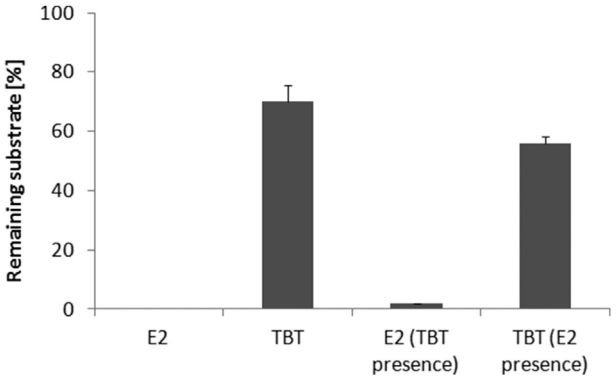

Fig. 2. Residues of TBT (2.5 mg l^{-1}) and/or E2 (10 mg l^{-1}) after 5 days of *M. robertsii* culture on Sabouraud medium.

The concentration of estradiol in the culture medium fell rapidly (Fig. 2). The subsequent change in the estradiol concentration was accompanied by the presence of some E2 metabolites. Estrone, estriol, estrone 3-sulphate, estradiol-3-sulphate, estradiol-17-glucuronide and estrone-3-glucoronide were found in the fungal culture.

The differences between the dynamics of TBT debutylation were observed in the absence and in the presence of estradiol (Fig. 2). The results showed that butyltin transformation in the presence of estrogen was better compared to the culture without the steroid. Approximately 44% of TBT was transformed during 5 days of incubation. On the other hand, the presence of TBT slightly decreased the rate of the estrogen transformation. Moreover, the conjugate formation was observed during the incubation of the fungus in the medium with both substrates.

3.3. M. robertsii phospholipid profile in the presence of TBT and E2

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were determined as major classes of *M. robertsii* membrane phospholipids (Fig. 3). The PC class represented 66.8% of the total phospholipid content, followed by PE (17.3%) and PG (8.2%), while PS and PI stayed on a similar level (approximately 3.5%). Upon exposure to E2, the PE and PC contents decreased and increased, respectively, compared with the control. After TBT treatment, the inverse dependence was observed.

PC and PE are key components of the membranes of eukaryotic cells (Fajardo et al., 2011). The ratio of these two phospholipid classes is influenced by toxic substances or changes in the culture conditions. Moreover, the PC/PE ratio has an effect on membrane fluidity. A choline molecule as a head of phospholipid has a larger size compared to the acyl chains, so the enrichment of the membrane with PC results in an increase in membrane fluidity. In the case of PE, the surface of the acyl chains is larger than the surface of the phospholipid heads (ethanolamine), which leads to the reduction in the membrane fluidity (Fajardo et al., 2011). The ratio of PC to PE in the examined samples was designated. In the biotic control, a value of the PC/PE ratio of 3.85 was estimated. An increase

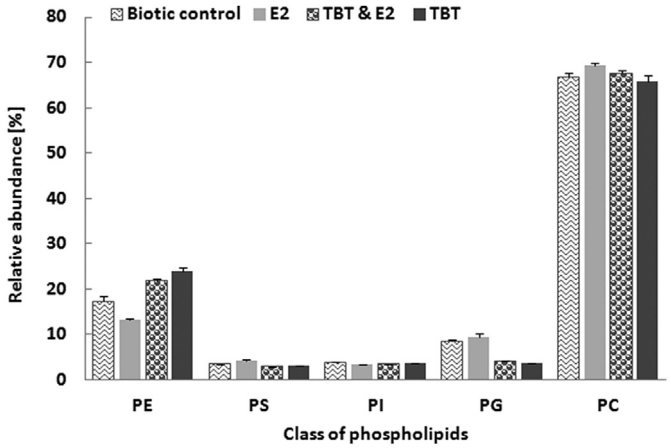

Fig. 3. Percentage composition of particular phospholipids classes of *M. robertsii* cell membranes treated with TBT and/or E2. PC phosphatidylcholine, PE phosphatidylethanolamine, PG phosphatidylglycerol, PI phosphatidylinositol, PS phosphatidylserine.

in the membrane fluidity was found in cultures supplemented with E2, where almost doubling the PC/PE ratio in relation to the control was determined. In contrast, a reduction of the membrane fluidity of the fungus in the presence of only TBT and with the addition of E2 was observed. In cultures with both test compounds, the most similar the PC/PE ratio to the control was defined.

Phospholipids of the fungal strain of *M. robertsii* are composed mainly of 16- and 18- carbon fatty acids (Fig. 4). Among the saturated fatty acids, the highest contents were recorded for palmitic (C16:0) and stearic (C18:0) acids. However, oleic (C18:1) and linoleic (C18:2) acids were recognized as the most abundant among unsaturated fatty acids. After the addition of the compounds, the biggest differences were observed for PC 18:1/18:1 and PC 18:2/18:2. The content of PC 18:1/18:1 decreased by approximately 31% after the exposure to TBT and increased by approximately 30% after the treatment with E2. On the other hand, the amount of PC 18:2/18:2 grew by approximately 16% due to the action of TBT and decreased by approximately 28% when treated with E2. TBT had an opposite effect on the phosphatidylethanolamine content (Fig. 3). An increase in the amount of phospholipids in the presence of TBT in maintained, in contrast to its decline in the presence of E2. The obtained results indicate the greatest influence of the TBT presence on the PE content.

In total, 62 different species of phospholipids in each sample were determined (data not shown). As a result of the principal component analysis (PCA), variability in the contents of the most abundant 14 phospholipids was specified in each extract (Fig. 5). A projection of the resulting sample scores for the first and second principal components showed the similarities and differences between the samples. The greatest differences were observed between the control and other samples, which were separated primarily along the PC1 axis; PC1 explained 92.3% of the variance of the dataset. The obtained results also indicate that the changes in the lipidome of the TBT and E2 treated samples occurred in the centre of both compounds when the compounds were introduced separately.

Moreover, the Double Bond Index (DBI), characterizing the degree of fatty acids unsaturation, remained at a constant level (~2.6) for all examined samples.

3.4. Changes in *M. robertsii* membrane permeability after the exposure to TBT and/or E2

The effects of tributyltin and estradiol on the fungal cell membrane integrity were checked. Fungal cultures after 5-day incubation on Sabouraud medium were stained with propidium iodide (PrI). The dye, after passing through the damaged cell membrane and binding to the DNA, was fluorescently detected (Wadhawan et al., 2011). The ratio of the red fluorescence surface area to the mycelium area was calculated. Changes in the membrane permeability of *M. robertsii* after a 5-day culture in four different test systems are shown in Fig. 6. In the presence of all toxic compounds, an increase in the membrane permeability was observed on the control background. The greatest penetration of cells by dye was recorded in TBT-exposed cultures, achieving more than 3500-fold fluorescence. For cells grown in the presence of only E2 or both E2 and TBT, lower fluorescence was observed, over 1000-fold and over 1100-hold compared to the control, respectively. The latest results are very similar, but due to the high standard deviations, their statistical significance is negligible. All of the observed fluorescence had a different character. The most intense red light of whole fungal hyphae was observed in the samples with the addition of TBT. On the other hand, in the samples with E2, the fluorescence occurred at specific points only, which was probably related to the staining of the cell nucleus. In the mycelium treated

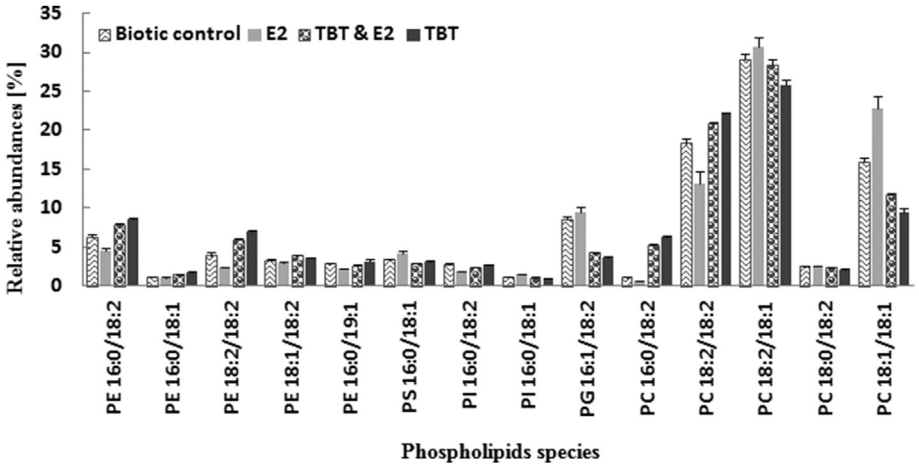

Fig. 4. The relative percentages of major *M. robertsii* phospholipids species after exposure to TBT and/or E2. PC phosphatidylcholine, PE phosphatidylethanolamine, PG phosphatidylglycerol, PI phosphatidylinositol, PS phosphatidylserine.

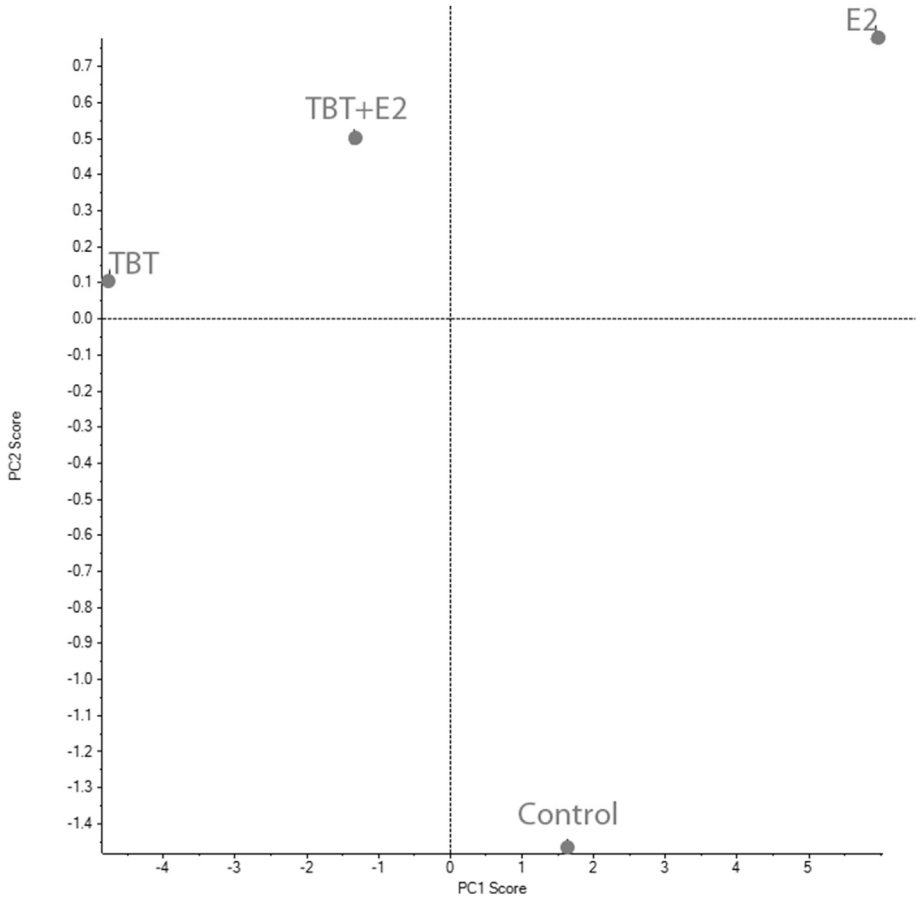

Fig. 5. Principal component analysis (PCA) of 14 phospholipid species in the *M. robertsii* mycelium treated with TBT and/or E2. Principal components of PC1 and PC2 accounted for 92.3% and 4.2%, respectively.

with both xenoestrogens, all hyphae showed lower fluorescence intensity.

4. Discussion

TBT degradation by microorganisms is an attractive method for the removal of organotins from polluted environments. However, cells exposed to TBT suffer oxidative stress. Therefore, the aim of this study was to increase the efficiency of TBT degradation by *M. robertsii* by the addition of an antioxidant compound that could increase its tolerance to oxidative stress. E2 was chosen as antioxidant compound.

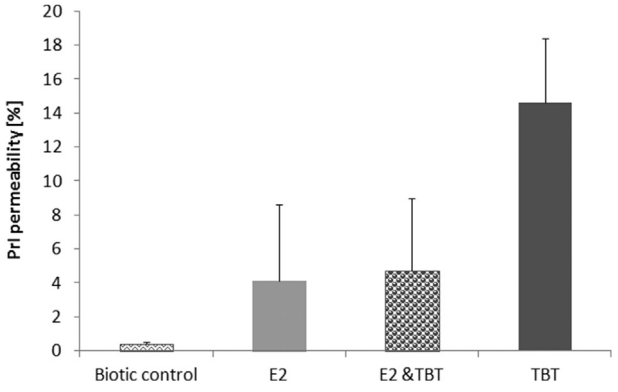

Fig. 6. The permeability of *M. robertsii* cell membranes after a 5-day culture on Sabouraud medium supplemented with xenoestrogens, in relation to propidium iodide.

The examined fungus was able to grow in the presence of TBT. However, TBT at an initial concentration of 2.5 mg l^{-1} caused growth inhibition compared to the control after 5-day of incubation. On the other hand, a decrease in the biomass of another TBT-degrading strain, *C. elegans*, cultured on Sabouraud medium for 7 days with the addition of 10 mg l^{-1} TBT reached 22% (Bernat and Długoński, 2007). A significant difference in the growth inhibition of both strains proves that the species *Metarhizium* is more sensitive to the presence of TBT in the environment. However, the simultaneous presence of both compounds (E2 and TBT) in the *M. robertsii* culture resulted in a strong increase in growth intensity, compared with the effect of TBT alone.

On the other hand, E2 did not inhibit the fungal growth. Interestingly, the cultures supplemented with both tested compounds presented better growth than those treated with TBT, suggesting that the estrogen partly reduced the negative effects induced by TBT.

In addition to the increase in the cell growth caused by the antioxidant compound, improved TBT degradation in an aqueous medium was observed in the presence of E2.

The examined strain was able to transform E2 into other metabolites. It seems that the oxidation of E2 into E1 diminishes the estrogenicity of the sample, although E1 still retains the estrogenicity level at 0.1–0.2 of the E2 equivalent (Khanal et al., 2006). The conjugation of parent estrogens represent potential “detoxification” reactions that may protect the cell from estrogen toxicity. In

mammals during conjugation, estrogens with higher polarity are produced and then excreted from the organisms with urine (Lai et al., 2002).

Interestingly, E2 counteracts the cell growth inhibition caused by the exposure to TBT in glucose-grown cells, indicating a protective effect of E2. Moreover, a decrease in the propidium iodide influx suggests that the presence of estrogen diminishes the membrane perturbation.

TBT alters lipid homeostasis by disturbing phospholipids and fatty acids composition (Bernat et al., 2014). The physical properties of the membrane are determined by the phospholipid composition (de Kroon, 2007; de Kroon et al., 2013). Among them, PC and PE play a crucial role in the maintenance of the proper fluidity and permeability of the membrane (Quartacci et al., 2001; Bou Khalil et al., 2010). It was documented that PC tends to form a bilayer, whereas PE has a strong propensity to build a non-bilayer hexagonal phase. The results obtained here revealed an increased level of PE in TBT supplemented mycelia combined with an enhanced propidium iodide influx, indicating strong membrane permeabilisation. However, the addition of E2 partly alleviated the toxic TBT influence on fungal phospholipids.

The treatment of rat hippocampal slices with E2 in a dose-dependent manner suppressed the neuronal cell death induced by TBT. E2 reduced TBT-induced neuronal injury via an ER-dependent non-genomic pathway. The attenuation of oxidative stress downstream of Akt activation is considered to be involved in the neuroprotection mediated by E2 (Ishihara et al., 2014). On the other hand, the suppressive effects of E2 on oxidative stress are believed to be receptor-independent and are attributed to the antioxidant properties of estrogen related to the presence of a hydroxyl group at the C3 position on the steroid A ring (Behl et al., 1997). Considering these findings, it can be assumed that a reduction in the growth inhibition influenced by TBT may be caused by the suppression of oxidative stress mediated by E2 or its metabolites. However, further studies are needed to reveal the mechanisms underlying the attenuation of oxidative stress by E2.

5. Conclusions

It has been reported that TBT induced oxidative stress by the overproduction of reactive oxygen species (ROS). The organotin presence causes cellular oxidative injury such as lipid peroxidation, protein oxidation and DNA damage.

The presented study indicates that the fungus *M. robertsii* is able to metabolize TBT. Moreover, the data obtained for fungal cultures incubated with TBT and E2, which is a known antioxidant, demonstrated higher biomass growth and an increased organotin degradation rate. The determination of phospholipids and the assessment of propidium iodide influx revealed a strong disturbance in the membrane integrity and the PC/PE ratio for TBT-stressed mycelia. However, a lower membrane permeability was found in fungal cells treated with the mixture of TBT and E2. Therefore, it cannot be excluded that the attenuation of oxidative stress induced by TBT might be involved in E2-mediated protection. It seems that lowering the ROS generation by E2 facilitates TBT degradation.

Acknowledgments

This study was supported by the National Science Centre in Cracow, Poland (Project No. UMO-2011/01/B/NZ9/02898).

References

Anastassiades, M., Lehotay, S.J., Stajnbaher, D., Schenck, F.J., 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J. AOAC Int. 86, 412–431.

Behl, C., Skutella, T., Lezoualc'h, F., Post, A., Widmann, M., Newton, C.J., Holsboer, F., 1997. Neuroprotection against oxidative stress by estrogens: structure-activity relationship. Mol. Pharmacol 51, 535–541.

Bernat, P., Długoński, J., 2007. Tributyltin chloride interactions with fatty acids composition and degradation ability of the filamentous fungus *Cunninghamella elegans*. Int. Biodeter. Biodegrad. 60, 133–136.

Bernat, P., Długoński, J., 2009. Isolation of *Streptomyces* sp. strain capable of butyltin compounds degradation with high efficiency. J. Hazard. Mater. 171, 660–664.

Bernat, P., Gajewska, E., Szewczyk, R., Słaba, M., Długoński, J., 2014. Tributyltin (TBT) induces oxidative stress and modifies lipid profile in the filamentous fungus *Cunnighamella elegans*. Environ. Sci. Pollut. Res. 21, 4228–4235.

Bernat, P., Szewczyk, R., Krupiński, M., Długoński, J., 2013. Butyltins degradation by *Cunninghamella elegans* and *Cochliobolus lunatus* co-culture. J. Hazard. Mater. 246–247, 277–282.

Bou Khalil, M., Hou, W., Zhou, H., Elisma, F., Swayne, L.A., Blanchard, A.P., Yao, Z., Bennett, S.A., Figeys, D., 2010. Lipidomics era, accomplishments and challenges. Mass Spectrom. Rev. 29, 877–929.

Culakova, H., Dzugasova, V., Gbelska, Y., Subik, J., 2012. CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine) producing ROS affects growth and viability of filamentous fungi. FEMS Microbiol. Lett. 328, 138–143.

de Kroon, A.I., 2007. Metabolism of phosphatidylcholine and its implications for lipid acyl chain composition in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 1771, 343–352.

de Kroon, A.I., Rijken, P.J., De Smet, C.H., 2013. Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. Prog. Lipid Res. 52, 374–394.

Dubascoux, S., Heroult, J., Le Hécho, I., Potin-Gautier, M., Lespes, G., 2008. Evaluation of a combined fractionation and speciation approach for study of size-based distribution of organotin species on environmental colloids. Anal. Bioanal. Chem. 390, 1805–1813.

Fajardo, V.A., McMeekin, L., LeBlan, P.J., 2011. Influence of phospholipid species on membrane fluidity, a meta-analysis for a novel phospholipid fluidity index. J. Membr. Biol. 244, 97–103.

Folch, J., Lees, M., Sloane-Stanley, G., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 199, 833–841.

Ishihara, Y., Fujitani, N., Kawami, T., Adachi, C., Ishida, A., Yamazaki, T., 2014. Suppressive effects of 17β-estradiol on tributyltin-induced neuronal injury via Akt activation and subsequent attenuation of oxidative stress. Life Sci. 99, 24–30.

Ishihara, Y., Kawami, T., Ishida, A., Yamazaki, T., 2012. Tributyltin induces oxidative stress and neuronal injury by inhibiting glutathione S-transferase in rat organotypic hippocampal slice cultures. Neurochem. Int. 60, 782–790.

Khanal, S.K., Xie, B., Thompson, M.L., Sung, S., Ong, S.K., Van Leeuwent, J., 2006. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. Environ. Sci. Technol. 40, 6537–6546.

Kumar, P., Kale, R.K., Baquer, N.Z., 2011. Estradiol modulates membrane-linked ATPases, antioxidant enzymes, membrane fluidity, lipid peroxidation, and lipofuscin in aged rat liver. J. Aging Res. 2011, 580245.

Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. 32, 113–132.

Lucas, S.D., Jones, D.L., 2006. Biodegradation of estrone and 17 beta-estradiol in grassland soils amended with animal wastes. Soil Biol. Biochem. 38, 2803–2815.

Pagliarani, A., Nesci, S., Ventrella, V., 2013. Toxicity of organotin compounds, shared and unshared biochemical targets and mechanisms in animal cells. Toxicol. In Vitro 27, 978–990.

Paraszkiewicz, K., Frycie, A., Słaba, M., Długoński, J., 2007. Enhancement of emulsifier production *by Curvularia lunata* in cadmium, zinc and lead presence. BioMetals 20, 797–805.

Quartacci, M.F., Cosi, E., Navari-Izzo, F., 2001. Lipids and NADPH dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. J. Exp. Bot 52, 77–84.

Różalska, S., Bernat, P., Michnicki, P., Długoński, J., 2015a. Fungal transformation of 17α-ethinylestradiol in the presence of various concentrations of sodium chloride. Int. Biodeter. Biodegrad. 103, 77–84.

Różalska, S., Soboń, A., Pawłowska, J., Wrzosek, M., Długoński, J., 2015b. Biodegradation of nonylphenol by a novel entomopathogenic *Metarhizium robertsii* strain. Bioresour. Technol. 191, 166–172.

Słaba, M., Bernat, P., Różalska, S., Nykiel, J., Długoński, J., 2013. Comparative study of metal induced phospholipid modifications in the heavy metal tolerant filamentous fungus *Paecilomyces marquandii* and implications for the fungal membrane integrity. Acta Biochim. Pol. 60, 695–700.

Su, K., Bremer, D.J., Jeannotte, R., Welti, R., Yang, C., 2009. Membrane lipid composition and heat tolerance in cool-season turfgrasses, including a hybrid bluegrass. J. Am. Soc. Hortic. Sci. 134, 511–520.

Wadhawan, T., Maruska, Z.B., Siripattanakul, S., Hill, C.B., Gupta, A., Prüb, B.M., McEvoy, J.M., Khan, E., 2011. A new method to determine initial viability of entrapped cells using fluorescent nucleic acid staining. Bioresour. Technol. 102, 1622–1627.

III. 3. Opis badania toksycznego oddziaływania di- i tributylocyny (w/bez obecności witaminy C) na wybrane składniki lipidomu grzybowego szczepu *M. robertsii* IM 6519

Obecność substancji o charakterze lipofilowym wpływa na skład ilościowy komponentów błon komórkowych, a także przyczynia się bezpośrednio do zmian płynności i przepuszczalności membran. Z drugiej strony, modyfikacje kluczowych składników błon komórkowych, tj. fosfolipidów, steroli i sfingolipidów wpływają pośrednio na właściwości fizyczne dwuwarstwy lipidowej, takie jak ładunek powierzchniowy, grubość, płynność, przepuszczalność i wewnętrzną krzywiznę (de Kroon i in., 2013; Róg i in., 2009). Co więcej, mikroorganizmy oporne na działanie ksenobiotyków wypracowały pewne mechanizmy adaptacyjne związane zazwyczaj ze strukturalnymi i funkcjonalnymi modyfikacjami ich błon (Angelova i Schmauder, 1999). Ponadto, elastyczność lipidomiczna uznawana jest za szczególną właściwość dla zachodzenia procesów biodegradacji ksenobiotyków (Murínová i Dercová, 2014). Dlatego też, za ostatni cel naukowy niniejszej pracy doktorskiej obrano toksyczne oddziaływanie DBT i TBT (w/bez obecności witaminy C jako najbardziej skutecznej substancji pod względem zwiększania wydajności usuwania MBT oraz ochrony komórek grzybowych przed stresem oksydacyjnym) na wybrane składniki lipidomu grzybowego *M. robertsii,* którego realizacja posłużyła identyfikacji zmian adaptacyjnych szczepu IM 6519 względem badanych związków.

W początkowym etapie, uwagę skupiono na badaniach jakościowych i ilościowych fosfolipidów, sfingolipidów, acylogliceroli i steroli komórkowych badanego szczepu. Analiza HPLC-MS/MS ukazała silnie zmodyfikowany profil fosfolipidowy szczepu *M. robertsii* IM 6519 inkubowanego w warunkach intensywnego napowietrzania ($pO_2 \geq 20\%$), eksponując podwyższoną do niemalże 80% zawartość fosfatydylocholin, znajdującą odzwierciedlenie w zwiększonej płynności błony, co najprawdopodobniej ułatwiło transfer masy, a w efekcie końcowym przyspieszyło proces biodegradacji butylowych związków cyny. W efekcie kolejnej analizy z zastosowaniem techniki HPLC-MS/MS scharakteryzowano skład jakościowy i ilościowy acylogliceroli badanego szczepu, identyfikując łącznie 17 rodzajów lipidów neutralnych, spośród których 5 stanowiły diacyloglicerole (DAG), a pozostałe 12 to triacyloglicerole (TAG). DAG stanowiły ogółem 80% wyizolowanych acylogliceroli, świadcząc o tym, iż szczep *M. robertsii* IM 6519 nie należy do mikroorganizmów olejodajnych akumulujących TAG. Wykorzystanie techniki wysokosprawnej chromatografii cieczowej

sprzężonej z tandemową spektrometrią mas umożliwiło również zidentyfikowanie sfingolipidów budujących błony komórkowe badanego szczepu. Spośród 17 rozpoznanych lipidów, 3 z nich stanowiły ceramidy, 2 dihydroceramidy, 10 sfingomielin, a pozostałymi była sfingozyna oraz dihydrofingozyna. Ostatnim komponentem błonowym poddanym analizie ilościowej był ergosterol jako główny sterol grzybowy. Pomijając zmiany proporcji między komponentami błonowymi, kluczową modyfikacją okazała się być wzmożona synteza fosfolipidów, sfingolipidów i ergosterolu przez komórki badanego szczepu grzybowego poddane ekspozycji na butylowe związki cyny.

Odrębna część eksperymentów prowadzonych z użyciem różnorodnych fluoroforów umożliwiła określenie wpływu badanych substancji na właściwości fizyko-chemiczne błon szczepu IM 6519. Na wstępie, wskazano na hiperpolaryzację błon jako potencjalny mechanizm przyspieszający wiązanie powierzchniowe TBT, a następnie jego biodegradację. Kolejne badania fluorescencyjne ujawniły usztywnienie błon komórkowych w regionie zarówno hydrofilowym jak i hydrofobowym dwuwarstwy lipidowej, będące efektem działania butylowych związków cyny. Natomiast ostatnie analizy ukazały istotne zmniejszenie przepuszczalności błon komórkowych względem jodku propidu po ekspozycji na badane związki. Podsumowując rozległy obszar badań nad składem i właściwościami błon komórkowych będących docelowym miejscem działania związków lipofilowych jakimi są butylocyny, wskazano kilka wyznaczników uniwersalnej reakcji komórek grzybowych *M. robertsii* na obecność tych związków. Wśród biomarkerów fosfolipidowych wyszczególniono a) stosunek fosfatydylocholin do fosfatydyloetanoloamin, b) stosunek zawartości 16- do 18-węglowych kwasów tłuszczowych, c) poziom PC 18:2 18:2 oraz d) wartość indeksu wiązań podwójnych, które ulegały istotnemu zmniejszeniu w obecności butylocyn. Z kolei, wśród zmian właściwości błonowych wyróżniono usztywnienie i zwiększenie integralności membran, co okazało się być zgodne ze wzmożoną syntezą ergosterolu – sterolu powszechnie uznawanego za element wzmacniający struktury błonowej. Jedną z prawdopodobnych przyczyn występowania zmian adaptacyjnych w postaci grubszych i gęściej upakowanych błon komórkowych może być tworzenie przestrzeni do tymczasowego przechowywania biodegradowanych związków.

Równocześnie wykonywane analizy nad oddziaływaniem witaminy C na zmodyfikowaną przez butylowe związki cyny błonę szczepu *M. robertsii* IM 6519 doprowadziły do wyłonienia biomarkerów, identycznych jak wyżej wymienione dla obecności butylowych związków cyny, z tą różnicą, iż ulegały one istotnemu zwiększeniu w trakcie

jednoczesnej ekspozycji grzybni na DBT/TBT i witaminę C zbliżając się do osiągnięcia wartości kontrolnych. Niewątpliwym skutkiem suplementacji przeciwutleniaczem było dążenie do przywrócenia równowagi zarówno w składzie błony jak i jej właściwościach dynamicznych, co hipotetycznie mogło przyspieszyć transport MBT i jego biodegradację, chroniąc komórki szczepu IM 6519 przed stresem oksydacyjnym i nitrozowym.

Wyniki uzyskane podczas realizacji ostatniego celu niniejszej pracy doktorskiej opublikowano w pracy oryginalnej „Lipidomic adaptations of the *Metarhizium robertsii* strain in response to butyltin compounds presence" (Biochimica et Biophysica Acta - Biomembranes, IF = 3,498, 35 pkt MNiSW), będącej pierwszym, szczegółowym doniesieniem charakteryzującym jakościowo i ilościowo kluczowe komponenty i podstawowe właściwości fizyko-chemiczne błon eukariotycznych inkubowanych w obecności toksycznego związku lipofilowego i przeciwutleniacza.

Stwierdzenia cząstkowe dotyczące badania toksycznego oddziaływania di- i tributylocyny (w/bez obecności witaminy C) na wybrane składniki lipidomu grzybowego szczepu *M. robertsii* IM 6519

1. Dodatkowa podaż tlenu do hodowli ($pO_2 \geq 20\%$) skutkuje silnie zmodyfikowanym – bogatym w fosfatydylocholiny – profilem fosfolipidowym szczepu *M. robertsii* IM 6519.
2. Zarówno DBT, jak i TBT indukują zintensyfikowaną biosyntezę fosfolipidów, sfingolipidów i ergosterolu.
3. W odpowiedzi na obecność tributylocyny błony komórkowe szczepu IM 6519 ulegają hiperpolaryzacji.
4. Ekspozycja na działanie butylowych związków cyny powoduje zmniejszenie płynności i przepuszczalności błon komórkowych badanego grzyba mikroskopowego.
5. W wyniku suplementacji podłoża wzrostowego witaminą C, zmodyfikowane obecnością DBT/TBT błony komórkowe *M. robertsii* dążą do osiągnięcia stanu fizjologicznego.
6. Swoistymi biomarkerami obecności DBT/TBT i witaminy C są: stosunek fosfatydylocholin do fosfatydyloetanoloamin; stosunek zawartości 16- do 18-węglowych kwasów tłuszczowych; poziom PC 18:2 18:2; wartość indeksu wiązań podwójnych; poziom ergosterolu; sztywność i integralność błon komórkowych.

Lipidomic adaptations of the *Metarhizium robertsii* strain in response to the presence of butyltin compounds

[Biochimica et Biophysica Acta – Biomembranes (2019), 1861:316-326]

BBA - Biomembranes 1861 (2019) 316–326

Contents lists available at ScienceDirect

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Lipidomic adaptations of the *Metarhizium robertsii* strain in response to the presence of butyltin compounds

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ARTICLE INFO

Keywords:
Bioreactor
Butyltin compounds biodegradation
Metarhizium robertsii
Membrane lipids
Ergosterol
Fungal membrane properties

ABSTRACT

Metarhizium robertsii, a butyltin-resistant filamentous fungus, can rapid and complete biodegradation of di- (DBT) and tributyltin (TBT) under conditions of intensive aeration and ascorbic acid supplementation. In this paper, lipidomic investigations were performed to find the membrane adaptations necessary for effective butyltins degradation. HPLC-MS/MS analysis showed that the phospholipid profile was greatly modified during *M. robertsii* batch cultivation ($pO_2 \geq 20\%$), contributing to increased membrane fluidity and facilitated mass transfer, which could enhance butyltins biodegradation. Intensified biosynthesis of phospholipids, sphingolipids and ergosterol by the mycelia exposed to butyltins was noted. $DIOC_6(3)$ fluorescence intensity for TBT-treated mycelium increased 9-fold pointing to membrane hyperpolarization. Fluorescent studies showed improved membrane rigidity and integrity in response to butyltins presence. Vitamin C supplementation restored membrane composition and dynamic properties, followed by supposed acceleration of transport of monobutyltin and its biodegradation thus protecting the *M. robertsii* cells against oxidative and nitrosative stress.

1. Introduction

Organotin compounds are industrial chemicals used as biocides, polyvinyl chloride stabilizers and catalysts for the manufacture of silicon and polyurethane foams [1]. Tri- and dialkyl compounds, including tributyltin (TBT) and dibutyltin (DBT), are characterized by high biological activity [2]. The toxicity of butyltins has been well described [1, 3, 4], but understanding of their interaction with cells is still limited.

In previous papers, we demonstrated that the *Metarhizium robertsii* strain can grow in the presence of TBT and DBT [5–7]. Moreover, we discovered that bioreactor ($pO_2 \geq 20\%$) application accelerated the biodegradation of the butyltins as much as 10-fold or 40-fold, respectively, for DBT [6] and TBT (data unpublished), compared to flask cultures. This phenomenon was directly associated with the modification of the mycelium structure during intensive aeration. Due to the 'hairy' morphology of the *M. robertsii* mycelium, exchange of nutrients and respiratory gases was facilitated (unlike the situation with densely interwoven pellets), which intensified butyltins biodegradation [6]. A strong dependence between fungal morphology and the productivity of both fungal biomass and secondary metabolites has been observed by Antecka et al. [8]. The size, shape and structure of mycelia have a significant impact on mass transfer within the culture medium. Small, dispersed fungal pellets are better supplied with oxygen and nutrients than large pellets, whose interior may contain inactive or dead biomass [8].

Oxygen supports microbial utilization of various xenobiotics [9–12]. The proper mass transfer between growth environment and a microbial cell with the involvement of membranes is important. Limitation of permeability disrupts whole-cell bioprocesses such as biocatalysis, fermentation, and bioremediation [13]. Therefore, modulated permeability of phospholipid membranes by oxygen supply could also contribute to enhanced biodegradation of lipophilic compounds.

The significance of antioxidant-mediated elimination of harmful substances by microorganisms has recently been identified [14, 15]. We have also reported that vitamin C supplementation accelerates the biodegradation of monobutyltin (MBT), i.e. the main byproduct of TBT and DBT biotransformation [6], which was an inducer of reactive oxygen and nitrogen species in the *M. robertsii* mycelium [7]. Antioxidants improve biodegradation of butyltins, polychlorobiphenyls and heavy metals via the protection of microbial cells from oxidative stress and membrane damage [6, 14, 15] but information about cell adaptation is unavailable.

The cell membrane is the prime target for any toxicants, especially those with a lipophilic structure. Alterations in both the composition and properties of biological membranes in response to membrane active substances, such as aromatic hydrocarbons and pharmaceuticals [16–21], are a common phenomenon. Xenobiotic-resistant

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https://doi.org/10.1016/j.bbamem.2018.06.007
Received 28 February 2018; Received in revised form 6 June 2018; Accepted 11 June 2018
Available online 14 June 2018
0005-2736/

microorganisms have developed some cell adaptations, usually related with structural and functional modifications of their membranes [22]. Moreover, lipidomic flexibility is thought to be an essential feature condition for chemicals biodegradation [23]. According to literature data focusing on organotin degraders, little information is available on fatty acid and/or phospholipid profiles of fungal (*Cunninghamella*, *Metarhizium*) and bacterial (*Pseudomonas*) strains treated with TBT [5, 24, 25]. A more comprehensive approach is needed to recognize and understand the microbial adaptation responses to the presence of organotins.

In this study, an attempt was made to identify lipidomic adaptations of the *Metarhizium robertsii* strain for effective butyltins biodegradation conducted at a bioreactor scale and mediated by vitamin C supplementation. The investigations involved analyses of the membrane components such as phospholipids, sphingolipids, neutral lipids and ergosterol as well as membrane properties such as potential, fluidity and permeability.

2. Materials and methods

2.1. Chemicals

$DBTCl_2$, TBTCl, vitamin C, acylglycerol standards, ergosterol (ERG) standard, and fluorescent probes were purchased from Sigma-Aldrich (Germany). The phospholipid (PL) and sphingolipid (SPL) standards were obtained from Avanti Polar Lipids (USA) and Cayman Chemical (USA), respectively. The other chemicals were obtained from J.T. Baker Chemical Co. (the Netherlands) or POCH S.A. (Poland). Stock solutions of TBTCl ($5\ mg\ ml^{-1}$) and $DBTCl_2$ ($10\ mg\ ml^{-1}$) were prepared in ethanol, while vitamin C was dissolved in distilled water to a final concentration of $10\ mg\ ml^{-1}$.

2.2. Microorganism and growth conditions

In this study, *Metarhizium robertsii* IM 6519, an *Ascomycete* strain obtained from the Department of Industrial Microbiology and Biotechnology (University of Lodz, Poland) was applied. The ability of the fungus to degrade butyltins was described in our previous reports [5–7].

Batch cultivations of IM 6519 strain in a 3.6-l bioreactor (Labfors 5; Iris 6 software; Infors AG, Switzerland) were conducted in accordance with the procedure by Siewiera et al. [6]. The cultures – with DBT ($20\ mg\ l^{-1}$) or TBT ($2.5\ mg\ l^{-1}$) introduced alone or as a mixture with vitamin C ($20\ mg\ l^{-1}$), or without the compounds (as a control culture) – were incubated for 24 h. Applied concentrations of the above-mentioned substances had been selected experimentally in previous studies, so as not to negatively affect the fungal growth (vitamin C) or to cause 50% inhibition of the biomass quantity (TBT). Despite the high tolerance of the tested strain to DBT (even to $100\ mg\ l^{-1}$), the chosen concentration was relatively low but probable to occur in reservoirs intended for purification. One tested system equated to one batch culture. Three subsamples were collected each time after 3 and 24 h of incubation. The choice of sampling time was dictated by the butyltins biodegradation curve. In a 3-hour culture of the *M. robertsii* strain, the levels of TBT and DBT, as well as MBT were still high, while after 24 h of incubation, the compounds amounts were residual [6, data unpublished].

2.3. Phospholipid analysis

Phospholipids were isolated according to the Folch method [26] with a few modifications. The mycelium (125 mg) was placed into an Eppendorf tube containing 333 µl of methanol, 666 µl of chloroform and glass beads. The homogenization process was performed thrice for 20 s at $30\ m\ s^{-1}$ with the use of FastPrep-24 (MP Biomedicals). After the removal of glass beads, 100 µl of 0.85% NaCl was added to the homogenate. The samples were vortexed for 1 min and centrifuged at $3000 \times g$ for 5 min. The bottom phase was transferred to a fresh Eppendorf tube and dried.

The obtained extract was dissolved in 1 ml of methanol and then analyzed using the LC-MS/MS technique (LC Agilent 1200 coupled with an AB Sciex QTRAP 4500 tandem mass spectrometer). A Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, Torrance, CA, USA) heated to 40 °C with a flow rate of $500\ µl\ min^{-1}$ was applied. Water and methanol were used as solvents, both supplemented with 5 mM ammonium formate. The solvent gradient started at 70% B, after 0.25 min increased to 95% B during 1 min, and maintained at 95% B for 7 min before returning to 70% B over 2 min. The mass spectrometer ion source worked in negative mode, spray voltage −4.500 V, curtain gas 25, nebulizer gas 60, auxiliary gas 50, and temperature 600 °C. The data analysis was performed with Analyst™ v1.6.2 software (AB Sciex, Framingham, MA, USA).

The Double Bond Index (DBI), whose values indicate the degree of fatty acid unsaturation, was calculated in accordance with Su et al. [27].

2.4. Quantification of neutral lipids

Tri- (TAGs) and diacylglycerols (DAGs) contained in lipid extracts (point 2.3.) were separated and analyzed with the use of the liquid chromatography technique with the LC-MS/MS system (as for phospholipid determination). Acylglycerols were detected using ammonium adducts of MRM scans including parent–daughter pairs. For quantification, a standard curve correlating the peak area to moles of TAGs or DAGs was with TAG 50:1 as a representative triglyceride and DAG 36:2 as a representative diglyceride. Chromatographic separation was conducted on a C18 column (the same model as above) heated to 40 °C with a flow rate of $0.6\ ml\ min^{-1}$. The mobile phases were water (A) and a mixture of acetonitrile:isopropyl alcohol (5:2, v:v) with 5 mM ammonium formate and 0.1% formic acid (B). During the analysis, the following gradient was applied: mobile phase B increased from 35% to 100% during 4 min and after 11 min it decreased to 35% during 2 min. The mass spectrometer worked in positive mode with curtain gas 25, ion spray voltage 5500, nebulizer gas 60, auxiliary gas 50, ion source temperature 600 °C, declustering potential 70 and entrance potential 10.

2.5. Ergosterol content

Quantitative analysis of ergosterol from lipid extracts (point 2.3.) was performed using a QTRAP 3200 (AB Sciex) mass spectrometer with the 1200 series HPLC system. Chromatographic separation was performed on a C18 column (the same model as above) heated to 40 °C with a flow rate of $0.8\ ml^{-1}$. The solvents were: water and methanol, both containing 5 mM ammonium formate. The following gradient was used: 40% solvent B from 0 to 1 min, 100% solvent B from 1 to 4 min, 40% solvent B from 4.0 to 4.1 min, 40% solvent B from 4.1 to 6 min. Atmospheric pressure chemical ionization (APCI) was applied, working in positive ion mode with MRM pairs: *m/z* 379.3–69.1 and 379.3–81.3.

2.6. Sphingolipid separation

Sphingolipids (SPLs) were isolated from the fungal membranes according to the Bielawski et al. method [28] with some modifications. Firstly, 125 mg of the biomass was placed into an Eppendorf tube containing 1 ml of the solvent mixture (isopropanol-water-ethyl acetate, 30:10:60, v:v:v) and glass beads. The sample was homogenized thrice for 20 s at $30\ m\ s^{-1}$ using a FastPrep-24. After the removal of glass beads, another 1 ml of the same mixture was added to the homogenate. The Eppendorf content was vortexed for 2 min and then centrifuged at $6000 \times g$ for 5 min. The upper layer was collected to a fresh Eppendorf tube. The precipitate was extracted again. SPL extract

was evaporated at 45 °C to dryness. The precipitate was dissolved in 300 µl of 5 mM ammonium formate in methanol containing 0.2% formic acid. After centrifugation (6000 ×*g*, 5 min), the supernatant was transferred into a dark glass vial.

SPLs were analyzed by the LC-MS/MS technique by using the QTRAP 4500 (AB Sciex) and an Agilent 1200 HPLC system (Santa Clara, CA, USA). The mass spectrometer worked in positive ionization mode, using the MRM pair as described earlier by Bielawski et al. [28].

2.7. Fluorescent studies

The changes in membrane potential were estimated using 3,3′-dihexyloxacarbocyanine iodide ($DIOC_6(3)$) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$) according to the Liao et al. method [29] with some modifications. Investigation of the surface and interior membrane fluidities was performed according to the modified methods of Krasnowska et al. [30], Hofstetter et al. [31] and Kuhry et al. [32] using the following fluorescent labels: *N*,*N*-dimethyl-6-propionyl-2-naphthylamine (Prodan), 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine (Laurdan). Permeability of the fungal membranes was examined using propidium iodide (PI) according to the Siewiera et al. [5] procedure with some modifications.

One mililiter of the culture was transferred into an Eppendorf tube and then centrifuged for 5 min at 6000 ×*g*. Subsequently, 2 µl of $DIOC_6(3)$ (1 µM in PBS), 2 µl $DiBAC_4(3)$ (1 mg ml^{-1} in EtOH), 2 µl of DPH (6 mM in TFH), 12 µl of Laurdan (0.5 mM in EtOH), 8 µl of Prodan (0.1 mM in EtOH), or 2 µl of PI (1 mg ml^{-1} in H_2O) solution was added to the mycelium suspended in 1 ml of PBS (pH 7). The samples labeled with $DIOC_6(3)$, $DiBAC_4(3)$ or PI were incubated for 5 min in the dark at room temperature, while the incubation of the samples labeled with DPH, Laurdan or Prodan was performed for 45 min in a water bath (37 °C). The pellets were washed twice with PBS to remove fluorophores residues and finally resuspended in 1 ml of the buffer.

The fluorescence intensity of all samples was measured with a FLUOstar Omega spectrofluorometer (BMG Labtech) with the following parameters: λ_{ex} = 540–10, λem = 630–10, gain = 2000 for PI; λ_{ex} = 485–12 nm, λ_{em} = 520 nm, gain = 1000 for $DIOC_6(3)$ and $DiBAC_4(3)$; λ_{ex} = 355 nm, λ_{em} = 460 nm, gain = 800 for DPH; λ_{ex} = 355 nm, λ_{em} = 510–10 nm, gain = 800 for Laurdan and Prodan. The target for all measurements was 70%.

The obtained values were decreased by the fluorescence intensity of the backgrounds (PBS without the pellets). The results were shown as a fluorescence unit per 1 mg of the fungal biomass.

2.8. Statistical analyses

Experiments were conducted in triplicate. Sample variability is given as a standard deviation (± SD). Statistical significance was tested with two-way ANOVA using treatment and time as factors on transformed data. The analyses were performed using Statistica 13.1 (StatSoft, USA). Values at $p < 0.05$ were considered significant.

3. Results and discussion

3.1. The phospholipid profile

Phospholipids (PLs) are crucial components of most eukaryotic cell membranes. Alternations in their structure affect the physical properties of the bilayer, such as surface charge, thickness, fluidity, and intrinsic curvature [33].

The main phospholipid classes determined in the *M. robertsii* membrane are presented in Table 1. Phosphatidylcholines (PC) constituted about 72.5–79.4% of the total PL content. Phosphatidylinositol (PI) followed by phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) were revealed to be less abundant PLs, representing about 8.5–9.7%, 5.2–6.1%, 2.9–7.9%, 2.5–4.2% and 0.4–0.6%, respectively. In our previous study, a different PL profile of the fungal strain was determined. First of all, the PE content (representing about 17.3%) was second in the phospholipid class in terms of abundance [5]. Moreover, the PG amount exceeded 8% of the total lipid content, while PI remained at a low level (about 3.5%). Divergences in the obtained results were caused by the varied cultures conditions, with emphasis on the cultivation period and oxygen supply. The influence of the culture scale and the growth phase on the lipid composition of microorganisms has been documented [34, 35]. The hairy, relaxed, non-spherical structure of the *M. robertsii* mycelium cultivated in batch conditions [6] seems to be related with an increased content of phosphatidylcholines. Lucchesi et al. [36] and Zink and Rabus [16] also reported higher pools of PC during the biodegradation of tetradecyltrimethylammonium bromide by a *P. putida* strain and alkylbenzenes utilization by an *A. aromaticum* strain. It appears that the modified membrane dynamics required for the transport of oxygen [37] facilitate the uptake of substrate accelerating the butyltins biodegradation by the homogenous *M. robertsii* culture.

Two-way ANOVA showed a significant increase in the amounts of PA species after exposure to both DBT (B_1: $F = 9.97$, $p < 0.05$) and TBT (B_2: $F = 76.96$, $p < 0.01$). Bernat et al. [24, 25] have recently shown an increased level of PA, a signal lipid formed in stress conditions, in TBT-treated cells of *Pseudomonas* sp. and *Cunninghamella elegans* strain. The exposure of the *M. robertsii* mycelium to TBT resulted in a significant decrease in the PI/PS ratios (B_2: $F = 28.30$, $p < 0.05$): from 3.5 to 1.9 and from 2.2 to 1.7 (Table 1). A reverse dependence i.e. a higher value of PI/PS in the *C. elegans* strain treated with TBT has been observed [30]. In our study, the values of the PC/PE ratios (equal to 27.5 and 28.7 for the 3- and 24-hour control membranes of the *M. robertsii* strain) were significantly lower, as much as 2 or 4 times, after treatment with DBT (B_1: $F = 99.40$, $p < 0.01$) and TBT (B_2: $F = 345.80$, $p < 0.01$), respectively. The reduction of the PC content observed simultaneously with membrane enrichment in PE is thought to be a common pattern of response to butyltins. Membrane dynamics depend on the proportions between the PC and PE levels. The PE headgroup has a smaller volume than PC. Therefore, an increased amount of PE causes dense packing of the hydrophilic zone of the cell membrane reducing its fluidity [33]. Bernat et al. [25] reported that the *C. elegans* bilayers were slightly more fluid in the presence of TBT. However, in our previous study on *M. robertsii* phospholipid adaptations during TBT treatment, a decrease in the PC/PE ratio was observed [5]. Literature data point to a reduction in the fluidity of microbial membranes induced by other lipophilic compounds such as carvedilol, trichlorophenol or perfluorooctanesulfonate [17, 18]. Accumulation of lipophilic compounds in the lipid bilayer enhances their availability for the cell [22]. Therefore, limited membrane fluidity may constitute an adaptive mechanism which ensures a gradual uptake of butyltin compounds.

The dominant PL species of the *M. robertsii* strain are shown in Table 2. The total amount of PC 18:2 18:2, PC 18:2 18:1 and PC 18:1 18:1 was almost 70%. The influence of the butyltins on the quantity of individual phospholipids was noticeable. First of all, two-way ANOVA indicated a significant decrease in the PC 18:2 18:2 amounts in the fungal membranes treated with the butyltins (B_1: $F = 51.20$, $p < 0.01$; B_2: $F = 238.03$, $p < 0.01$), which is probably another phospholipid marker of the presence of the compounds. Moreover, an inversely proportional dependence between PC 18:2 18:2 and PC 18:1 18:1 levels was observed. The quantities of PC containing two linoleic acids decreased from 43.7% to 27.9% and 26.1% in the presence of DBT and TBT presence, respectively, whereas PC containing two oleic acids increased from 10.6% to 18.2% and 13.4% after exposure to DBT and TBT, respectively (Table 2). The same dependence was observed during our previous experiments [5]. Therefore, the presence of the butyltins may be responsible for increased oxidation of one of the double bonds in linoleic acid or reduced activity of Δ12-desaturase, the enzyme responsible for introducing a double bond into oleic acid [38]. On the

Table 1
Phospholipid composition, PC/PE and PI/PS ratios determined in the *M. robertsii* cells after 3 h and 24 h of cultivation with DBT or TBT introduced alone or as a mixture with vitamin C, or without the tested compounds.

Phospholipid class	Culture										Two-way ANOVA			
	3 h					24 h								
	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	B_1, T, $B_1 \times T$	V_1, T, $V_1 \times T$	B_2, T, $B_2 \times T$	V_2, T, $V_2 \times T$
PA														
Relative abundance (%)	6.1 ± 2.1	7.5 ± 1.6	6.9 ± 2.0	8.2 ± 1.1	10.9 ± 0.4	5.2 ± 2.4	6.9 ± 1.2	7.3 ± 0.7	9.2 ± 0.2	9.4 ± 1.2	*, ns, ns	ns, ns, ns	**, ns, ns	**, ns, **
PC														
Relative abundance (%)	79.4 ± 25.1	78.1 ± 14.3	74.5 ± 1.8	65.4 ± 8.5	72.0 ± 0.9	72.5 ± 7.9	77.0 ± 18.2	78.91 ± 6.4	66.7 ± 0.6	71.8 ± 8.5	ns, *, **	*, ns, ns	**, ns, *	*, ns, ns
PE														
Relative abundance (%)	2.9 ± 1.0	3.1 ± 0.6	5.5 ± 0.1	9.6 ± 1.8	4.2 ± 0.3	7.9 ± 1.7	3.6 ± 1.1	2.8 ± 0.1	8.0 ± 1.2	6.5 ± 2.7	**, **, *	**, **, *	**, ns, ns	*, ns, ns
PG														
Relative abundance (%)	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	**, *, ns	*, ns, ns	**, ns, *	*, *, ns
PI														
Relative abundance (%)	8.5 ± 3.8	8.2 ± 1.8	9.2 ± 1.9	10.5 ± 0.3	9.2 ± 0.2	9.7 ± 1.2	9.5 ± 2.1	8.8 ± 0.8	9.5 ± 0.1	8.9 ± 1.2	ns, ns, ns	ns, ns, ns	ns, ns, ns	ns, ns, ns
PS														
Relative abundance (%)	2.5 ± 1.5	2.3 ± 0.5	3.0 ± 0.4	5.6 ± 0.5	2.9 ± 0.2	4.2 ± 0.3	2.2 ± 0.1	1.6 ± 0.2	5.7 ± 0.1	2.5 ± 0.9	ns, ns, *	*, *, ns	**, ns, ns	**, ns, ns
Σ of PL (mg g d.w.$^{-1}$)	27.0 ± 9.1	54.6 ± 3.0	48.3 ± 10.3	50.1 ± 3.5	33.8 ± 0.1	29.9 ± 3.1	58.6 ± 13.4	37.8 ± 3.0	49.2 ± 0.7	37.6 ± 5.5	*, ns, ns	ns, ns, ns	**, ns, ns	**, ns, ns
PC/PE	27.5	13.3	25.2	6.7	17.0	28.7	21.9	27.8	8.3	11.2	**, *, *	**, *, ns	**, ns, ns	*, ns, ns
PI/PS	3.5	3.0	3.5	1.9	3.2	2.2	4.2	5.3	1.7	3.7	ns, ns, *	ns, *, ns	**, *, ns	**, ns, ns

Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

Table 2
Relative abundance (%) of major phospholipid species, C16/C18 ratio and DBI values determined in 3- and 24-h mycelium of the *M. robertsii* strain incubated with or without the tested compounds.

Phospholipid species	Culture								Two-way ANOVA			
	3 h				24 h							
	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	B_1, T, $B_1 \times T$	V_1, T, $V_1 \times T$	B_2, T, $B_2 \times T$	V_2, T, $V_2 \times T$
PE 16:0 18:2	0.8 ± 0.2	1.7 ± 0.2	0.9 ± 0.1	3.5 ± 1.2	1.3 ± 0.1	2.7 ± 0.8	1.1 ± 0.1	1.0 ± 0.1	2.5 ± 0.4	2.3 ± 0.7	ns, ns, *	*, ns, *	ns, ns, ns	ns, ns, ns
PS 16:0 18:1	1.2 ± 0.5	1.9 ± 0.1	1.2 ± 0.2	3.6 ± 0.5	1.6 ± 0.1	2.3 ± 0.1	1.1 ± 0.2	0.8 ± 0.1	4.1 ± 0.1	1.6 ± 0.5	ns, ns, **	**, **, ns	**, *, ns	**, ns, ns
PA 16:0 18:1	1.8 ± 0.1	2.4 ± 0.1	2.2 ± 0.5	2.7 ± 0.1	3.4 ± 0.1	1.4 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	** , **, ns	ns, ns, ns	** , ** , **	** , ** , **
PI 16:0 18:1	2.4 ± 0.5	3.8 ± 0.1	2.4 ± 0.2	3.4 ± 0.5	2.5 ± 0.2	2.5 ± 0.2	2.1 ± 0.2	2.3 ± 0.1	2.9 ± 0.1	2.3 ± 0.1	ns, *, **	** , ** , **	ns, ns, ns	*, ns, ns
PI 16:0 18:2	2.6 ± 0.3	1.5 ± 0.1	1.9 ± 0.5	2.2 ± 0.3	2.0 ± 0.1	3.1 ± 0.2	1.9 ± 0.1	2.0 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	**, *, ns	ns, ns, ns	** , ns, *	ns, *, ns
PC 16:0 18:2	3.2 ± 0.2	2.2 ± 0.2	3.2 ± 0.1	2.9 ± 1.2	3.2 ± 0.1	3.4 ± 0.8	4.3 ± 0.1	4.0 ± 0.1	3.6 ± 0.4	3.6 ± 0.7	ns, **, **	ns, ** , *	ns, *, ns	ns, **, ns
PC 18:1 18:1	10.6 ± 0.1	18.2 ± 0.4	12.9 ± 1.5	13.4 ± 0.9	13.5 ± 0.2	7.2 ± 0.1	8.0 ± 0.3	8.3 ± 0.2	16.0 ± 1.3	15.4 ± 0.1	**, **, **	**, **, **	**, ns, **	ns, *, ns
PC 18:2 18:1	18.7 ± 1.2	21.2 ± 0.1	19.3 ± 0.7	18.2 ± 1.3	20.1 ± 0.3	15.6 ± 0.1	18.1 ± 0.1	17.8 ± 0.5	18.1 ± 0.7	19.7 ± 0.8	**, **, ns	*, *, ns	ns, ns, ns	*, ns, ns
PC 18:2 18:2	43.7 ± 3.2	27.9 ± 0.3	38.6 ± 1.3	26.1 ± 1.3	29.1 ± 0.5	43.5 ± 0.1	42.9 ± 0.1	45.6 ± 0.2	22.3 ± 0.7	26.9 ± 0.5	**, **, **	**, **, **	**, ns, ns	**, **, ns
C16/C18	0.11	0.14	0.12	0.18	0.16	0.13	0.13	0.12	0.21	0.18	*, ns, *	*, ns, ns	**, *, ns	*, ns, ns
DBI	3.1	2.7	3.0	2.7	2.8	3.1	3.1	3.1	2.5	2.7	**, **, **	**, **, **	**, ns, *	**, **, ns

Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

other hand, Masia et al. [39] reported that 18:2-enriched membranes enhanced the resistance of *S. cerevisiae* cells against TBT action.

In microorganisms, membrane fluidity could be regulated by the manipulation of the length and saturation degree of acyl chains. Enrichment in unsaturated phospholipids as well as reduction of their chain length cause an increase in membrane liquidity [33]. According to the two-way ANOVA analysis, the exposure of the *M. robertsii* strain to the butyltins resulted in a significant rise in the C16/C18 ratio: by 20% for DBT (B_1: $F = 9.12$, $p < 0.05$) and by 50% for TBT (B_2: $F = 95.12$, $p < 0.01$). In contrast, the double bond index (DBI) – a value describing the degree of fatty acid unsaturation – was significantly lower in the presence of DBT (B_1: $F = 74.77$, $p < 0.01$) or TBT (B_2: $F = 325.33$, $p < 0.01$) (Table 2). The alterations of the above membrane parameters could constitute a universal response of the fungus to the butyltins action. Modifications of fatty acids play a role in microbial cell adaptations to the presence of toxic substances such as terpenes and ethanol [40, 41]. The butyltins may affect the thickness and density of the tested membranes.

Vitamin C supplementation protected the *M. robertsii* strain from the peroxidation of its lipids [6], which could have been related with radical exchange reactions between the antioxidant and phospholipids [42]. Other antioxidants such as isobornylphenols, β-carotene, or dl-α-tocopherylacetate affected the fatty acid profile or the phospholipids from the outer membrane layer [43, 44]. However, no literature data focusing on the phospholipid alternations after vitamin C supplementation are available. Introduction of the antioxidant into the *M. robertsii* culture conducted in the presence of DBT or TBT resulted in important changes in the fungal membrane composition. Two-way ANOVA showed significant alterations in the quantities of PC (V_1: $F = 8.24$, $p < 0.05$; V_2: $F = 43.04$, $p < 0.01$) and PE (V_1: $F = 14.38$, $p < 0.05$; V_2: $F = 9.40$, $p < 0.05$) after simultaneous exposure of the fungus to vitamin C and DBT or TBT (Table 1). Moreover, the addition of the antioxidant led to significant changes in the amounts of PL species such as PC 18:2 18:2 (V_1: $F = 210.69$, $p < 0.01$; V_2: $F = 41.30$, $p < 0.01$), PI 16:0 18:1 (V_1: $F = 37.06$, $p < 0.01$; V_2: $F = 14.30$, $p < 0.05$) and PS 16:0 18:1 (V_1: $F = 24.10$, $p < 0.01$; V_2: $F = 78.29$, $p < 0.01$) as well as to certain values such as the C16/C18 ratio (V_1: $F = 12.71$, $p < 0.05$; V_2: $F = 10.47$, $p < 0.05$) and DBI (V_1: $F = 463.50$, $p < 0.01$; V_2: $F = 43.80$, $p < 0.01$) (Table 2). The modifications of the above-mentioned parameters generating values similar to the control seem to be a typical model of the fungal response to the presence of vitamin C. It appears that ascorbic acid mitigates the butyltins interactions with the *M. robertsii* membranes, restoring their homeostasis.

A strong impact of the butyltins on the phospholipid concentration was recognized (Table 1). After DBT exposure, PL quantity (27.0–29.9 mg g d.w.$^{-1}$ for control membranes) was enhanced 2-fold (B_1: $F = 13.20$, $p < 0.05$). For TBT-treated cells, the PL content increased by 65–86% (B_2: $F = 35.80$, $p < 0.01$). Lipid accumulation after TBT exposure was recorded repeatedly [45–48]. It can be suggested that intensified lipid production is associated with the creation of an additional place for the storage of lipophilic compounds, especially because, in the presence of vitamin C (an accelerator of MBT biodegradation), a considerable decrease in the amounts of the *M. robertsii* lipids was observed. Endo et al. [49] mentioned the ability of membrane lipids to accumulate chemicals.

3.2. Tri- and diacylglycerol content

Neutral lipids stored in lipid particles are considered the main reserve material (triacylglycerols, TAGs), cellular signaling molecules and an important element of the phospholipid biosynthesis pathway (diacylglycerols, DAGs) [50].

Among the 17 different species of neutral lipids determined in each sample (data not shown), 5 were DAGs representing about 80% of the total acylglycerols, while the other 12 species were TAGs. The main

species of the *M. robertsii* neutral lipids are presented in Supplementary Table 1. The highest contents were recorded for DAG 16:0 16:0, DAG 18:0 18:1 and DAG 18:1 18:1. Besides DAG 18:1 18:2, the level of which was significantly lower in the presence of TBT (B_2: $F = 80.25$, $p < 0.01$), the other acylglycerols were unchanged after the treatment with butyltins. The presence of vitamin C influenced the amounts of TAG 16:0 18:1 18:1 (V_2: $F = 11.06$, $p < 0.05$), DAG 16:0 18:0 (V_2: $F = 12.55$, $p < 0.05$) and DAG 18:1 18:2 (V_1: $F = 9.18$, $p < 0.05$). However, markers of the fungal response to the tested compounds were not indicated.

Increased TAG storage by *Gobiocypris rarus* [48] and mouse liver [47] cells after their exposure to TBT has been observed. An opposite dependence, i.e. a lower TAG content in *Ciona intestinalis*, was found by Puccia et al. [46]. The *M. robertsii* strain is not an oleaginous microorganism, which accumulates high amounts of TAGs [51]. Moreover, two-way ANOVA showed no significant influence of either butyltins or vitamin C on the total levels of tri- and diacylglycerols in the fungal cells (Supplementary Table 1).

3.3. Ergosterol content

Sterols, which are membrane reinforcers, maintain the domain structure of cell membranes and are responsible for preserving a state of fluidity adequate for its function [52]. The results obtained during the quantitative analysis of ergosterol (ERG), the main sterol found in fungi, are shown in Fig. 1. In the control samples, the ERG amounts were estimated to be 172.3 μg g d.w.$^{-1}$ and 141.4 μg g d.w.$^{-1}$ for 3- and 24-hour mycelia, respectively. Two-way ANOVA showed a significant increase in the ERG content in the mycelium exposed to the butyltins. The sterol content was 16% and 42% higher, respectively, after 3 and 24 h of cultivation in the presence of DBT (B_1: $F = 9.82$, $p < 0.05$) as well as approximately 70% and > 2-fold higher, respectively, after 3- and 24-hour incubation in the presence of TBT (B_2: $F = 107.06$, $p < 0.01$). On the other hand, the addition of vitamin C caused a significant decrease in the level of sterols (V_1: $F = 8.61$, $p < 0.05$; V_2: $F = 33.85$, $p < 0.01$): 77% and 65% in 3- and 24-hour mycelia, respectively. In contrast to the increased biosynthesis of ergosterol, which seems to be a common pattern of the *M. robertsii* response to the butyltins, decreased sterol production was assumed to be the fungal marker for the presence of vitamin C. Little information is available about ERG level modifications in the presence of organotin. However, there are reports proving that TBT induces more intense sterol biosynthesis by mice liver cells [47] and muscle tissues of *Gobiocypris rarus* [48].

The mechanistic role of ergosterol in membrane rigidity and the resistance of *S. cerevisiae* to cycloheximide, another lipophilic compound, was described by Abe and Hiraki [53]. On the other hand, dependence between the ERG content and the hyphal diameter of fungi was demonstrated by Klamer and Bååth [54]. Besides the defensive function, it was assumed that a higher sterol level could facilitate butyltins transport due to the thinner hyphae of the *M. robertsii* strain.

3.4. Sphingolipid composition

Followed by glycerophospholipids and sterols, sphingolipids (SPLs) are the most abundant of the eukaryotic membrane lipids. They play an important role in a variety of biological processes [52]. However, their impact on membrane properties is still unclear.

Among the 17 identified sphingolipids of the *M. robertsii* strain, 3 were ceramides (Cers) containing 16, 18 and 20 carbon atoms, 2 were dihydroceramides (dhCers) with 16- and 18-carbon chain lengths, 10 were sphingomyelins (SMs) composed of 14, 16, 18, 20, 22 and 24-carbon chains and the remaining 2 were sphingosine (Sph) and dihydrosphingosine (dhSph) (data not shown).

The SPLs most abundantly occurring in the fungal membranes were sphingomyelins, which represented 99.4% of the total SPL quantity (Table 3). The other lipids were found in trace amounts (< 0.3%). The levels of C22-SM (B_2: $F = 21.64$, $p < 0.01$), C20:1-SM (B_2: $F = 10.40$, $p < 0.05$) and C22:1-SM (B_2: $F = 18.08$, $p < 0.05$) were significantly modified by the presence of TBT.

SM containing very long saturated acyl chains exhibit a strong affinity to sterols, leading to the formation of liquid ordered domains [33]. Moreover, Gabrielska et al. [55] reported that adsorption of organometallic compounds depends on the quantitative proportions between sphingomyelin and cholesterol. A simultaneous increase in the C22-SM and ERG contents in the *M. robertsii* strain is another proof for the stiffening of its membranes in response to the presence of TBT.

Sphingolipid turnover as well as ceramide accumulation related with the sphingomyelinase activity are usually found in response to various stress stimuli, including oxidative stress [56]. Although MBT (a major byproduct of DBT and TBT conversion) generates reactive oxygen and nitrogen species [7], the ceramides identified in the *M. robertsii* membranes were found to be quantitatively negligible. Moreover, due to the low basal level of Cer, a SPL demonstrating the ability of relocation in the bilayer [57], its effect on membrane permeability was also omitted.

Two-way ANOVA showed significant changes in the quantities of C22-SM (V_1: $F = 23.87$, $p < 0.01$) and C20:1-SM (V_1: $F = 8.19$,

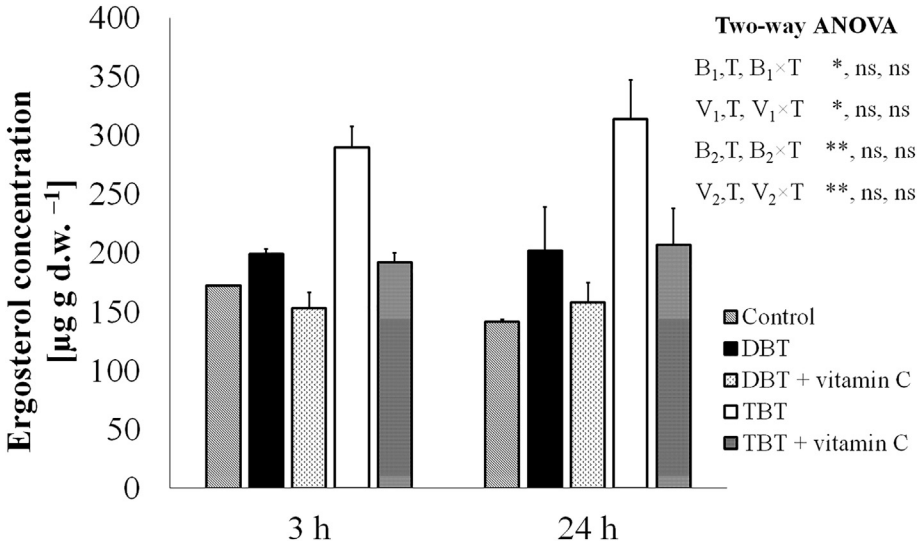

Fig. 1. Ergosterol concentration (μg g d.w.$^{-1}$) in fungal cells cultivated with the addition of DBT/TBT and vitamin C, or without the tested compounds. Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

Table 3
Relative abundance (%) of major sphingolipids identified in the *M. robertsii* membranes after 3 h and 24 h of cultivation with or without the tested compounds.

Sphingolipids	Culture								Two-way ANOVA				
	3 h				24 h								
	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	B_1, T, $B_1 \times T$	V_1, T, $V_1 \times T$	B_2, T, $B_2 \times T$	V_2, T, $V_2 \times T$
C20:1-SM	3.5 ± 0.2	4.0 ± 0.2	3.4 ± 0.1	3.8 ± 0.2	4.5 ± 0.3	4.0 ± 0.2	2.8 ± 0.1	2.9 ± 0.0	3.0 ± 0.1	3.3 ± 0.1	ns, ns, **	*, **, ns	*, *, **	*, **, ns
C24:1-SM	0.9 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	0.7 ± 0.0	1.1 ± 0.0	ns, ns, ns	ns, ns, *	ns, ns, ns	ns, ns, ns
C22-SM	39.8 ± 2.4	36.8 ± 2.3	45.7 ± 3.0	45.0 ± 1.9	41.6 ± 2.4	36.8 ± 2.7	37.7 ± 2.4	36.6 ± 2.9	45.0 ± 2.3	48.1 ± 3.7	ns, ns, **	**, ns, **	** , ns, ns	ns, ns, *
C22:1-SM	53.8 ± 4.8	56.8 ± 4.3	49.5 ± 4.0	49.1 ± 4.4	50.9 ± 3.2	56.9 ± 4.3	57.5 ± 4.7	58.1 ± 5.6	49.5 ± 3.7	46.7 ± 4.2	ns, ns, ns	ns, *, ns	*, ns, ns	ns, ns, ns
Σ of SPL ($\mu g\ g\ d.w.^{-1}$)	23.2 ± 1.4	88.9 ± 4.4	194.1 ± 17.5	38.2 ± 1.1	19.9 ± 6.0	22.0 ± 1.8	98.7 ± 3.9	113.2 ± 9.1	35.6 ± 2.1	112.9 ± 4.5	**, **, **	**, **, **	**, **, ns	**, **, **

Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

$p < 0.05$; V_2: $F = 18.69$, $p < 0.01$) in the *M. robertsii* strain cultivated in the presence of vitamin C. To date, the influence of antioxidants on the modification of fungal sphingolipids has not been described. However, Babenko et al. [58] mentioned the action of vitamin E in the abolition of increased Cers levels in liver cells, while Uchida et al. [59] reported that vitamin C stimulates SPL production by human keratinocyte.

DBT was found to activate sphingolipid biosynthesis by the *M. robertsii* cells much more efficiently than TBT. The amount of SPLs increased significantly from 23.2 to 38.2 or to 88.9 $\mu g\ g\ d.w.^{-1}$ as well as from 22.0 to 35.6 or to 98.7 $\mu g\ g\ d.w.^{-1}$ after 3- and 24-hour incubation with TBT (B_2: $F = 1395.04$, $p < 0.01$) and DBT (B_1: $F = 10{,}957.35$, $p < 0.01$), respectively (Table 3). On the other hand, the addition of vitamin C resulted in even more enhanced lipid production (V_1: $F = 739.8$, $p < 0.01$; V_2: $F = 1134.17$, $p < 0.01$). It seems that intensified SPL biosynthesis could be a typical reaction of the *M. robertsii* cells to the action of the tested compounds.

3.5. Membrane potential

Positively-charged lipophilic compounds interact with plasma membranes. The action of the butyltins on the *M. robertsii* cell polarization was investigated using two fluorophores. The first – $DIOC_6(3)$ – has a cationic character. Therefore, it penetrates hyperpolarized cells, in a different way than $DiBAC_4(3)$, which enters depolarized cells because of its anionic nature [60].

$DIOC_6(3)$ fluorescence intensity for the control cells was observed to amount to 935.1 U mg^{-1} and 1669.0 U mg^{-1}, whereas $DiBAC_4(3)$ fluorescence intensity was equal to 7346.2 U mg^{-1} and 6007.2 U mg^{-1} for 3-hour and 24-hour mycelia, respectively (Fig. 2). The presence of TBT significantly influenced the measured fluorescence intensity. $DIOC_6(3)$ fluorescence values for 3- and 24-hour mycelia were, respectively, 9-fold and 5-fold higher (B_2: $F = 3482.30$, $p < 0.01$) than for the control mycelium (Fig. 2a). On the other hand, the fluorescence values of the cells labeled with $DiBAC_4(3)$ decreased (B_2: $F = 520.43$, $p < 0.01$) 5 and 2.5 times, respectively (Fig. 2b), which confirmed the membrane hyperpolarization.

Enhanced negative charges of *S. cerevisiae* membranes treated with cationic amiodarone were described by Maresova et al. [19]. According to the authors, membrane hyperpolarization was associated with calcium influx. Moreover, Elicharova and Sychrova [61] reported that hyperpolarization of *C. glabrata* membranes induced by fluconazole resulted in an inability of the yeast to export potassium and toxic alkali-metal cations. Bernat et al. [62] focused on an increased potassium content in *C. elegans* cells exposed to TBT. Diffusion of organotin across the membrane may reduce K+ leakage [63]. Therefore, it cannot be excluded that the increased membrane potential of the *M. robertsii* cells is related with disturbances in the activity of ion pumps caused by the butyltins absorption and their penetration into the bilayer.

On the other hand, hyperpolarization of the *M. robertsii* membranes could be associated with a strong affinity of positively-charged TBT to negatively charged phospholipids. The results from fluorescence studies were consistent with the changes in the level of negatively charged PL. The sum of PA, PG, PS and PI contents increased from 17.4% to 25% and 23.7% after 3 h incubation with TBT and a mixture of TBT and vitamin C, respectively, as well as from 20.7% to 25.2% after 24-hour treatment with TBT (data not shown). The amounts of PC, PE and SM were not included due to the zwitterionic character of their molecules. On the other hand, it could not be excluded that the enhancement of negative charges on the fungal membrane surface constitutes one of the adaptive changes accelerating TBT attachment followed by its biodegradation. Maresova et al. [19] also suggested that hyperpolarization of *S. cerevisiae* membranes induced by amiodarone is connected with the transport of this cationic drug across the yeast membrane.

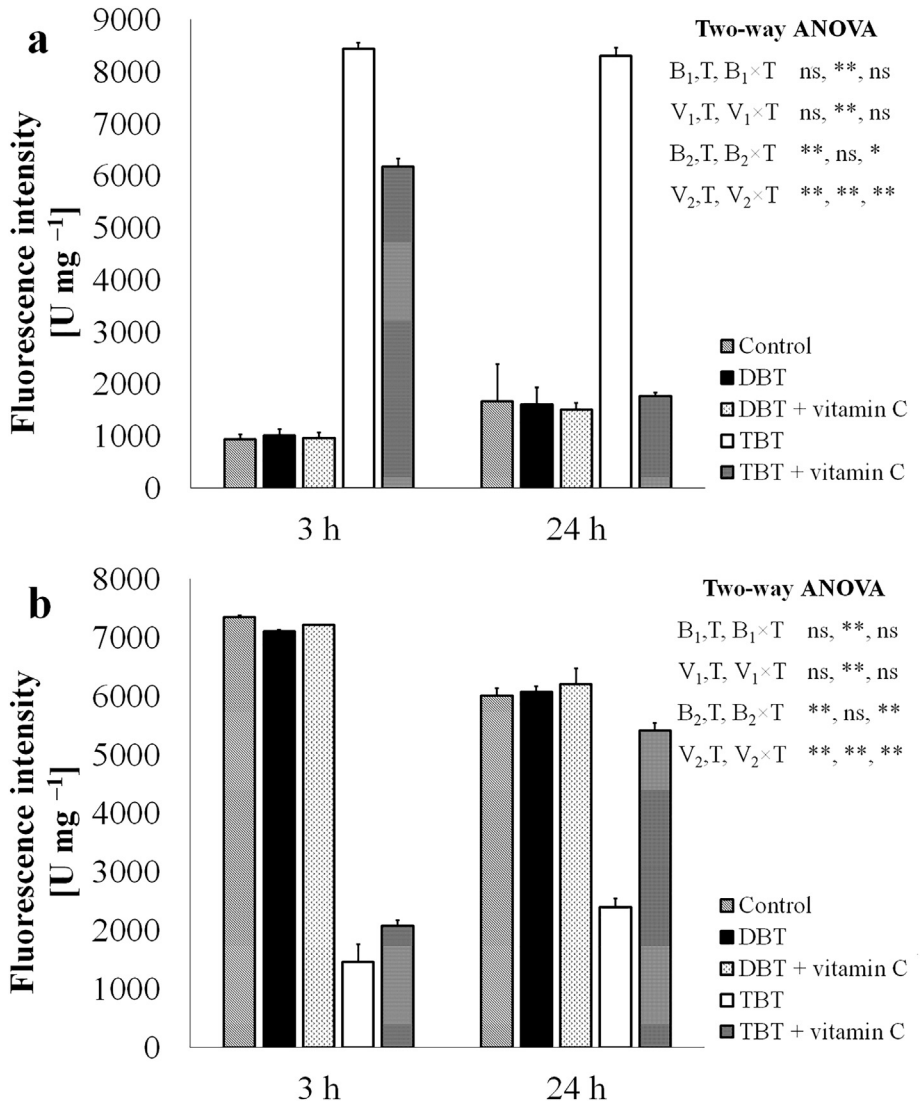

Fig. 2. Fluorescence intensity of the *M. robertsii* mycelium labeled with $DIOC_6(3)$ (a) and $DiBAC_4(3)$ (b). Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

3.6. Membrane fluidity

Cell membrane fluidity can be affected by the presence of lipophilic compounds. Application of the various fluorophores (Prodan, Laurdan and DPH) allows the comparison of membrane physicochemical characteristics at different depths of a bilayer.

Prodan can be located simultaneously in both polar and apolar regions of the membrane but usually it is closer to the aqueous phase [64]. Fluorescence intensity of *M. robertsii* membranes labeled with Prodan was similar for 3-h and 24-h cells and amounted to about 700 U mg^{-1} (Fig. 3a). After exposure to the butyltins, a significant decrease in the exterior membrane fluidity (B_1: $F = 484.95$, $p < 0.01$; B_2: $F = 659.12$, $p < 0.01$) was observed. Prodan fluorescence values for 3-h mycelia were 56% and 78% lower, respectively, for DBT- and TBT-treated cells. Literature data confirming the action of the butyltins on outer membrane fluidity are not available. The influence of a new dibutyltin complex with citric acid on the bilayers surface was suggested by Pruchnik et al. [65]. Slight changes in membrane fluidity are probably caused by a partial penetration of the lipid head group region. However, the size of DBT and TBT molecules is much smaller and this facilitates diffusion. Incorporation of DBT in the outer layer as well as homogeneous distribution of MBT in the bilayer have been proved [4, 66]. On the other hand, the presence of sterols can lead to the relocation of Prodan [64]. Therefore, decreased fluorophore fluorescence is probably caused not only by filling of the *M. robertsii* outer membrane with MBT and DBT but also by increased ERG content in TBT-treated cells.

Laurdan contains the same chromophore as Prodan but it enters deeper in the headgroup region until its fluorescent moiety is found at the level of the glycerol backbone [64]. The dye fluorescence values for the control cells were 452.5 U mg^{-1} and 512.2 U mg^{-1} after 3 h and 24 h of incubation, respectively (Fig. 3b). Significantly weakened fluorescence intensity of Laurdan followed by limited membrane fluidity in the area of fatty acids was detected in *M. robertsii* cells exposed to butyltins. The membranes were 62% and 54% more rigid, respectively, in 3- and 24-hour mycelia exposed to DBT (B_1: $F = 111.67$, $p < 0.01$) as well as 33% and 43% more rigid, respectively, after 3 and 24 h of incubation in the presence of TBT (B_2: $F = 36.92$, $p < 0.01$). Bonarska et al. [67] also observed stiffening of erythrocyte membranes exposed to TBT and DBT. The slight differences between the results obtained for Prodan- and Laurdan-labeled cells probably result from the similar membrane packing by butyltins and sterol as well as lack of the spatial specificity of Prodan.

DPH, the last of the used chromophores, is located within the

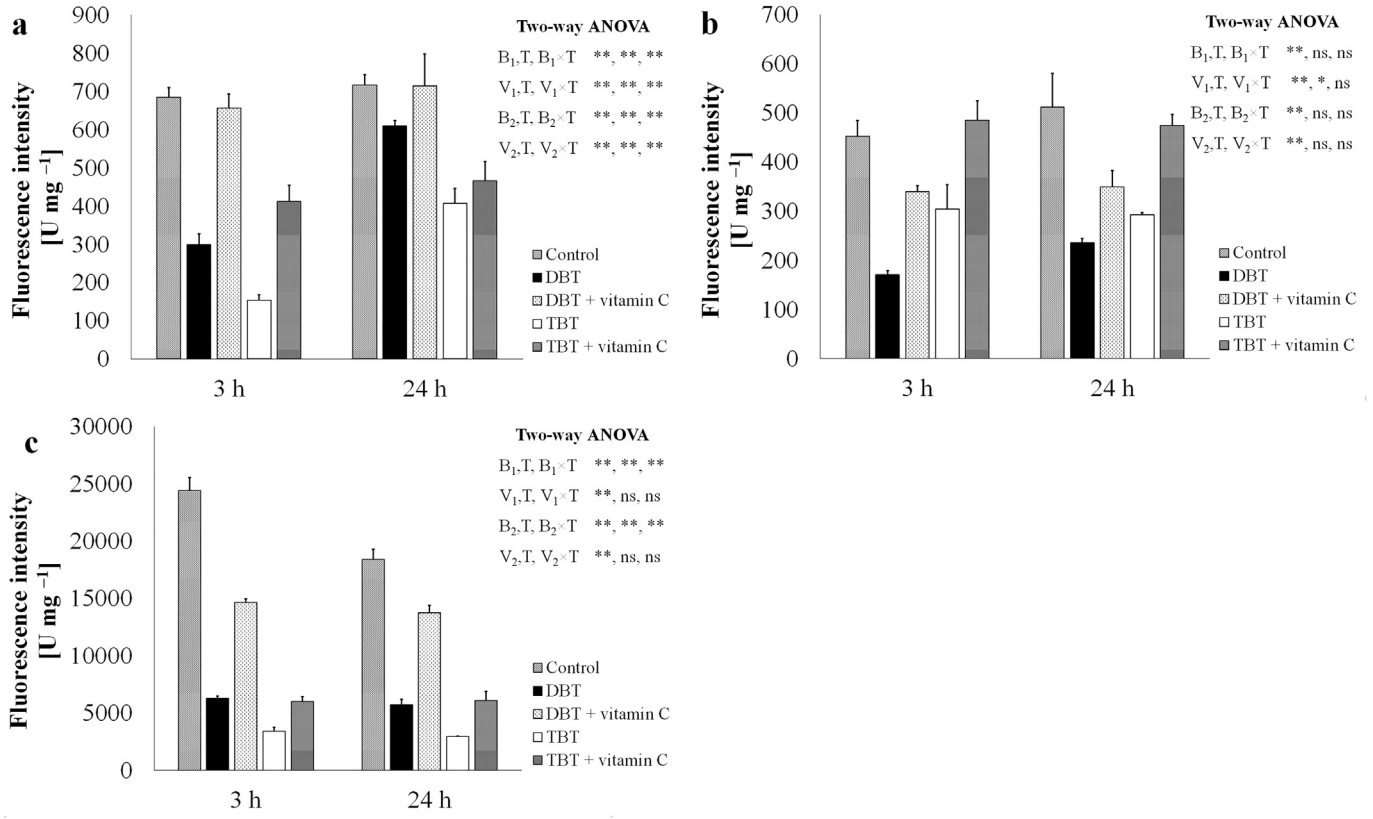

Fig. 3. Fluorescence intensity of the *M. robertsii* mycelium labeled with Prodan (a), Laurdan (b) and DPH (c). Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

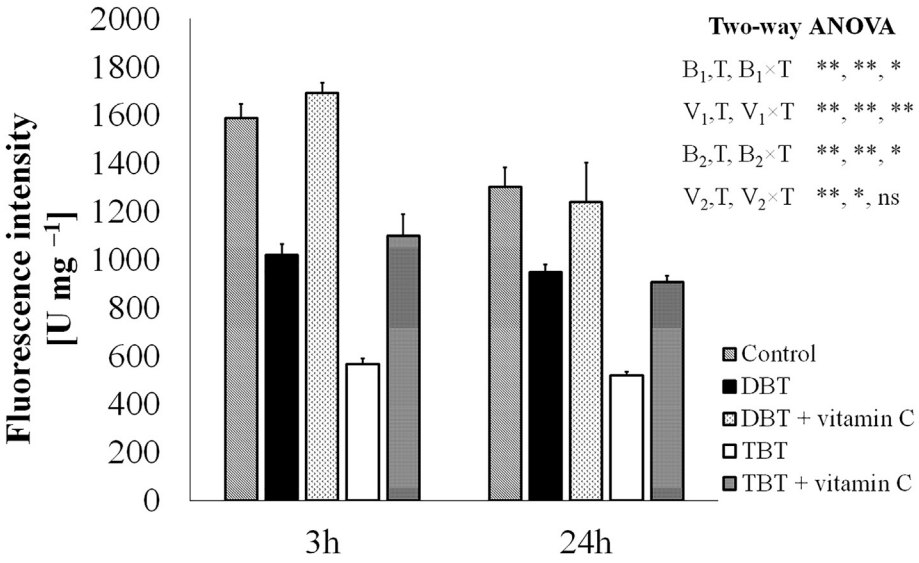

Fig. 4. Fluorescence intensity of the *M. robertsii* mycelium labeled with PI. Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors; B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time. p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

hydrophobic membrane interior [64]. Fluorescence intensity of the 3-hour and 24-hour fungal cells labeled with DPH amounted, respectively, to 24,414.2 U mg^{-1} and 18,395.1 U mg^{-1} (Fig. 3c). After exposure to butyltins, a significant reduction of the membrane fluidity (69–74% and ~85%, respectively, in the presence of DBT (B_1: $F = 824.37$, $p < 0.01$) and TBT (B_2: $F = 1259.54$, $p < 0.01$)) was observed. An increase in membrane rigidity as a result of organotins incorporation between hydrophobic alkyl chains has also been observed in erythrocytes, liposomes, *Bacillus* sp. cells and *C. maltose* protoplasts [66–69].

Membrane stiffening has also been noticed during bacterial biodegradation of other hydrophobic xenobiotics such as p-nitrophenol or phenanthrene [20, 21]. According to this research, a highly ordered membrane structure either is used for quick adaptation to toxic substances [21] or is a result of the uptake of these compounds inside the membrane [20].

The addition of vitamin C caused an increase in the liquidity of the *M. robertsii* membranes at all tested depths. Significantly higher fluorescence intensities for the Prodan - (V_1: $F = 148.05$, $p < 0.01$; V_2: $F = 56.24$, $p < 0.01$), Laurdan- (V_1: $F = 114.03$, $p < 0.01$; V_2:

F = 65.93, $p < 0.01$), and DPH-labeled (V_1: F = 1049.39, $p < 0.01$; V_2: F = 74.28, $p < 0.01$) fungal membranes were observed after the antioxidant supplementation. This could be explained by an increased phosphatidylcholine content and decreased ergosterol level. To date, no reports have been published on the alterations in the properties of microbial membranes caused by the presence of ascorbic acid. Unlike the membrane stiffening, which appears to be a universal reaction of the *M. robertsii* strain to butyltins, its increased liquidity could be another fungal marker for the presence of vitamin C.

3.7. Membrane permeability

The lipophilicity of the compounds contributes to changes in plasma membrane permeability. The influence of the tested compounds on the *M. robertsii* cell membrane integrity is presented in Fig. 4. The values of fluorescence intensity for the control 3-hour and 24-hour fungal cells labeled with propidium iodide were estimated at 1587.2 U mg^{-1} and 1302.9 U mg^{-1}, respectively. TBT action had a greater impact on the degree of cell penetration by the dye than DBT. Permeability of the fungal membranes exposed to DBT was significantly lower – 60% (B_1: F = 164.84, $p < 0.01$), whereas TBT-treated membranes were less permeable – 27-36% (B_2: F = 755.90, $p < 0.01$).

Butyltin compounds are known as membrane permeability disrupters [70–72]. In our previous study [5], we observed a negative impact of TBT on *M. robertsii* membrane integrity. However, optimization of the fungal growth by the application of intensified culture oxygenation, which resulted in accelerated butyltins biodegradation [6], suggests mitigation of the cell damage. Moreover, the presented results obtained during fluorescence experiments were consistent with the quantitative analysis of ergosterol. Sterols increase the mechanical strength of a membrane [52], simultaneously reducing its permeability for di- and triorganotin compounds. The tight structure of the *M. robertsii* membrane, in combination with intensified phospholipid synthesis, seems to confirm the hypothesis of the formation of a temporary storage for butyltins in the fungal bilayer.

Triphenyltin (TPT) is the most often described organotin which affects membrane integrity [73–75]. According to the authors, permeability of *B. brevis*, *S. maltophilia* and *B. thuringiensis* membranes increases during TPT biodegradation. Despite the similar molecular weights of TPT and TBT, ring moieties and butyl chains probably influence the membrane properties in a different way. Therefore, these results should not be compared.

Supplementation with vitamin C resulted in a significant increase in the membrane permeability for PI, 66% and 31% respectively, after 3 and 24 h of incubation in the presence of DBT (V_1: F = 145.26, $p < 0.01$) as well as by 94% and 75%, respectively in 3- and 24-h mycelia exposed to TBT (V_2: F = 177.27, $p < 0.01$). In contrast to the higher membrane integrity, which could be a marker of the *M. robertsii* response to the butyltins action, its increased permeability seems to be the fungal pattern for vitamin C supplementation. Although vitamin C is described as a substance limiting membrane permeability through a leveling of oxidative stress effects [76, 77], MBT levels (an ROS and RNS inductor [7]) in the *M. robertsii* cultures supplemented with vitamin C were negligible, which excludes peroxidative damage as a reason for increased fungal membrane permeability. Considering the facts that MBT is distributed homogeneously in the bilayer [66] and it is transported across the membrane by passive diffusion [70], it can be assumed that the relaxed membrane structure of the *M. robertsii* strain caused by the presence of vitamin C is necessary for facilitated diffusion of MBT.

4. Conclusions

The phospholipid composition of the *M. robertsii* membranes altered during intensive aeration resulted in changes of bilayer dynamics contributing to a facilitated uptake of butyltins. Hyperpolarization of the fungal membrane probably accelerates surface attachment of TBT. Both DBT and TBT induce intensified lipid and ergosterol biosynthesis. A rigid, compacted, less permeable membrane seems to be used for the butyltins confinement. Vitamin C supplementation restores the balance of the fungal membrane. A more fluid and permeable structure probably supports transport and biodegradation of MBT, avoiding the effects of oxidative and nitrosative stress.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2018.06.007.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

The authors are grateful to J. Nykiel-Szymańska MSc, University of Lodz, for her help with the statistical analysis of the data. This study was supported by the National Science Centre, Poland (Project No. 2015/19/N/NZ9/00459).

References

[1] A.C.A. Sousa, M.R. Pastorinho, S. Takahashi, S. Tanabe, History on organotin compounds, from snails to humans, Environ. Chem. Lett. 12 (2014) 117–137.
[2] M. Hoch, Organotin compounds in the environment - an overview, Appl. Geochem. 16 (2001) 719–743.
[3] Y. Kotake, Molecular mechanisms of environmental organotin toxicity in mammals, Biol. Pharm. Bull. 35 (2012) 1876–1880.
[4] A. Pagliarani, N. Salvatore, V. Vittoria, Toxicity of organotin compounds: shared and unshared biochemical targets and mechanisms in animal cells, Toxicol. In Vitro 27 (2013) 978–990.
[5] P. Siewiera, P. Bernat, S. Różalska, J. Długoński, Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*, Int. Biodeterior. Biodegrad. 104 (2015) 258–263.
[6] P. Siewiera, S. Różalska, P. Bernat, Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation, Environ. Sci. Pollut. Res. 24 (2017) 12118–12127.
[7] P. Siewiera, S. Różalska, P. Bernat, Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin, Chemosphere 185 (2017) 96–104.
[8] A. Antecka, M. Bizukojc, S. Ledakowicz, Modern morphological engineering techniques for improving productivity of filamentous fungi in submerged cultures, World J. Microbiol. Biotechnol. 32 (2016) 193.
[9] S.R. Geed, M.K. Kureel, B.S. Giri, R.S. Singh, B.N. Rai, Performance evaluation of malathion biodegradation in batch and continuous packed bed bioreactor (PBBR), Bioresour. Technol. 227 (2017) 56–65.
[10] Z. Křesinová, L. Linhartová, A. Filipová, M. Ezechiáš, P. Mašín, T. Cajthaml, Biodegradation of endocrine disruptors in urban wastewater using *Pleurotus ostreatus* bioreactor, New Biotechnol. (2017), http://dx.doi.org/10.1016/j.nbt.2017.05.004.
[11] F. Moscoso, F.J. Deive, M.A. Longo, M.A. Sanromán, Insights into polyaromatic hydrocarbon biodegradation by *Pseudomonas stutzeri* CECT 930: operation at bioreactor scale and metabolic pathways, Int. J. Environ. Sci. Technol. 12 (2015) 1243–1252.
[12] M. Yadav, N. Srivastva, R.S. Singh, S.N. Upadhyay, S.K. Dubey, Biodegradation of chlorpyrifos by *Pseudomonas* sp. in a continuous packed bed bioreactor, Bioresour. Technol. 165 (2014) 265–269.
[13] R.R. Chen, Permeability issues in whole-cell bioprocesses and cellular membrane engineering, Appl. Microbiol. Biotechnol. 74 (2007) 730–738.
[14] B.L. Ponce, V.K. Latorre, M. González, M. Seeger, Antioxidant compounds improved PCB-degradation by *Burkholderia xenovorans* strain LB400, Enzym. Microb. Technol. 49 (2011) 509–516.
[15] M. Słaba, E. Gajewska, P. Bernat, M. Fornalska, J. Długoński, Adaptive alterations in the fatty acids composition under induced oxidative stress in heavy metal-tolerant filamentous fungus *Paecilomyces marquandii* cultured in ascorbic acid presence, Environ. Sci. Pollut. R. 20 (2013) 3423–3434.
[16] K.G. Zink, R. Rabus, Stress-induced changes of phospholipids in betaproteobacterium *Aromatoleum aromaticum* strain EbN1 due to alkylbenzene growth substrates, J. Mol. Microbiol. Biotechnol. 18 (2010) 92–101.
[17] T.T. Liao, L. Wang, R.W. Jia, X.H. Fu, H. Chua, Lipophilic organic pollutants induce changes in phospholipid and membrane protein composition leading to *Vero cell* morphological change, J. Environ. Sci. Health B 49 (2014) 760–768.
[18] K. Zawadzka, P. Bernat, A. Felczak, K. Lisowska, Microbial detoxification of carvedilol, a β-adrenergic antagonist, by the filamentous fungus *Cunninghamella echinulate*, Chemosphere 183 (2017) 18–26.
[19] L. Maresova, S. Muend, Y.Q. Zhang, H. Sychrova, R. Rao, Membrane

hyperpolarization drives cation influx and fungicidal activity of amiodarone, J. Biol. Chem. 284 (2009) 2795–2802.
[20] A. Kallimanis, S. Frillingos, C. Drainas, A.I. Koukkou, Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3, Appl. Microbiol. Biotechnol. 76 (2007) 709–717.
[21] M. Kulkarni, A. Chaudhari, Biodegradation of *p*-nitrophenol by *P. putida*, Bioresour. Technol. 97 (2006) 982–988.
[22] B. Angelova, H.P. Schmauder, Lipophilic compounds in biotechnology – interactions with cells and technological problems, J. Biotechnol. 67 (1999) 13–32.
[23] S. Murínová, K. Dercová, Response mechanisms of bacterial degraders to environmental contaminants on the level of cell walls and cytoplasmic membrane, Int. J. Microbiol. (2014), http://dx.doi.org/10.1155/2014/873081.
[24] P. Bernat, P. Siewiera, A. Soboń, J. Długoński, Phospholipids and protein adaptation of *Pseudomonas* sp. to the xenoestrogen tributyltin chloride (TBT), World J. Microbiol. Biotechnol. 30 (2014) 2343–2350.
[25] P. Bernat, E. Gajewska, R. Szewczyk, M. Słaba, J. Długoński, Tributyltin (TBT) induces oxidative stress and modifies lipid profile in the filamentous fungus *Cunninghamella elegans*, Environ. Sci. Pollut. Res. 21 (2013) 4228–4235.
[26] J. Folch, M. Lees, G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 199 (1957) 833–841.
[27] K. Su, D.J. Bremer, R. Jeannotte, R. Welti, C. Yang, Membrane lipid composition and heat tolerance in cool-season turfgrasses, including a hybrid bluegrass, J. Am. Soc. Hortic. Sci. 134 (2009) 511–520.
[28] J. Bielawski, Z.M. Szulc, Y.A. Hannun, A. Bielawska, Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry, Methods 39 (2006) 82–91.
[29] R.S. Liao, R.P. Rennie, J.A. Talbot, Assessment of the effect of amphotericin B on the vitality of *Candida albicans*, Antimicrob. Agents Chemother. 43 (1999) 1034–1041.
[30] E.K. Krasnowska, E. Gratton, T. Parasassi, Prodan as a membrane surface fluorescence probe: partitioning between water and phospholipid phases, Biophys. J. 74 (1998) 1984–1993.
[31] S. Hofstetter, C. Denter, R. Winter, L.M. Mcmullen, M.G. Gänzle, Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp, J. Microbiol. Methods 91 (2012) 93–100.
[32] J.G. Kuhry, P. Fonteneau, G. Duportail, C. Maechling, G. Laustriat, TMA-DPH: a suitable fluorescence polarization probe for specific plasma membrane fluidity studies in intact living cells, Cell Biophys. 5 (1983) 129–140.
[33] A.I. de Kroon, P.J. Rijken, C.H. De Smet, Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective, Prog. Lipid Res. 52 (2013) 374–394.
[34] M.R. Miller, S.Y. Quek, K. Staehler, T. Nalder, M.A. Packer, Changes in oil content, lipid class and fatty acid composition of the microalga *Chaetoceros calcitrans* over different phases of batch culture, Aquac. Res. 45 (2014) 1634–1647.
[35] B. Qiao, H. Lu, Y.X. Cao, R. Chen, Y.J. Yuan, Phospholipid profiles of *Penicillium chrysogenum* in different scales of fermentations, Eng. Life Sci. 13 (2013) 496–505.
[36] G.I. Lucchesi, A.S. Liffourrena, P.S. Boeris, M.A. Salvano, Adaptative response and degradation of quaternary ammonium compounds by *Pseudomonas putida* A ATCC 12633, in: Méndez-Vilas (Ed.), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 2010, pp. 1297–1303.
[37] M.N. Möller, Q. Li, M. Chinnaraj, H.C. Cheung, J.R.Jr. Lancaster, A. Denicola, Solubility and diffusion of oxygen in phospholipid membranes, Biochim. Biophys. Acta 1858 (2016) 2923–2930.
[38] S.L. Pereira, A.E. Leonard, P. Mukerji, Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes, Prostaglandins Leukot. Essent. Fatty Acids 68 (2003) 97–106.
[39] A. Masia, S.V. Avery, M.A. Zoroddu, G.M. Gadd, Enrichment with a polyunsaturated fatty acid enhances the survival of *Saccharomyces cerevisiae* in the presence of tributyltin, FEMS Microbiol. Lett. 167 (1998) 321–326.
[40] T.N. Dinh, K. Nagahisa, T. Hirasawa, C. Furusawa, H. Shimizu, Adaptation of *Saccharomyces cerevisiae* cells to high ethanol concentration and changes in fatty acid composition of membrane and cell size, PLoS ONE 3 (7) (2008) e2623.
[41] F. Dubois-Brissonnet, M. Naïtali, A.A. Mafu, R. Briandet, Induction of fatty acid composition modifications and tolerance to biocides in *Salmonella enterica* serovar *Typhimurium* by plant-derived terpenes, Appl. Environ. Microbiol. 77 (3) (2011) 906–910.
[42] P. Lambelet, F. Saucy, J. Löliger, Radical exchange reactions between vitamin E, vitamin C and phospholipids in autoxidizing polyunsaturated lipids, Free Radic. Res. 20 (1994) 1–10.
[43] K.M. Marakulina, R.V. Kramor, Y.K. Lukanina, I.G. Plashchina, A.V. Polyakov, I.V. Fedorova, I.Y. Chukicheva, A.V. Kutchin, L.N. Shishkina, Effect of the nature of phospholipids on the degree of their interaction with isobornylphenol antioxidants, Russ. J. Phys. Chem. A 90 (2016) 286–292.
[44] V. Sánchez, M. Lutz, Fatty acid composition of microsomal phospholipids in rats fed different oils and antioxidant vitamins supplement, Nutr. Biochem. 9 (1998) 155–163.
[45] G. Janer, J.C. Navarro, C. Porte, Exposure to TBT increases accumulation of lipids and alters fatty acid homeostasis in the ramshorn snail *Marisa cornuarietis*, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146 (2007) 368–374.
[46] E. Puccia, C.M. Messina, M.V. Cangialosi, P. D'Agati, C. Mansueto, C. Pellerito, L. Nagy, V. Mansueto, M. Scopelliti, T. Fiore, L. Pellerito, Lipid and fatty acid variations in *Ciona intestinalis* ovary after tri-n-butyltin (IV) chloride exposure, Appl. Organomet. Chem. 19 (2005) 23–29.
[47] Y. Wang, J. Zhang, M. Pan, Tributyltin targets hepatic transcriptional regulation of lipid metabolism in mice, Toxicol. Environ. Chem. 99 (2017) 492–504.
[48] J. Zhang, C. Zhang, D. Ma, M. Liu, S. Huang, Lipid accumulation, oxidative stress and immune-related molecules affected by tributyltin exposure in muscle tissues of rare minnow (*Gobiocypris rarus*), Fish Shellfish Immunol. 71 (2017) 10–18.
[49] S. Endo, B.I. Escher, K.U. Goss, Capacities of membrane lipids to accumulate neutral organic chemicals, Environ. Sci. Technol. 45 (2011) 5912–5921.
[50] T. Czabany, K. Athenstaedt, G. Daum, Synthesis, storage and degradation of neutral lipids in yeast, Biochim. Biophys. Acta 1771 (2007) 299–309.
[51] Q. Gao, Y. Shang, W. Huang, C. Wang, Glycerol-3-phosphate acyltransferase contributes to triacylglycerol biosynthesis, lipid droplet formation, and host invasion in *Metarhizium robertsii*, Appl. Environ. Microbiol. 79 (2013) 7646–7653.
[52] T. Róg, M. Pasenkiewicz-Gierula, I. Vattulainen, M. Karttunen, Ordering effects of cholesterol and its analogues, Biochim. Biophys. Acta 1788 (2009) 97–121.
[53] F. Abe, T. Hiraki, Mechanistic role of ergosterol in membrane rigidity and cycloheximide resistance in *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1788 (2009) 743–752.
[54] M. Klamer, E. Bååth, Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9, Soil Biol. Biochem. 36 (2004) 57–65.
[55] J. Gabrielska, S. Przestalski, A. Miszta, M. Soczyńska-Kordala, M. Langner, The effect of cholesterol on the adsorption of phenyltin compounds onto phosphatidylcholine and sphingomyelin liposome membranes, Appl. Organomet. Chem. 18 (2004) 9–14.
[56] J.S. Won, I. Singh, Sphingolipid signaling and redox regulation, Free Radic. Biol. Med. 40 (2006) 1875–1888.
[57] P. Pullmannová, L. Pavlíková, A. Kováčik, M. Sochorová, B. Školová, P. Slepička, J. Maixner, J. Zbytovská, K. Vávrová, Permeability and microstructure of model stratum corneum lipid membranes containing ceramides with long (C16) and very long (C24) acyl chains, Biophys. Chem. 224 (2017) 20–31.
[58] N.A. Babenko, L.K. Hassouneh, V.S. Kharchenko, V.V. Garkavenko, Vitamin E prevents the age-dependent and palmitate-induced disturbances of sphingolipid turnover in liver cells, Age (Dordr.) 34 (2012) 905–915.
[59] Y. Uchida, M. Behne, D. Quiec, P.M. Elias, W.M. Holleran, Vitamin C stimulates sphingolipid production and markers of barrier formation in submerged human keratinocyte cultures, J. Invest. Dermatol. 117 (2001) 1307–1313.
[60] C. Laflamme, J. Ho, M. Veillette, M.C. de Latrémoille, D. Verreault, A. Mériaux, C. Duchaine, Flow cytometry analysis of germinating *Bacillus* spores, using membrane potential dye, Arch. Microbiol. 183 (2005) 107–112.
[61] H. Elicharova, H. Sychrova, Fluconazole affects the alkali-metal-cation homeostasis and susceptibility to cationic toxic compounds of *Candida glabrata*, Microbiology 8 (2014) 1705–1713.
[62] P. Bernat, M. Słaba, J. Długoński, Action of tributyltin (TBT) on the lipid content and potassium retention in the organotins degradating fungus *Cunninghamella elegans*, Curr. Microbiol. 59 (2009) 315–320.
[63] J.S. White, J.M. Tobin, Role of speciation in organotin toxicity to the yeast *Candida maltose*, Environ. Sci. Technol. 38 (2004) 3877–3884.
[64] A.P. Demchenko, Y. Mély, G. Duportail, A.S. Klymchenko, Monitoring biophysical properties of lipid membranes by environment-sensitive fluorescent probes, Biophys. J. 96 (2009) 3461–3470.
[65] H. Pruchnik, T. Kral, M. Hof, Interaction of new butyltin citrate complex with lipid model membrane and DNA, J. Therm. Anal. Calorim. 118 (2014) 967–975.
[66] A. Ambrosini, E. Bertoli, G. Zolese, Effect of organotin compounds on membrane lipids: fluorescence spectroscopy studies, Appl. Organomet. Chem. 10 (1996) 53–59.
[67] D. Bonarska-Kujawa, H. Kleszczyńska, S. Przestalski, The location of organotins within the erythrocyte membrane in relation to their toxicity, Ecotoxicol. Environ. Saf. 78 (2012) 232–238.
[68] J.D. Martins, A.S. Jurado, A.J. Moreno, V.M. Madeira, Comparative study of tributyltin toxicity on two bacteria of the genus *Bacillus*, Toxicol. In Vitro 19 (2005) 943–949.
[69] J.S. White, J.M. Tobin, Inorganic tin and organotin interactions with *Candida maltosa*, Appl. Microbiol. Biotechnol. 63 (2004) 445–451.
[70] W.R. Cullen, F.G. Herring, B.U. Nwata, The effect of organotin compounds on the permeability of model biological membranes, Appl. Organomet. Chem. 11 (1997) 369–379.
[71] B.R.C.M.K. Heywood, P.C. Waterfield, Organotin biocides XV: modelling the interactions of triorganotins with cell membranes, Appl. Organomet. Chem. 3 (1989) 443–450.
[72] M. Tsukazachi, H. Satsu, A. Mori, Y. Sugita-Konishi, M. Shimizu, Effect of tributyltin on barrier functions in human intestinal cells, Biochem. Biophys. Res. Commun. 315 (2004) 991–997.
[73] J. Gao, J. Ye, J. Ma, L. Tang, J. Huang, Biosorption and biodegradation of triphenyltin by *Stenotrophomonas maltophilia* and their influence on cellular metabolism, J. Hazard. Mater. 276 (2014) 112–119.
[74] J. Huang, J. Ye, J. Ma, J. Gao, S. Chen, X. Wu, Triphenyltin biosorption, dephenylation pathway and cellular responses during triphenyltin biodegradation by *Bacillus thuringiensis* and tea saponin, Chem. Eng. J. 249 (2014) 167–173.
[75] J. Ye, H. Zhao, H. Yin, H. Peng, L. Tang, J. Gao, Y. Ma, Triphenyltin biodegradation and intracellular material release by *Brevibacillus brevis*, Chemosphere 105 (2014) 62–67.
[76] H.S. Al-Shmgani, R.M. Moate, P.D. Macnaughton, J.R. Sneyd, A.J. Moody, Effects of hyperoxia on the permeability of 16HBE14o-cell monolayers–the protective role of antioxidant vitamins E and C, FEBS J. 280 (2013) 4512–4521.
[77] K. Singh, V. Mhatre, M. Bhori, T. Marar, Vitamins E and C reduce oxidative stress and mitochondrial permeability transition caused by camptothecin – an *in vitro* study, Toxicol. Environ. Chem. 95 (2013) 646–657.

Supplementary

Table 1. Relative abundance (%) of acylglycerol species, total levels of tri- and diacylglycerols as well as the TAG/DAG ratio determined in the *M. robertsii* cells after 3 h and 24 h of incubation with the butyltins and vitamin C.

Acylglycerol species	Culture										Two-way ANOVA			
	3 h					24 h								
	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	Control	DBT	DBT + vitamin C	TBT	TBT + vitamin C	B_1,T, B_1×T	V_1,T, V_1×T	B_2,T, B_2×T	V_2,T, V_2×T
TAG 16:0 18:1 18:1	3.3±0.3	2.9±0.1	3.6±0.1	2.8±0.2	2.2±0.1	2.5±0.1	3.3±1.1	2.4±0.1	2.7±0.4	2.6±0.7	ns, ns, *	ns, ns, *	ns, ns, ns	*, ns, ns
DAG 16:0 18:0	9.0±2.5	10.5±0.5	8.5±1.6	9.4±0.3	9.9±0.7	10.2±1.0	10.0±0.7	10.8±0.1	14.4±8.1	8.6±1.3	ns, ns, ns	ns, ns, ns	ns, ns, ns	*, ns, *
DAG 18:1 18:2	9.0±7.5	4.3±0.1	3.7±0.5	3.0±0.3	3.2±1.1	2.8±0.8	7.2±4.0	3.8±0.8	3.2±0.3	2.5±0.4	ns, *, **	*, ns, ns	**, **, **	ns, ns, ns
DAG 18:1 18:1	16.7±4.2	16.5±2.0	14.1±1.3	14.6±4.0	14.9±2.0	12.2±1.0	12.3±0.7	17.1±0.1	13.7±8.1	13.6±1.3	ns, **, ns	ns, *, **	ns, **, ns	ns, *, ns
DAG 18:0 18:1	17.4±4.5	17.5±0.1	17.3±0.4	18.9±2.3	20.1±1.6	17.2±0.1	17.4±2.5	19.0±0.6	17.7±1.0	20.8±2.7	ns, ns, ns	ns, ns, ns	ns, ns, ns	ns, ns, ns
DAG 16:0 16:0	27.7±2.0	33.4±2.5	35.4±0.4	35.1±1.0	36.8±1.5	40.9±5.3	32.6±2.0	32.5±3.3	32.6±1.9	36.7±0.5	ns, **, **	ns, ns, ns	ns, **, **	ns, ns, ns
Σ TAGs (%)	20.2±2.8	17.8±0.2	21.0±0.2	19.1±0.9	15.1±0.0	16.8±0.3	20.6±4.2	16.8±0.4	18.4±2.4	17.8±2.7	ns, ns, ns	ns, ns, ns	ns, ns, ns	ns, ns, ns
Σ DAGs (%)	79.8±2.8	82.2±0.2	79.0±0.2	80.9±0.9	84.9±0.0	83.2±0.3	79.4±0.2	83.2±0.4	81.6±2.4	82.2±2.7	ns, ns, ns	ns, ns, ns	ns, ns, ns	ns, ns, ns
DAG/TAG	3.9	4.6	3.8	4.2	5.6	4.9	3.9	4.9	4.4	4.6	ns, ns, ns	ns, ns, ns	ns, ns, ns	ns, ns, ns

Results were tested by two-way ANOVA with treatment (B or V) and time (T) factors;
B – butyltin [B_1 – DBT, B_2 – TBT; analyses with regard to control]; V – vitamin C [analyses with regard to DBT (V_1) or TBT (V_2)]; T – time.
p significance was shown: ** – $p < 0.01$, * – $p < 0.05$, ns – not significant.

VI. STWIERDZENIA KOŃCOWE

1. Optymalizacja warunków wzrostu szczepu *M. robertsii* IM 6519 – a w szczególności intensywne natlenienie hodowli ($pO_2 \geq 20\%$) – prowadzi do szybkiej i całkowitej eliminacji butylowych związków cyny ze środowiska wzrostu.

2. Procesy leżące u podstaw efektywnej biodegradacji butylocyn to modyfikacje a) morfologiczne grzybni, b) profilu fosfolipidowego i c) dynamiki dwuwarstwy lipidowej ułatwiające transfer masy, a także kometabolizm oraz zaangażowanie monooksygenaz cytochromu P450.

3. Witamina C to najbardziej skuteczna substancja pod względem zwiększania wydajności usuwania MBT oraz ochrony błon komórkowych przed stresem oksydacyjnym, stanowiąca najlepszy (spośród badanych antyoksydantów) suplement hodowli grzybowej.

4. Występowanie inhibicji kompetycyjnej kompleksu enzymatycznego CYP450 wyklucza zastosowanie estrogenów naturalnych jako intensyfikatorów procesu biodegaradacji DBT.

5. Nadprodukcja reaktywnych form tlenu i azotu w badanej grzybni indukowana jest obecnością MBT – substancji uznawanej za najmniej toksyczną spośród butylowych związków cyny.

6. Brak udziału katalazy i dysmutazy ponadtlenkowej w usuwaniu nadtlenku wodoru i anionorodnika ponadtlenkowego potwierdza pośrednio efektywność estronu i 17β-estradiolu w usuwaniu reaktywnych form tlenu.

7. Hiperpolaryzacja błony grzybowej (przypuszczalnie) przyspiesza wiązanie powierzchniowe TBT.

8. Sztywność i integralność błon komórkowych o zwiększonej zawartości fosfolipidów, sfingolipidów oraz ergosterolu to zmiany adaptacyjne szczepu IM 6519 względem DBT i TBT.

9. Dodatek witaminy C ogranicza zmiany badanych komponentów i właściwości fizyko-chemicznych membran szczepu *M. robertsii* IM 6519 wywołane ekspozycją na butylowe związki cyny, przywracając równowagę błony grzybowej.

10. Wzrost płynności i przepuszczalności błony komórkowej szczepu IM 6519 suplementowanego witaminą C (przypuszczalnie) wspiera transport i biodegradację MBT, zmniejszając skutki stresu oksydacyjnego.

11. Swoistymi biomarkerami obecności DBT/TBT i witaminy C są: stosunek fosfatydylocholin do fosfatydyloetanoloamin; stosunek zawartości 16- do 18-węglowych kwasów tłuszczowych; poziom PC 18:2 18:2; wartość indeksu wiązań podwójnych; poziom ergosterolu; sztywność i integralność błon komórkowych.

Wpływ osiągniętych wyników na rozwój dyscypliny wraz z omówieniem potencjalnego zastosowania praktycznego

Badania wykonane w trakcie tworzenia niniejszej rozprawy doktorskiej umożliwiły stworzenie nieopisanego wcześniej systemu biologicznego służącego do szybkiej i całkowitej eliminacji butylowych związków cyny z użyciem szczepu grzybowego *M. robertsii* IM 6519 inkubowanego w warunkach intensywnego napowietrzania oraz suplementacji podłoża wzrostowego witaminą C.

Przeprowadzone analizy zwracają uwagę na istotę dostępu tlenu dla prawidłowego przebiegu bioprocesów tlenowych. Zastosowany model badawczy wydaje się być silnie aerobowym mikroorganizmem wymagającym znacznych dostaw tlenu do hodowli, co skutkuje zmianami morfologicznymi grzybni niewątpliwie ułatwiającymi transfer tlenu i dwutlenku węgla, substancji odżywczych oraz butylowych związków cyny pomiędzy strzępkami, a podłożem wzrostowym. Ponadto, uzyskane wyniki sugerują weryfikację toksyczności powstających metabolitów, które są zazwyczaj uznawane za mniej toksyczne niż związki macierzyste, co może stanowić błędne założenie, tak jak to miało miejsce w przypadku MBT. Co więcej, niezwykle ważnym aspektem okazał się być trafny wybór substancji o aktywności antyoksydacyjnej. Pozytywny wpływ przeciwutleniacza na przebieg procesu biodegradacji butylocyn, równoznaczny z ograniczeniem stresu oksydacyjnego i ochroną błony komórkowej wyklucza się wraz z wyborem antyoksydantu przekształcanego przez ten sam układ enzymatyczny co związek ulegający biodegradacji. Dlatego też, wytypowane do badań estrogeny naturalne jak i witamina E nie sprawdziły się w roli suplementów przyspieszających tempo usuwania butylowych związków cyny. Ostatecznie, analizy lipidomiczne umożliwiły wskazanie zmian adaptacyjnych na poziomie molekularnym (m. in. w składzie fosfolipidów i poziomie ergosterolu oraz w właściwościach fizyko-chemicznych błon komórkowych), związanych bezpośrednio z efektywnością biodegradacji butylowych związków cyny przez szczep *M. robertsii* IM 6519.

Badania wykonane podczas realizacji niniejszej pracy doktorskiej mają charakter podstawowy, jednakże zauważalny jest potencjał przeniesienia opracowanego modelu biologicznego z warunków laboratoryjnych na ewentualne aplikacje praktyczne w obszarze utylizacji osadów ściekowych stanowiących rezerwuar butylowych związków cyny. Co więcej, wykorzystanie powyższych wskazówek dotyczących optymalizacji procesu biodegradacji, wyboru antyoksydantów oraz możliwych manipulacji w składzie i właściwościach błon

komórkowych przyczyniających się do zwiększenia wydajności biodegradacji butylowych związków cyny przez szczep *M. robertsii* IM 6519 może posłużyć podczas kreowania innych systemów biologicznych do usuwania związków o podobnym, szkodliwym działaniu.

V. STRESZCZENIE

Butylowe związki cyny charakteryzują się różnorodnymi mechanizmami toksyczności, a ze względu na ich zdolność do akumulacji, pozostają długoterminowymi zanieczyszczeniami środowiska naturalnego. Niestety, substancje te stanowią zagrożenie nie tylko dla mikroorganizmów środowiskowych, organizmów morskich, ale także dla człowieka, będącego ostatnim ogniwem łańcucha pokarmowego. Konwencjonalne metody oczyszczania skażonych środowisk obarczone są wysokimi kosztami oraz stosunkowo niską skutecznością, przez co procesy biotechnologiczne wykorzystujące mikroorganizmy degradujące ksenobiotyki zyskały na wartości.

Niniejsza praca doktorska dotyczy oddziaływania wybranych substancji o właściwościach antyoksydacyjnych: witamin C i E oraz estrogenów naturalnych - 17β-estradiolu i estronu na proces biodegradacji butylowych związków cyny, z jednoczesnym uwzględnieniem modyfikacji wybranych elementów lipidomu mikroskopowego grzyba strzępkowego *Metarhizium robertsii*.

W trakcie realizacji niniejszej rozprawy doktorskiej przeprowadzono optymalizację biodegradacji dibutylocyny (DBT) przez szczep grzybowy *M. robertsii* IM 6519, doprowadzając do szybkiej i całkowitej eliminacji badanego związku ze środowiska wzrostu. Następnie, odkryto procesy leżące u podstaw efektywnego rozkładu butylowych związków cyny, takie jak zmiany morfologiczne grzybni indukowane intensywnym napowietrzaniem, kometabolizm oraz udział monooksygenaz cytochromu P450 w tym procesie. Z kolei identyfikacja hydroksylowanej monobutylocyny (MBTOH), jako metabolitu pośredniego powstającego w trakcie reakcji debutylacji DBT, umożliwiła zaproponowanie szlaku biotransformacji związku macierzystego. Kolejny cel naukowy niniejszej pracy dotyczył wpływu kilku substancji o właściwościach antyoksydacyjnych na wydajność rozkładu butylowych związków cyny przez badany grzyb strzępkowy. W rezultacie przeprowadzonych analiz wskazano na witaminę C jako najlepszy suplement hodowli szczepu *M. robertsii* IM 6519 wyjaśniając pozytywne oddziaływanie przeciwutleniacza poprzez ograniczenie peroksydacji błon komórkowych. Negatywny wpływ estrogenów naturalnych na wydajność biodegradacji DBT został natomiast wyjaśniony występowaniem zjawiska inhibicji kompetycyjnej monooksygenaz cytochromu P450. Detekcja *in situ* reaktywnych form tlenu i azotu w badanej grzybni dostarczyła dowodów, iż przyczyną ich nadprodukcji jest obecność MBT (substancji uznawanej za najmniej toksyczną spośród butylowych związków cyny), a nie

DBT (jak początkowo uważano). Z kolei, wykluczenie udziału enzymów antyoksydacyjnych takich jak katalaza i dysmutaza ponadtlenkowa w usuwaniu nadtlenku wodoru i anionorodnika ponadtlenkowego potwierdziło pośrednio efektywność estronu i 17β-estradiolu w usuwaniu reaktywnych form tlenu. Podczas realizacji ostatniego celu naukowego niniejszej pracy doktorskiej zidentyfikowano zmiany adaptacyjne wybranych komponentów i właściwości fizyko-chemicznych błon grzybowych *M. robertsii* stanowiących swoiste biomarkery obecności DBT/TBT i witaminy C. Wśród wyznaczników uniwersalnej reakcji komórek poddanych ekspozycji na badane związki wyróżniono stosunek fosfatydylocholin do fosfatydyloetanoloamin, stosunek zawartości 16- do 18-węglowych kwasów tłuszczowych, poziom PC 18:2 18:2, wartość indeksu wiązań podwójnych, poziom ergosterolu oraz zwiększoną sztywność i integralność błon komórkowych.

Analizy przeprowadzone w trakcie tworzenia niniejszej rozprawy doktorskiej umożliwiły stworzenie nieopisanego wcześniej systemu biologicznego służącego do szybkiej i całkowitej eliminacji butylowych związków cyny z użyciem szczepu grzybowego *M. robertsii* IM 6519 inkubowanego w warunkach intensywnego napowietrzania oraz suplementacji podłoża wzrostowego witaminą C.

Badania wykonane podczas realizacji niniejszej pracy doktorskiej mają charakter podstawowy, jednakże zauważalny jest potencjał przeniesienia opracowanego modelu biologicznego z warunków laboratoryjnych na ewentualne aplikacje praktyczne w obszarze utylizacji osadów ściekowych stanowiących rezerwuar butylowych związków cyny. Co więcej, uzyskane wyniki mogą posłużyć podczas kreowania innych systemów biologicznych do usuwania związków o podobnym, szkodliwym działaniu.

VI. ABSTRACT

Butyltin compounds are characterized by a variety of toxicity mechanisms. Moreover, due to their ability to accumulate, they remain long-term pollutants of the natural environment. Unfortunately, these substances pose a threat not only to environmental microorganisms and marine organisms but also to humans, who are the last link in the food chain. Conventional purification methods of contaminated environments are burdened with high costs and relatively low efficiency, therefore biotechnological processes using xenobiotic-degrading microorganisms have gained in value.

This doctoral thesis concerns the analysis of the effect of the compounds with antioxidant properties: vitamins C and E, natural estrogens - 17β-estradiol and estrone on the process of butyltins biodegradation with simultaneous consideration of the modification of selected elements of the lipidome of the microscopic filamentous fungus *Metarhizium robertsii*.

During the doctoral thesis realization, optimization of dibutyltin (DBT) biodegradation by a fungal strain *M. robertsii* IM 6519 was carried out, leading to rapid and complete elimination of the compound from the growth environment. Subsequently, the processes underlying the effective decomposition of butyltin compounds, such as morphological mycelium modifications induced by intensive aeration, cometabolism and participation of cytochrome P450 monooxygenases, were discovered. Moreover, identification of the hydroxylated monobutyltin (MBTOH), as an intermediate metabolite formed during the DBT debutilation made it possible to propose a biotransformation pathway of the parent compound. The subsequent aim of the doctoral thesis concerned an impact of few compounds with antioxidant properties on the efficiency of butyltins decomposition by the tested filamentous fungus. As a result of the performed analyses, vitamin C was indicated as the best culture supplement for the *M. robertsii* IM 6519 strain explaining the positive effect of the antioxidant by limiting the cell membranes peroxidation. On the other hand, a negative effect of the natural estrogens on the efficiency of DBT biodegradation was explained by the occurrence of the competitive inhibition of cytochrome P450 monooxygenases. *In situ* detection of reactive oxygen and nitrogen species in the tested mycelium providing evidence that their overproduction is caused by the presence of MBT (a substance considered as the least toxic among butyltin compounds) and not DBT (as initially thought). In turn, the exclusion of participation of antioxidative enzymes such as catalase and superoxide dismutase in the removal of hydrogen peroxide and superoxide anion indirectly proved the effectiveness of

estrone and 17β-estradiol in removal of reactive oxygen species. During realization of the last aim of the doctoral thesis, adaptive modifications of chosen components and physico-chemical properties of the *M. robertsii* membranes were identified and some of them were indicated as specific biomarkers of the presence of DBT/TBT and vitamin C. A ratio of phosphatidylcholines to phosphatidylethanolamines, ratio of 16- to 18-carbon fatty acids, PC 18:2 18:2 content, double bonds index, ergosterol level and increased stiffness and integrity of the cell membranes were indicated as determinants of the universal response of the fungal cells exposed to the tested compounds.

The analyses conducted during the creation of the doctoral thesis made it possible to create a previously unwritten biological system for the rapid and complete elimination of butyltin compounds by the application of a fungal *M. robertsii* IM 6519 strain incubated under conditions of intense aeration and vitamin C supplementation.

The research carried out during the realization of the doctoral thesis is basic, but a potential of transferring of the developed biological model from laboratory conditions to possible practical applications (i.e. for the utilization of sewage sludge constituting a reservoir of butyltin compounds) is visible. Moreover, the obtained results can be used for creating other biological systems to remove compounds with similar harmful effects.

VII. LITERATURA

1. Angelova B, Schmauder HP. 1999. Lipophilic compounds in biotechnology – interactions with cells and technological problems. J. Biotechnol., 67:13-32.

2. Azzi A. 2007. Molecular mechanism of α-tocopherol action. Free Radic Biol Med., 43:16-21.

3. Bernat P, Długoński J. 2006. Acceleration of tributyltin chloride (TBT) degradation in liquid cultures of the filamentous fungus *Cunninghamella elegans*. Chemosphere, 62(1):3-8.

4. Bernat P, Długoński J. 2009. Isolation of *Streptomyces* sp. strain capable of butyltin compounds degradation with high efficiency. J Hazard Mater., 171:660-664.

5. Bernat P, Siewiera P, Soboń A, Długoński J. 2014. Phospholipids and protein adaptation of *Pseudomonas* sp. to the xenoestrogen tributyltin chloride (TBT). World J Microbiol Biotechnol., 30:2343-2350.

6. Bernat P, Szewczyk R, Krupiński M, Długoński J. 2013 Butyltins degradation by *Cunninghamella elegans* and *Cochliobolus lunatus* co-culture. J Hazard Mater., 246-247:277-282.

7. Bonarska-Kujawa D, Kleszczyńska H, Przestalski S. 2012. The location of organotins within the erythrocyte membrane in relation to their toxicity. Ecotoxicol Environ Saf., 4/2012. DOI: 10.1016/j.ecoenv.2011.11.027.

8. Bouchard N, Pelletier É, Fournier M. 1999. Effects of butyltin compounds on phagocytic activity of hemocytes from three marine bivalves. Environ Toxicol Chem., 18(3):519-522.

9. Buettner GR. 1993. The pecking order of free-radicals and antioxidants: lipid-peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophys., 300:535-543.

10. Chantong B, Kratschmar DV, Lister A, Odermatt A. 2014. Dibutyltin promotes oxidative stress and increases inflammatory mediators in BV-2 microglia cells. Toxicol Lett., 230(2):177-187.

11. Chen C, Chen L, Huang Q, Chen Z, Zhang W. 2019. Organotin contamination in commercial and wild oysters from China: Increasing occurrence of triphenyltin. Mar Pollut Bull., 139:174-180.

12. Chiavarini S, Ubaldi C, Cannarsa S. 2014. Biocides in antifouling paints: environmental concentration levels and distribution. Energia, Ambiente e Innovazione 1/2014. DOI: 10.12910/EAI2014-45.

13. de Carvalho Oliveira R, Santelli RE. 2010. Occurrence and chemical speciation analysis of organotin compounds in the environment: a review. Talanta, 82(1):19-24.

14. de Castro IB, Perina FC, Fillmann G. 2012. Organotin contamination in South American coastal areas. Environ Monit Assess., 184(3):1781-9179.

15. de Kroon A, Rijken PJ, De Smet CH. 2013. Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. Prog. Lipid Res., 52:374-394.

16. Du J, Chadalavada S, Chen Z, Naidu R. 2014. Environmental remediation techniques of tributyltin contamination in soil and water: A review. CHEM ENG J., 235:141-150.

17. Farr CH, Reinisch K, Holson JF, Neubert D. 2001. Potential teratogenicity of dibutyltin dichloride and other dibutyltin compounds. Teratog. Carcinog. Mutagen., 21(6):405-415.

18. Ferreira M, Blanco L, Garrido A, Vieites JM, Cabado AG. 2013. *In vitro* approaches to evaluate toxicity induced by organotin compounds tributyltin (TBT), dibutyltin (DBT), and monobutyltin (MBT) in neuroblastoma cells. J. Agric. Food Chem., 61(17):4195-4203.

19. Filipkowska A, Kowalewska G, Pavoni B. 2014. Organotin compounds in surface sediments of the Southern Baltic coastal zone: a study on the main factors for their accumulation and degradation. Environ Sci Pollut Res., 21(3):2077-2087.

20. Frouin H Lebeuf M, Saint-Louis R, Hammill M, Pelletier E, Fournier M. 2008. Toxic effects of tributyltin and its metabolites on harbour seal (*Phoca vitulina*) immune cells *in vitro*. Aquat Toxicol., 90(3):243-251.

21. Gennari A, Viviani B, Galli CL, Marinovich M, Pieters R, Corsini E. 2000. Organotins induce apoptosis by disturbance of [Ca^{2+}] and mitochondrial activity, causing oxidative stress and activation of caspases in rat thymocytes. Toxicol. Appl. Pharmacol., 169(2):185-190.

22. Gumy C, Chandsawangbhuwana C, Dzyakanchuk AA, Kratschmar DV, Baker ME, Odermatt A. 2008. Dibutyltin disrupts glucocorticoid receptor function and impairs glucocorticoid-induced suppression of cytokine production. PLoS One, 3(10),e3545.

23. Hassan AT, Qurban M, Manikandan K, Tawabini B, Basheer C, Periyadan K. 2019. Assessment of the organotin pollution in the coastal sediments of the Western Arabian Gulf, Saudi Arabia. Mar Pollut Bull., 139:174-180.

24. Hoch M. 2001. Organotin compounds in the environment - An overview. Appl Geochem. 6(7-8):719-743.

25. Ishihara Y, Kawami T, Ishida A, Yamazaki T. 2012. Tributyltin induces oxidative stress and neuronal injury by inhibiting glutathione S-transferase in rat organotypic hippocampal slice cultures. Neurochem. Int., 60:782-790.

26. Jenkins SM, Ehman K, Barone S Jr. 2004. Structure–activity comparison of organotin species: dibutyltin is a developmental neurotoxicant *in vitro* and *in vivo*. Behav Brain Res., 151:1-12.

27. Kannan K, Falandysz J. 1997. Butyltin residues in sediment, fish, fish-eating birds, harbour porpoise and human tissues from the Polish coast of the Baltic Sea. Mar Pollut Bull., 34(3):203-207.

28. Li Y, Schellhorn HE. 2007. New developments and novel therapeutic perspectives for vitamin C. J Nutr., 137(10):2171-2184.

29. Liao X, Fang W, Lin L, Lu H-L, Leger RJ St. 2013. *Metarhizium robertsii* produces an extracellular invertase (MrINV) that plays a pivotal role in rhizospheric interactions and root colonization. Plos One, 8:1-14.

30. Mino Y, Amano F, Yoshioka T, Konishi Y. 2008. Determination of organotins in human breast milk by gas chromatography with flame photometric detection. J Health Sci., 54(2):224-228.

31. Moser VC, McGee JK, Ehman KD. 2009. Concentration and persistence of tin in rat brain and blood following dibutyltin exposure during development. J Toxicol Environ Health A., 72(1):47-52.

32. Murínová S, Dercová K. 2014. Response mechanisms of bacterial degraders to environmental contaminants on the level of cell walls and cytoplasmic membrane. Int. J. Microbiol. DOI: 10.1155/2014/873081.

33. Nesci S, Ventrella V, Trombetti F, Pirini M, Borgatti AR, Pagliarani A. 2011. Tributyltin (TBT) and dibutyltin (DBT) differently inhibit the mitochondria Mg-ATPase activity in mussel digestive gland. Toxicol in Vitro, 25:117-124.

34. Niki E, Yoshida Y, Saito Y, Noguchi N. 2005. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun., 338(1):668-676.

35. Okoro HK, Fatoki OS, Adekola FA, Ximba BJ, Snyman RG. 2011. Sources, environmental levels and toxicity of organotin in marine environment-a review. Asian J. Chem., 23(2):473-482.

36. Pagliarani A, Nesci S, Ventrella V. 2013. Toxicity of organotin compounds: shared and unshared biochemical targets and mechanisms in animal cells. Toxicol In Vitro, 27:978-990.

37. Ponce BL, Latorre VK, González M, Seeger M. 2011. Antioxidant compounds improved PCB-degradation by *Burkholderia xenovorans* strain LB400. Enzym Microb Technol., 49(6-7):509-516.

38. Potin O, Veignie E, Rafin C. 2004. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. FEMS Microbiol Ecol., 51(1):71-78.

39. Raederstorff D, Wyss A, Calder PC, Weber P, Eggersdorfer M. 2015. Vitamin E function and requirements in relation to PUFA. Br J Nutr., 114(8):1113-1122.

40. Rantakokko P, Turunen A, Verkasalo PK, Kiviranta H, Männistö S, Vartiainen T. 2008. Blood levels of organotin compounds and their relation to fish consumption in Finland. Sci Total Environ., 399(1-3):90-95.

41. Róg T, Pasenkiewicz-Gierula M, Vattulainen I, Karttunen, M. 2009. Ordering effects of cholesterol and its analogues. Biochim. Biophys. Acta, 1788:97-121.

42. Różalska S, Soboń A, Pawłowska J, Wrzosek M, Długoński J. 2015. Biodegradation of nonylphenol by a novel entomopathogenic *Metarhizium robertsii* strain. Bioresour. Technol., 191:166-172.

43. Ruiz-Larrea MB, Martín C, Martínez R, Navarro R, Lacort M, Miller NJ. 2000. Antioxidant activities of estrogens against aqueous and lipophilic radicals; differences between phenol and catechol estrogens. Chem Phys Lipids, 105(2):179-188.

44. Samanta SK, Singh OV, Jain RK. 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol., 20:243-248.

45. Sasan RK, Bidochka MJ. 2011. The insect-pathogenic fungus *Metarhizium robertsii* (*Clavicipitaceae*) is also an endophyte that stimulates plant root development. Am J Bot., 99:101-107.

46. Słaba M, Gajewska E, Bernat P, Fornalska M, Długoński J. 2013. Adaptive alterations in the fatty acids composition under induced oxidative stress in heavy metal-tolerant filamentous fungus *Paecilomyces marquandii* cultured in ascorbic acid presence. Environ Sci Pollut R., 20:3423-3434.

47. Sontag TJ, Parker RS. 2002. Cytochrome P450 ω-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. J Biol Chem., 277(28):25290–25296.

48. Sousa ACA, Pastorinho MR, Takahashi S, Tanabe S. 2014. History on organotin compounds, from snails to human. Environ. Chem. Lett., 12:117-137.

49. Sroka Z, Gamian A, Cisowski W. 2005. Niskocząsteczkowe związki przeciwutleniające pochodzenia naturalnego. Postepy Hig Med Dosw., 59:34-41.

50. Toste R, Fernandez MA, Pessoa IA, Parahyba MA, Dore MP. 2011. Organotin pollution at Arraial do Cabo, Rio de Janeiro State, Brazil: Increasing levels after the TBT ban. Braz. J. Oceanogr., 59(1):111-117.

51. Tsang CK, Lau PS, Tam NFY, Wong YS. 1999. Biodegradation capacity of tributyltin by two *Chlorella* species. Environ Pollut., 105:289-297.

52. Tsuchiya Y, Nakajima M, Yokoi T. 2005. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. Cancer Lett., 227(2):115-124.

53. Ueno S, Kashimoto T, Susa N, Shiota Y, Okuda M, Mutoh K, Hoshi F, Watanabe K, Tsuda S, Kawazoe S, Suzuki T, Sugiyama M. 2003. Effects of butyltin compounds on mitochondrial respiration and its relation to hepatotoxicity in mice and Guinea pigs. Toxicol. Sci., 75(1):201-207.

54. Yi AX, Leung KMY, Lam MHW, Lee J-S, Giesy JP. 2012. Review of measured concentrations of triphenyltin compounds in marine ecosystems and meta-analysis of their risks to humans and the environment. Chemosphere, 89(9):1015-1025.

Całkowity dorobek naukowy

Publikacje

1. Bernat P, **Siewiera P**, Soboń A, Długoński J. 2014. Phospholipids and protein adaptation of *Pseudomonas* sp. to the xenoestrogen tributyltin chloride (TBT). World Journal of Microbiology and Biotechnology, 30(9):2343-2350.

   IF = 1,779, MNiSW = 20

2. **Siewiera P**, Bernat P, Różalska S, Długoński J. 2015. Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*. International Biodeterioration & Biodegradation, 104:258-263.

   IF = 2,429, MNiSW = 30

3. Bernat P, Paraszkiewicz K, **Siewiera P**, Moryl M., Płaza G, Chojniak J. 2016. Lipid composition in a strain of *Bacillus subtilis*, a producer of iturin A lipopeptides that are active against uropathogenic bacteria. World Journal of Microbiology and Biotechnology, 32(10):157.

   IF = 1,658, MNiSW = 20

4. Paraszkiewicz K. Bernat P, **Siewiera P**, Moryl M, Sas Paszt L, Trzciński P, Jałowiecki Ł, Płaza G. 2017. Agricultural potential of rhizospheric *Bacillus subtilis* strains exhibiting varied efficiency of surfactin production. Scientia Horticulturae 225:802-809.

   IF = 1,760, MNiSW = 35

5. **Siewiera P**, Różalska S, Bernat P. 2017. Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin. Chemosphere, 185, 96-104.

   IF = 4,427, MNiSW = 35

6. **Siewiera P**, Różalska S, Bernat P. 2017. Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation. Environmental Science and Pollution Research, 24:12118–12127.

   IF = 2,800, MNiSW = 30

7. Nykiel-Szymańska J, **Stolarek P**, Bernat P. 2017. Elimination and detoxification of 2,4-D by *Umbelopsis isabellina* with the involvement of cytochrome P450. Environmental Science and Pollution Research, 25(3):2738-2743.

   IF = 2,800, MNiSW = 30

8. Bernat P, Nykiel-Szymańska J, **Stolarek P**, Słaba M, Szewczyk R, Różalska S. 2018. 2,4-dichlorophenoxyacetic acid-induced oxidative stress: metabolome and membrane modifications in *Umbelopsis isabellina*, a herbicide degrader. PLoS One, 13(6):e0199677.

   IF = 2,766, MNiSW = 35

9. Bernat P, Nykiel-Szymańska J, Gajewska E, Różalska S, **Stolarek P**, Dackowa J, Słaba M. 2018. *Trichoderma harzianum* diminished oxidative stress caused by dichlorophenoxyacetic acid (2,4-D) in wheat, with insights from lipidomics. Journal of Plant Physiology 229:158-163.

   IF = 2,833, MNiSW = 35

10. **Stolarek P**, Różalska S, Bernat P. 2019. Lipidomic adaptations of the *Metarhizium robertsii* strain in response to butyltin compounds presence. Biochimica et Biophysica Acta - Biomembranes, 1861(1):316-326.

    IF = 3,438, MNiSW = 35

Sumaryczny IF = 26,089
Łączna liczba punktów MNiSW = 305
Liczba cytowań = 35
Index H = 3

Wartości IF oraz punktację MNiSW podano zgodnie z rokiem opublikowania.

Komunikaty zjazdowe

a) prezentacje ustne

1. **Siewiera P**, Nykiel J, Bernat P, Długoński J. 2014. Mikrobiologiczna degradacja związków cyny. Mała wielka nauka. Mikro, nano i co dalej?

2. Nykiel J, **Siewiera P**. 2014. Wpływ alachloru na przepuszczalność błony komórkowej i system antyoksydacyjny grzyba strzępkowego *Paecilomyces marquandii*. Mała wielka nauka. Mikro, nano i co dalej?

3. **Siewiera P**, Nykiel J, Bernat P, Długoński J. 2015. Wpływ tributylocyny (TBT) na skład fosfolipidowy i przepuszczalność błon komórkowych wybranych szczepów grzybów mikroskopowych Puzzel 2015. IV Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.

4. Nykiel J, **Siewiera P**, Długoński J. 2015. Odpowiedź systemu antyoksydacyjnego z rodzaju *Trichoderma* na stres wywołany obecnością alachloru. Puzzel 2015. IV Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych.

5. **Siewiera P**, Bernat P. 2015. Spektrometria mas narzędziem w profilowaniu lipidomu wybranych grzybów strzępkowych. Mała Wielka Nauka. Innowacje i idee przyszłości.

6. Nykiel J, **Siewiera P**, Słaba M. 2015. Eliminacja alachloru przez mikroskopowy grzyb strzępkowy *Trichoderma atroviride*. Mała Wielka Nauka. Innowacje i idee przyszłości.

7. Nykiel J, **Siewiera P**, Różalska S, Słaba M. 2015. Detection of reactive oxygen species and elevation of antioxidant enzymes activity in microscopic filamentous fungus exposed to alachlor. VII International Conference of Biotechnology Students & XVII National Academic Seminar of Biotechnology Students.

8. **Siewiera P**, Nykiel J, Bernat P. 2016. Biodegradacja związków cynoorganicznych przez wybrane szczepy bakteryjne i grzybowe z uwzględnieniem roli cytochromu P-450. V Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych, Puzzel 2016.

9. Nykiel J, **Siewiera P**, Słaba M. 2016. Eliminacja alachloru przez mikroskopowe grzyby strzępkowe. V Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych, Puzzel 2016.

10. **Siewiera P**, Nykiel J, Bernat P. 2016. Rola przeciwutleniaczy w procesie eliminacji związków o właściwościach prooksydacyjnych. Mała wielka nauka. W labiryncie nauki.

11. Nykiel J, **Siewiera P**, Bernat P, Słaba M. 2016. Eliminacja 2,4-D przez grzyby strzępkowe z rodzaju *Fusarium*. Mała wielka nauka. W labiryncie nauki.

12. Bernat P, **Siewiera P**, Szewczyk R, Długoński J. 2016. Lipidomic study in biodegradation of toxic pollutants by microorganisms. European Congress on Biotechnology.

13. **Siewiera P**, Nykiel-Szymańska J, Bernat P. 2017. Oddziaływanie tributylocyny (TBT) na profil fosfolipidowy, skład kwasów tłuszczowych i przepuszczalność błon komórkowych wybranych grzybów mikroskopowych. VI Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych Puzzel 2017.

14. Nykiel-Szymańska J, **Siewiera P**, Różalska S, Słaba M. 2017. Detekcja reaktywnych form tlenu oraz pomiar aktywności enzymów antyoksydacyjnych u wybranych szczepów *Trichoderma* poddanych działaniu alachloru. VI Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych Puzzel 2017.

15. Nykiel-Szymańska J, **Siewiera P**, Słaba M. 2017. Wpływ miedzi na eliminację alachloru przez mikroskopowy grzyb strzępkowy *Trichoderma koningii*. I sesja Młodych Mikrobiologów Środowiska Łódzkiego.

16. Bernat P, Nykiel-Szymańska J, **Siewiera** P, Dackowa J., Pietrzak M. 2017. Lipidomic adaptations in the filamentous fungus, auxin herbicides degrader. 6th Central European Congress of Life Science EUROBIOTECH.

17. Długoński A, **Siewiera P**, Bernat P, Słaba M, Długoński J. 2017. The significance of interdisciplinary research in the field of landscape architecture and environmental biotechnology. A case study of green space revitalization in the City of Lodz (Poland). 6th Central European Congress of Life Science EUROBIOTECH.

18. Bernat P, Nykiel-Szymańska J, **Siewiera** P, Dackowa J, Pietrzak M. 2017. Changes in lipidome composition of *Mucorales* treated with toxic lipophilic compounds. 4th Workshop on Microbiology in Health and Environmental Protection MIKROBIOT 2017.

19. **Stolarek P**, Nykiel-Szymańska J, Bernat P. 2017. Analiza jakościowa i ilościowa sfingolipidów grzybni *Metarhizium robertsii* poddanej ekspozycji na dibutylocynę (DBT). Mała Wielka Nauka „Pasja, Wiedza, Nauka".

20. Nykiel-Szymańska J, **Stolarek P**, Rożalska S, Słaba M. 2017. Detekcja markerów stresu oksydacyjnego podczas eliminacji metolachloru przez wybrane szczepy *Trichoderma*. Mała Wielka Nauka „Pasja, Wiedza, Nauka".

21. **Stolarek P**, Nykiel-Szymańska J, Bernat P. 2018. Interakcja związków lipofilnych z błoną komórkową szczepu *Metarhizium robertsii*. Konferencja Młodych Naukowców nt.: NOWE WYZWANIA DLA POLSKIEJ NAUKI.

22. **Stolarek P**, Nykiel-Szymańska J, Bernat P. 2018. Ingerencja związków cynoorganicznych w szlaki syntezy fosfolipidów błonowych grzyba strzępkowego *Metarhizium robertsii*. Konferencja Młodych Naukowców nt.: NOWE WYZWANIA DLA POLSKIEJ NAUKI

23. Nykiel-Szymańska J, **Stolarek P**, Bernat P, Słaba M. 2018. Aktywność systemu antyoksydacyjnego mikroskopowych grzybów strzępkowych podczas eliminacji ksenobiotyków. Konferencja Młodych Naukowców nt.: NOWE WYZWANIA DLA POLSKIEJ NAUKI.

24. Nykiel-Szymańska J, **Stolarek P**, Bernat P, Słaba M. 2018. Rola cytochromu P450 w eliminacji wybranych herbicydów przez mikroskopowe grzyby strzępkowe z rodzaju *Trichoderma* i *Umbelopsis*. Konferencja Młodych Naukowców nt.: NOWE WYZWANIA DLA POLSKIEJ NAUKI.

25. **Stolarek P**, Nykiel-Szymańska J, Bernat P. 2018. Zawartość kwasów tłuszczowych oraz ergosterolu w grzybni szczepu *Metarhizium robertsii* inkubowanej w obecności tributylocyny odzwierciedleniem płynności błony. Cykl konferencji interdyscyplinarnych „Wiedza kluczem do sukcesu".

26. **Stolarek P**, Nykiel-Szymańska J, Bernat P. 2018. Wpływ butylowych związków cyny na syntezę acylogliceroli przez komórki grzybowe *Metarhizium robertsii*. Cykl konferencji interdyscyplinarnych „Wiedza kluczem do sukcesu".

27. Nykiel-Szymańska J, **Stolarek P**, Bernat P, Słaba M. 2018. Biodegradacja i detoksykacja alachloru przez mikroskopowy grzyb strzępkowy *Trichoderma koningii*. Cykl konferencji interdyscyplinarnych „Wiedza kluczem do sukcesu".

28. Nykiel-Szymańska J, **Stolarek** P, Bernat P, Słaba M. 2018. Ocena zmian w profilu fosfolipidowym grzybów strzępkowych z rodzaju *Trichoderma* sp. i *Umbelopsis* sp. podczas eliminacji chlorowanych związków organicznych. Cykl konferencji interdyscyplinarnych „Wiedza kluczem do sukcesu".

b) postery

1. **Siewiera P**, Dudzik M, Bernat P, Soboń A, Długoński J. 2013. Changing in membrane lipids of *Penicillium chrysogenum* in the presence of tributyltin chloride – a reactive oxygen species (ROS) inducer. XV National Academic Seminar of Biotechnology Students & V International Conference of Biotechnology Students.

2. Dudzik M, Bernat P, **Siewiera P**, Soboń A, Długoński J. 2013. Degradation of natural estrogen by the microscopic fungus *Metarhizium robertsii*. XV National Academic Seminar of Biotechnology Students & V International Conference of Biotechnology Students.

3. Bernat P, **Siewiera P**, Szewczyk R, Długoński J. 2013. Changes in phospholipids composition of filamentous fungus *Cunninghamella elegans* in response to tributyltin chloride (TBT). Warsztaty Polskiego Towarzystwa Mykologicznego „GRZYBY – organizmy kluczowe dla życia na Ziemi".

4. Dudzik M, **Siewiera P**, Bernat P, Długoński J. 2013. Elimination of natural estrogen and identification of the metabolites produced by microscopic fungus *Penicillium chrysogenum* IM 879. Warsztaty Polskiego Towarzystwa Mykologicznego „GRZYBY – organizmy kluczowe dla życia na Ziemi".

5. **Siewiera P**, Nykiel J, Soboń A, Bernat P, Długoński J. 2014. Tributyltin (TBT) degradation by organotin-decomposing factor, secreted by *Pseudomonas* sp.. XVI National Academic Seminar of Biotechnology Students & VI International Conference of Biotechnology Students.

6. Abu Aitah M, Kowalczyk K, **Siewiera P**, Bernat P, Długoński J. 2014. The effect of NaCl on the phospholipid composition of the fungus *Metarhizium robertsii*. XVI National Academic Seminar of Biotechnology Students & VI International Conference of Biotechnology Students.

7. **Siewiera P**, Nykiel J, Bernat P, Długoński J. 2015. Zmiany w profilu fosfolipidowym odzwierciedleniem płynności błony grzyba strzępkowego *Metarhizium robertsii* w odpowiedzi na obecność tributylocyny. IV Konferencja Biologii Molekularnej.

8. Nykiel J, **Siewiera P**, Piątek M.A, Słaba M, Długoński J. 2015. Udział cytochromu P450 w eliminacji alachloru przez grzyb strzępkowy *Peacilomyces marquandii*. IV Konferencja Biologii Molekularnej.

9. **Siewiera P**, Nykiel J, Bernat P, Różalska S, Długoński J. 2015. Wpływ 17β-estradiolu na komórki grzybowe *Metrahizium robertsii* poddane ekspozycji na tributylocynę. BioOpen. I Ogólnopolska Konferencja Doktorantów Nauk o Życiu.

10. Nykiel J, **Siewiera P**, Machała K, Słaba M, Długoński J. 2015. Eliminacja alachloru przez mikroskopowe grzyby strzępkowe w obecności chlorku sodu oraz metali ciężkich. BioOpen. I Ogólnopolska Konferencja Doktorantów Nauk o Życiu.

11. **Siewiera P**, Nykiel J, Bernat P, Długoński J. 2015. Oddziaływanie modulatorów hormonalnych na profil fosfolipidowy grzyba strzępkowego *Metarhizium robertsii*. IV Międzyuczelniane Sympozjum Biotechnologiczne „Symbioza".

12. Nykiel J, **Siewiera P**, Soboń A, Słaba M, Bernat P, Długoński J. 2015. Wpływ alachloru na profil lipidowy grzyba *Paecilomyces marquandii* w warunkach zróżnicowanego zasolenia. IV Międzyuczelniane Sympozjum Biotechnologiczne „Symbioza".

13. **Siewiera P**, Nykiel J, Bernat P. 2015. Effect of medium composition on dibutyltin degradation by the microscopic fungus *Metarhizium robertsii*. VII International Conference of Biotechnology Students & XVII National Academic Seminar of Biotechnology Students.

14. **Siewiera P**, Paraszkiewicz K, Bernat P. 2015. Wpływ składu podłoża wzrostowego na produkcję surfaktyny, fengicyny i ituriny przez glebowy szczep *Bacillus* sp. IM 13. Mikrobiologia w medycynie, przemyśle i ochronie środowiska. III edycja.

15. **Siewiera P**, Paraszkiewicz K, Bernat P. 2015. Wykorzystanie makucha rzepakowego oraz mikroskopowych grzybów strzępkowych do usuwania wybranych barwników przemysłowych ze środowisk wodnych. Mikrobiologia w medycynie, przemyśle i ochronie środowiska. III edycja.

16. Rutkowska J, Kuśmierska A, **Siewiera P**, Bernat P, Długoński J. 2015. Charakterystyka profilu fosfolipidowego drożdży *Pichia anomala* z wykorzystaniem tandemowej spektrometrii mas. I Ogólnopolska Konferencja Młodych Naukowców „Azymut 2015".

17. **Siewiera P**, Nykiel J, Bernat P. 2016. Significance of macronutrients in the growth and dibutyltin degradation by fungal cells in the presence of antioxidants. International Student Conference of Cell Biology.

18. Nykiel J, **Siewiera** P, Słaba M. 2016. Enhanced activity of laccase in the filamentous fungus *Trichoderma koningii* under alachlor exposure. International Student Conference of Cell Biology.

19. **Siewiera P**, Nykiel J, Bernat P. 2016. Vitamins or estrogens – which are more useful for fungal conversion of dibutyltin – a reactive oxygen species (ROS) inducer? I BioChemMed SESSION.

20. Nykiel J, **Siewiera P**, Bernat P, Słaba M. 2016. Phospholipids modification in *Trichoderma* strains under alachlor exposure. I BioChemMed SESSION.

21. **Siewiera P**, Nykiel-Szymańska J, Bernat P. 2017. Rola cytochromu P-450 w procesie eliminacji dibutylocyny przez szczep grzybowy *Metarhizium robertsii* inkubowany w obecności wybranych estrogenów naturalnych. VI Konferencja Biologii Molekularnej.

22. Nykiel-Szymańska J, **Siewiera P**, Słaba M. 2017. Detekcja markerów stresu oksydacyjnego podczas usuwania ksenobiotyków i metali ciężkich przez grzyby strzępkowe. VI Konferencja Biologii Molekularnej.

23. **Siewiera P**, Nykiel-Szymańska J, Bernat P. 2017. Aktywność systemu antyoksydacyjnego szczepu *Metarhizium robertsii* poddanego ekspozycji na dibutylocynę (DBT) i estrogeny naturalne. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu – BIOOPEN.

24. Nykiel-Szymańska J, **Siewiera P**, Bernat P, Słaba M. 2017. Eliminacja kwasu 2,4-dichlorofenoksyoctowego przez grzyb strzępkowy *Mortierella isabellina*. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu – BIOOPEN.

25. **Siewiera P**, Nykiel-Szymańska J, Różalska S, Bernat P. 2017. Detekcja *in situ* reaktywnych form tlenu i azotu w grzybni *Metarhizium robertsii* traktowanej dibutylocyną (DBT). I sesja Młodych Mikrobiologów Środowiska Łódzkiego.

26. **Siewiera P**, Nykiel-Szymańska J, Bernat P. 2017. Efficient dibutyltin (DBT) elimination by the microscopic fungus *Metarhizium robertsii* under conditions of intensive aeration and ascorbic acid supplementation. 6th Central European Congress of Life Science EUROBIOTECH.

27. Nykiel-Szymańska J, **Siewiera P**, Datskowa J, Bernat P. 2017. 6th Elimination of 2,4-dichlorophenoxyacetic acid by the microscopic filamentous fungus *Umbelopsis isabellina*. Central European Congress of Life Science EUROBIOTECH.

28. **Siewiera P**, Nykiel-Szymańska J, Bernat P. 2017. Estrogen protects the filamentous fungus *Metarhizium robertsii* against oxidative and nitrosative stress. 4th Workshop on Microbiology in Health and Environmental Protection MIKROBIOT 2017.

29. Nykiel-Szymańska J, **Siewiera P**, Bernat P. 2017. The evaluation of antioxidant system of filamentous fungus *Umbelopsis isabellina* exposed to 2,4-D. 4th Workshop on Microbiology in Health and Environmental Protection MIKROBIOT 2017.

30. Nykiel-Szymańska J, **Siewiera P**, Słaba M. 2017. Elimination of metolachlor by selected *Trichoderma* strains. 4th Workshop on Microbiology in Health and Environmental Protection MIKROBIOT 2017.

31. Bryl W, Dackowa J, Janczyk B, Kopa P, Salamon J, **Siewiera P**, Długoński A. 2017. Microbiological quantitative analysis of the Łódź city greenery soils as the base for revitalization works. 4th Workshop on Microbiology in Health and Environmental Protection MIKROBIOT 2017.