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mgr Jakub Maculewicz

Biokoncentracja związków chemicznych obdarzonych ładunkiem – analityka, ocena metodami *in vitro* oraz *in vivo*

Praca doktorska wykonana pod opieką
dr hab. inż. Anny Białk-Bielińskiej, prof. UG
w Katedrze Analizy Środowiska,
Wydziału Chemii, Uniwersytetu Gdańskiego

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Streszczenie

Biokoncentracja, rozumiana jako proces nagromadzania związków chemicznych w organizmach, stanowi istotny czynnik oceny skutków zanieczyszczenia środowiska. Mimo to wiedza na ten temat dla wielu związków chemicznych zwłaszcza tych obdarzonych ładunkiem, jest wciąż ograniczona. Celem niniejszej pracy doktorskiej była kompleksowa ocena potencjału tego typu indywiduów chemicznych do biokoncentracji. Jako modelowe związki chemiczne wybrano ciecze jonowe (ILs) oraz wybrane produkty transformacji (TPs) leków. Cele szczegółowe obejmowały zarówno zagadnienia analityki chemicznej, jak i eksperymentalne testy biokoncentracji metodami *in vitro* i *in vivo*. W ramach badań *in vitro* dokonano oceny potencjału do biokoncentracji 27 ILs oraz 7 TPs z wykorzystaniem lipidów osadzonych na nośniku stałym. Z kolei testy *in vivo* z wykorzystaniem omułka *Mytilus trossulus* pozwoliły na potwierdzenie czy wybrane kationy ILs mogą ulegać biokoncentracji w tkankach organizmów wodnych. Uzyskane wyniki wskazują, że ILs mogą cechować się wysokim powinowactwem do fosfolipidów tworzących błony komórkowe. W przypadku kationów ILs potwierdzono, że kluczowym parametrem determinującym ten proces jest długość łańcucha bocznego, która bezpośrednio przekłada się na hydrofobowość związku. Co więcej, choć aniony wykazują przeważnie słabsze oddziaływanie z fosfolipidami niż kationy, to jednak silnie zasocjowane z kationami mają wpływ jako przeciwjony. Przeciwnie do ILs, w toku badań potwierdzono, że analizowane TPs nie ulegają biokoncentracji opartej o oddziaływania z lipidami. Ponadto, w ramach niniejszej pracy opracowano i w pełni zwalidowano pierwszą procedurę oznaczania mieszaniny kationów imidazoliowych w tkankach bezkręgowców morskich, pozwalającą na oznaczanie tych związków w stężeniach na poziomie ng/g. Dzięki zastosowaniu tej metodyki możliwe było przeprowadzenie eksperymentu, w którym po raz pierwszy wykazano, że kationy imidazoliowe mogą ulegać biokoncentracji w tkankach bezkręgowców morskich. Wyniki badań uzyskane w niniejszej rozprawie doktorskiej obejmują zatem niezwykle istotne informacje w kontekście oceny ryzyka środowiskowego związków chemicznych obdarzonych ładunkiem, ale także dostarczają poddaną pełnej walidacji procedurę analityczną, pozwalającą na oznaczanie kationów imidazoliowych w złożonych matrycach biologicznych.

Słowa kluczowe: biokoncentracja, ciecze jonowe, farmaceutyki, *in vitro*, *in vivo*, analityka, omułek, *Mytilus trossulus*

Abstract

Bioconcentration, understood as the process of accumulation of chemical compounds in organisms, is an important factor in assessing the effects of environmental pollution. However, the knowledge on this topic for many chemical compounds, especially for charged species, is still limited. The aim of this dissertation was to comprehensively assess the potential for bioconcentration of such chemical entities. Ionic liquids (ILs) and selected transformation products (TPs) of pharmaceuticals were chosen as model chemicals. Specific objectives included both analytical tasks and experimental tests of bioconcentration using *in vitro* and *in vivo* methods. *In vitro* tests assessed the potential for bioconcentration of 27 ILs and seven TPs using solid-supported lipid membranes. In contrast, *in vivo* tests using the mussels *Mytilus trossulus* allowed confirmation of whether selected ILs cations can bioconcentrate in the tissues of aquatic organisms. The results indicate that ILs may have a high affinity to the cell membrane phospholipids. In the case of ILs cations, it was confirmed that the key parameter determining this process is the length of the side chain, which directly corresponds to the hydrophobicity of the compound. Moreover, although anions generally present weaker interactions with phospholipids than cations, when strongly associated with cations they have an effect as counter-ions. Contrary to ILs, the performed study confirmed that the analysed TPs do not undergo bioconcentration based on interactions with lipids. Furthermore, within the scope of the present work, the first procedure for the analysis of a mixture of imidazolium cations in marine invertebrate tissues was developed and fully validated, allowing the determination of these compounds at the concentrations on the ng/g level. By using this method, it was possible to carry out an *in vivo* experiment, in which it was demonstrated for the first time, that imidazolium cations can bioconcentrate in marine invertebrate tissues. The obtained results therefore include highly important information in the context of environmental risk assessment of charged chemicals, but also provide a fully validated analytical procedure for the determination of imidazolium cations in the complex biological matrices.

Keywords: bioconcentration, ionic liquids, pharmaceuticals, *in vitro, in vivo*, analytics, mussel, *Mytilus trossulus*

Publikacje wchodzące w skład rozprawy doktorskiej

P1

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P2

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P3

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P7

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Całkowity IF: 62; Suma punktów MEiN: 1200

Spis treści

1. Wstęp

Proces biokoncentracji obejmuje wchłanianie substancji chemicznych ze środowiska wodnego poprzez zewnętrzne powłoki ciała oraz narządy oddechowe (Ivanciuc et al., 2006). Biokoncentracja jest jedną z głównych składowych zjawiska bioakumulacji, które uwzględnia także absorpcję przez przewód pokarmowy wraz z transferem troficznym, określaną jako biomagnifikacja. W wyniku biomagnifikacji stężenie danej substancji wzrasta na kolejnych poziomach łańcucha pokarmowego, osiągając maksymalną wartość na jego szczycie (Yarsan i Yipfel, 2013). Wszystkie te procesy można opisać matematycznie za pomocą odpowiednich współczynników: współczynnika biokoncentracji (ang. *Bioconcentration Factor*, BCF), współczynnika bioakumulacji (ang. *Bioaccumulation Factor*, BAF) oraz współczynnika biomagnifikacji (ang. *Biomagnification Factor*, BMF) (Arnot i Gobas, 2006).

Biokoncentracja, jako zjawisko niosące za sobą poważne implikacje zarówno dla środowiska, jak i ludzi, stanowi przedmiot licznych badań eksperymentalnych i teoretycznych. W wyniku tego procesu, nawet śladowe ilości szkodliwych substancji obecne w środowisku mogą ostatecznie osiągać niebezpieczne poziomy stężeń w tkankach organizmów. Konsekwencje ekologiczne mogą dotyczyć nie tylko organizmów bezpośrednio podatnych na biokoncentrację, ale także, na skutek biomagnifikacji, całych łańcuchów troficznych. Co więcej, wiele organizmów narażonych na zjawisko biokoncentracji, zwłaszcza w przypadku fauny i flory morskiej, stanowi istotną część ludzkiej diety. W związku z tym, związki chemiczne nagromadzone w tkankach tych organizmów, mogą także stanowić poważne zagrożenie dla zdrowia publicznego (Armitage i in., 2013).

Standardowa metoda szacowania biokoncentracji w organizmach wodnych opiera się na doświadczeniach *in vivo* z wykorzystaniem ryb (np. OECD 305 (OECD, 2012)). Zastosowanie tej metody wymaga znaczących nakładów finansowych, jest czasochłonne oraz musi być poddane rygorom postepowania bioetycznego. Biorąc pod uwagę już ogromną i wciąż rosnącą liczbę związków chemicznych, dla których należy dokonać oceny ryzyka środowiskowego, wydajność metod *in vivo* jest zbyt mała, aby zapewnić odpowiednie dane dla wszystkich, potencjalnie szkodliwych substancji chemicznych (Weisbrod i in., 2009). W związku z tym, konieczne jest poszukiwanie alternatywnych rozwiązań, umożliwiających znacznie tańsze i szybsze określanie potencjału

do biokoncentracji, zachowując przy tym wysokie odwzorowanie procesów zachodzących w organizmach żywych.

Chociaż alternatywne rozwiązania, zarówno w przypadku metod *in vitro* oraz *in silico*, istnieją i są stosowane od wielu lat, większość dostępnych modeli wciąż opiera się na relatywnie prostej relacji aktywności biologicznej, proporcjonalnej do zlogarytmizowanej wielkości współczynnika podziału oktanol-woda (log K_{ow}, log P) lub współczynnika dystrybucji (log D), mającego zastosowanie w przypadku związków dysocjujących. Należy jednak mieć świadomość, że współczynniki te opierają się na hydrofobowości, traktując ją jako kluczową właściwość, odpowiadającą za ewentualną biokoncentrację (Fu i in., 2009). Wątpliwe jest jednak, że tak uproszczony model będzie w stanie odpowiednio odtworzyć oddziaływanie związków chemicznych ze złożonymi strukturami biologicznymi. Choć szacunki na podstawie log K_{ow} mogą dawać przybliżony obraz ich zachowania w organizmach, często są one zaniżone, zwłaszcza w przypadku dużej części związków obdarzonych ładunkiem, które mogą wykazywać znacznie większy, niż oczekiwano, potencjał do biokoncentracji (Bittermann i in., 2018; Dołżonek i in., 2017; Treu i in., 2015).

Na frakcję lipidową biorącą udział w procesie nagromadzania związków chemicznych w organizmach żywych składają się tłuszcze zapasowe i fosfolipidy (Weisbrod i in., 2009). Należy jednak pamiętać, że znacznie różnią się one od siebie zarówno strukturą, jak i funkcjonalnością, dlatego traktowanie ich jako jednej fazy lipidowej jest dużym uproszczeniem. Fosfolipidy zbudowane są z części hydrofilowej, zawierającej czwartorzędową resztę amonową (np. cholinę) i ujemnie naładowaną resztę fosforanową, połączoną z dwoma hydrofobowymi resztami kwasów tłuszczowych. Ich główna funkcja to tworzenie błon komórkowych. Z kolei lipidy magazynujące odpowiadają przede wszystkim za gromadzenie zapasów energetycznych. Wśród nich główną grupę stanowią triacyloglicerole, czyli estry glicerolu i trzech kwasów tłuszczowych (Lehner i Kuksis, 1996).

Powinowactwo związków obdarzonych ładunkiem do błon komórkowych może być wzmacniane przez oddziaływania elektrostatyczne z grupami hydrofilowymi fosfolipidów (Bitterman i in., 2016). Stąd wyprowadzanie zależności wyłącznie w oparciu o wielkość log K_{ow}, która nie uwzględnia tego rodzaju oddziaływania może być obarczone sporym błędem. Ponadto, należy podkreślić, że oddziaływania związków jonowych lub jonogennych z błonami biologicznymi są zależne nie tylko

od umiejscowienia ładunku w cząsteczce, ale także od całej struktury związku i jego właściwości sterycznych (Armitage i in., 2013).

Warto także podkreślić, że lipidy nie są jedyną frakcją biologiczną, która może odpowiadać za biokoncentrację. Istotną rolę mogą także pełnić białka w tkance mięśniowej czy we krwi, do których istotne powinowactwo wykazują takie ksenobiotyki jak perfluorowane związki alkilowe, pochodne kwasu benzoesowego, niektóre pestycydy i leki (Henneberger i in., 2016a, 2016b; Endo i in., 2012, 2013; Endo i Goss, 2011; Ng i Hungerbühler, 2013). Dlatego bardzo istotne jest uwzględnienie przy szacowaniu biokoncentracji znacznie bardziej złożonych zależności i właściwości niż hydrofobowość oceniana za pomocą log K_{ow}. Stało się to główną przesłanką do realizacji niniejszej pracy doktorskiej, której zasadniczym celem było określenie potencjału do biokoncentracji związków chemicznych obdarzonych ładunkiem. Do badań wybrano ciecze jonowe (ang. *Ionic Liquids*, ILs), stanowiące szeroką grupę soli ciekłych w temperaturze pokojowej, składających się z organicznych kationów i anionów o zróżnicowanej budowie chemicznej, a także produkty transformacji (TPs) leków, jako związki obdarzone ładunkiem o szczególnym znaczeniu w kontekście oceny ich ryzyka środowiskowego.

Niniejsza praca stanowi cykl 7 powiązanych ze sobą tematycznie publikacji, z czego trzy pierwsze to prace przeglądowe [**P1**-**P3**] omówione w części literaturowej, a kolejne 4 publikacje [**P4**-**P7**] to prace eksperymentalne, w których opublikowano wyniki przeprowadzonych badań, co także zostało omówione w dalszej części pracy.

2. Część literaturowa

2.1. Współczesne metody *in vitro* badania zjawiska biokoncentracji

Zjawisko biokoncentracji antropogenicznych związków chemicznych zostało obszernie przedyskutowane w opublikowanej pracy przeglądowej [**P1**] z uwzględnieniem opisu współcześnie stosowanych metod *in vitro* wykorzystywanych do jego oceny. Należy podkreślić, że kompleksowa analiza procesu biokoncentracji wymaga uwzględnienia wszystkich trzech głównych frakcji biologicznych odpowiedzialnych za to zjawisko, czyli fosfolipidów, tłuszczy zapasowych oraz białek.

Wśród narzędzi służących do określania powinowactwa do fosfolipidów tworzących błony komórkowe, wyróżnić należy metody wykorzystujące liposomy. Są to zbudowane z dwuwarstwy fosfolipidowej pęcherzyki, spontanicznie formujące się w roztworach wodnych (Alves i in., 2013; Balon i in., 1999; Decker i in., 2012; Escher i Schwarzenbach, 1996). Określanie powinowactwa związków chemicznych do sztucznych błon biologicznych przy użyciu liposomów jest jednak dość złożone, a najczęściej stosowaną techniką, wykorzystywaną w tym celu, jest dializa równowagowa, która jest czasochłonna (Escher i Schwarzenbach, 1996; Escher i in., 2000). Dodatkowo liposomy są stosunkowo niestabilne w buforze, co wymaga częstego powtarzania czasochłonnej procedury ich przygotowania. Pomimo wspomnianych wad, liposomy pozostają jednak najbardziej precyzyjnym narzędziem do określania powinowactwa do fosfolipidów, cechującym się najbardziej zbliżonymi właściwościami do rzeczywistych błon komórkowych.

Alternatywne rozwiązanie stanowią lipidy osadzone na nośniku stałym (ang. *Solid-Supported Lipid Membranes,* SSLM), które są coraz częściej wykorzystywane do określania powinowactwa związków do błon komórkowych, szczególnie w badaniach farmaceutycznych i środowiskowych (Golius i in., 2016; Morandi i in., 2016; Dołżonek i in., 2017; Timmer i Droge, 2017; Droge, 2018). SSLM to mikroziarna krzemionkowe pokryte niekowalencyjnie związanymi dwuwarstwami fosfatydylocholiny, które oferują szybkie osiągnięcie stanu równowagi pomiędzy frakcją związaną i niezwiązaną z lipidem, dzięki czemu pozwalają na wydajne prowadzenie badań przesiewowych. Innym rozwiązaniem opartym o lipidy osadzone na nośniku stałym jest chromatografia cieczowa z zastosowaniem unieruchomionej sztucznej błony biologicznej (ang. *Immobilized Artificial Membrane,* IAM) (Tsopelas i in., 2018, 2017; Droge i in., 2017;

Timmer i Droge, 2017). Chromatografia IAM wykorzystuje krzemionkowe fazy stacjonarne, na których występują chemicznie związane fosfolipidy (Tsopelas i in., 2016).

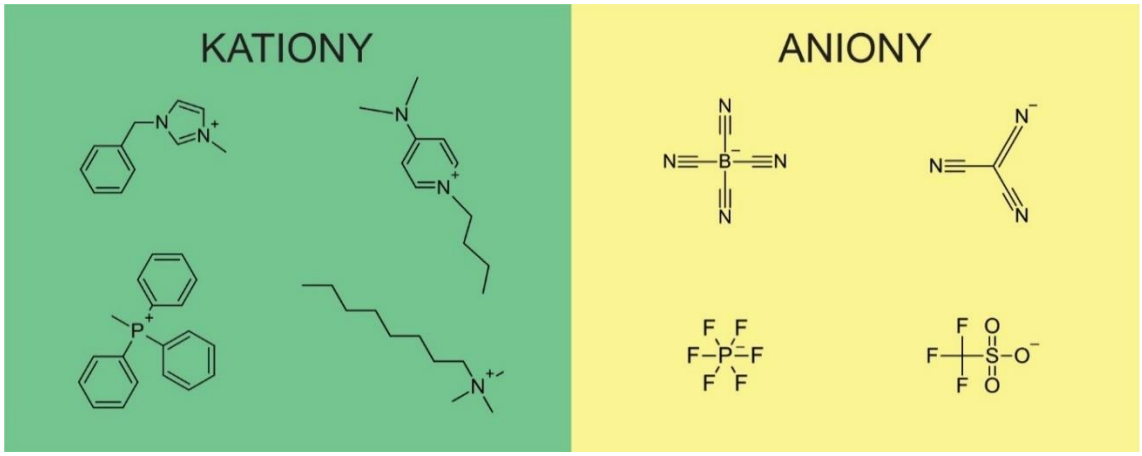
Tłuszcze zapasowe są kolejną frakcją, która może gromadzić zanieczyszczenia środowiskowe, aczkolwiek ich oddziaływania ze związkami chemicznymi opierają się na mniej złożonych mechanizmach niż w przypadku białek i fosfolipidów (Endo i Goss, 2013). Powinowactwo związków chemicznych do tłuszczów zapasowych zwykle szacuje się na podstawie współczynnika podziału trioleina – woda (K_{tw}), wyznaczanego analogicznie do współczynnika podziału oktanol – woda (Chiou, 1985). Bardziej nowatorskie podejście, wykorzystujące silikonowe mikrorurki wykonane z polidimetylosiloksanu (PDMS), zaproponowano w pracy Mayer i in. (2009). Metoda ta całkowicie oddziela fazę wodną od olejowej, zapobiegając niepożądanym efektom, takim jak minimalne rozpuszczanie się oleju w wodzie.

O ile lipidy bardzo często poruszane są w literaturze w odniesieniu do procesu biokoncentracji, to znacznie rzadziej dyskutowanym aspektem jest wiązanie się substancji z białkami. Te z kolei, będąc podstawowym składnikiem budulcowym organizmów, mogą także odgrywać ważną rolę w absorpcji zanieczyszczeń środowiskowych (Escher i in., 2011; Henneberger i in., 2016a, 2016b; Ng i Hungerbühler, 2013). Co więcej, znaczenie białek może być szczególnie duże w przypadku związków jonowych (Henneberger i in., 2016b). W tym kontekście wyróżnić należy dwie różne klasy białek: białka krwi, w tym albumina i alfa-1-kwaśna glikoproteina, znane ze swoich właściwości wiążących oraz białka budulcowe takie jak np. aktyna, miozyna, keratyna czy kolagen. Podobnie jak w przypadku liposomów, dializa równowagowa jest często wykorzystywana do wyznaczenia masy analitu we frakcji niezwiązanej w celu dalszej ilościowej oceny powinowactwa związków chemicznych do białek (Oemisch i in., 2014). Inną popularną techniką wykorzystywaną w celu analizy wiązania związków chemicznych z białkami jest ultrafiltracja (Fung i in., 2003; Singh i Mehta, 2006). Alternatywnym narzędziem są białka osadzone na nośniku stałym, gdzie wykorzystywane są różne frakcje białkowe kowalencyjnie związane z powierzchnią mikroziaren krzemionkowych (Loidl-Stahlhofen i in., 2001). Białka stosowane są również jako fazy stacjonarne w kolumnach chromatograficznych (Kim i in., 2004; Mallik i in., 2005).

2.2. Ciecze jonowe i produkty transformacji leków jako modelowe związki obdarzone ładunkiem

2.2.1. Ciecze jonowe

Zgodnie z definicją, ciecze jonowe (ang. *Ionic Liquids,* ILs) to sole, które osiągają stan ciekły już w temperaturze pokojowej (Beil i in., 2021; Guo i in., 2015). Od początku XXI wieku związki te zyskały ogromną popularność w środowisku naukowym, stanowiąc obiecującą alternatywę dla wielu klasycznych rozwiązań w chemii. Ich struktura opiera się zazwyczaj na złożonym kationie organicznym połączonym z nieorganicznym lub organicznym anionem (Guo i in., 2015). Przykładowe struktury organicznych jonów ILs zostały przedstawione na **Rysunku 1.** Wśród wielu właściwości, które sprawiły, że ILs stały się tak popularne, wyróżnić należy niską prężność par, co czyni je doskonałą alternatywą dla lotnych rozpuszczalników. Ich ważne cechy obejmują również wysoki zakres stabilności termicznej, a także niepalność (Singh i Savoy, 2020). Pośród licznych potencjalnych zastosowań, ILs w ostatnich latach zostały zaproponowane jako: wydajne ekstrahenty biomasy (Sen Tan i in., 2020), dodatki do hybrydowych elektrolitów w bateriach litowo-metalowych (Yang i in., 2020), zrównoważone inhibitory korozji (Verma i in., 2021), dodatki do smarów (Oulego i in., 2018), absorbenty CO_2 (Aghaie i in., 2018), polimery stosowane jako sorbenty w technice ekstrakcji do fazy stałej (SPE) (Liu i in., 2021) lub medium do przygotowania biopolimerów umożliwiających dostarczanie genów lub leków do tkanek (Chen i in., 2018).



Rysunek 1. Przykładowe wzory strukturalne ILs

Należy jednak nadmienić, że od wielu lat prowadzone są, m.in. w Katedrze Analizy Środowiska Wydziału Chemii Uniwersytetu Gdańskiego, szczegółowe badania

nad toksycznością ILs, która według wielu doniesień może być znacząca zarówno dla mikroorganizmów, takich jak bakterie i glony, jak i dla bezkręgowców wodnych, czy też ryb (Beil i in., 2021; Latała i in., 2005; Latała i in., 2009; Thamke i in., 2022). Co ważne, choć w literaturze naukowej dostępna jest dość znacząca liczba danych na temat toksyczności ILs, wciąż brakuje szczegółowych informacji na temat dokładnych mechanizmów odpowiedzialnych za ich szkodliwy wpływ na organizmy (Costa i in., 2017).

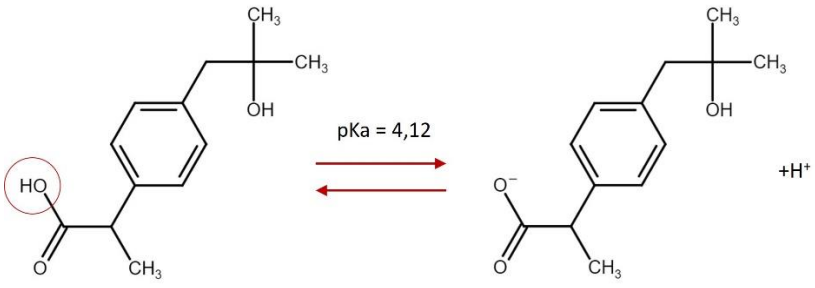
Chociaż zainteresowanie środowisk naukowych potencjalnym zastosowaniem ILs jest ogromne, nie znajduje to jeszcze odpowiedniego odzwierciedlenia w przemysłowej produkcji tych związków, a tym samym obecności w środowisku. Warto jednak zauważyć, że współcześnie wykrywa się w ekosystemach wodnych czy też produktach spożywczych kationy analogiczne do tych stosowanych w ILs - głównie czwartorzędowe sole amoniowe (np. Bertuzzi i in., 2014; Hepperle i in., 2013; Friedle i in., 2012; Xiang i in., 2015; Ferrer i in., 2001). Ich szerokie zastosowanie, szczególnie w środkach powierzchniowo czynnych i dezynfekcyjnych, sprawia, że mogą z łatwością przedostawać się do środowiska. W pracy Pati i in. (2020) wykryto stężenie tych soli w ściekach na poziomie kilku µg/L. Z kolei Li i Brownawell (2009) oznaczyli te związki w osadach ujść rzek w stężeniach sięgających 74 µg/g. Najnowsze doniesienia literaturowe dotyczące pionierskich prac nad identyfikacją obecności cieczy jonowych w środowisku, a także osiągnięcia analityczne dotyczące oznaczania śladowych ilości tych związków w różnych matrycach zostały szczegółowo omówione w pracy [**P2**].

Według doniesień z ostatnich lat, niektóre ILs mogą również wykazywać wysoki potencjał do biokoncentracji. Przeprowadzone badania wykazały, że związki te cechują się dużym powinowactwem do błon fosfolipidowych i białek krwi, co może skutkować ich akumulacją w organizmach (Dołżonek i in., 2017; Kowalska i in., 2022). Doniesienia z ostatnich lat wskazują, że problem środowiskowy mogą stanowić także kationy imidazoliowe, które nie są jeszcze tak powszechnie stosowane. W pracy Leitch i in. (2021) wykazano, że kation 1-oktylo-3-metyloimidazoliowy został wykryty we krwi pacjentów z autoimmunologiczną chorobą wątroby, pierwotną marskością żółciową (z ang. *Primary Biliary Cholangitis,* PBC), co może sugerować jego właściwości hepatotoksyczne. W związku z tym badania nad biokoncentracją ILs stanowią niezwykle istotną kwestię również w zakresie ich potencjalnej toksyczności i dlatego stały się zasadniczym przedmiotem podjętych w ramach niniejszej pracy doktorskiej badań.

2.2.2. Produkty transformacji leków

Leki stanowią grupę związków o szczególnym znaczeniu w kontekście oceny ryzyka środowiskowego. W ostatnich dziesięcioleciach zaczęto dostrzegać zagrożenie stwarzane przez te substancje, co zaowocowało dużą liczbą badań mających na celu lepsze zrozumienie ich wpływu na ekosystemy. Wiadomo, że związki te są powszechnie obecne w środowisku, a wiele prac naukowych szczegółowo analizuje ich mechanizmy działania na organizmy niebędące przedmiotem leczenia (Grabarczyk i in., 2020; Białk-Bielińska i in., 2022; Li i in., 2021; Świacka i in., 2021). Badania z ostatnich lat sugerują ponadto, że same leki stanowią zaledwie część problemu, a istotnym zagrożeniem mogą być także produkty ich przemian (ang. *Transformation Products*, TPs). Do TPs zalicza się metabolity wydalane przez organizm poddany terapii leczniczej, ale także produkty przemian biotycznych i abiotycznych zachodzących po opuszczeniu organizmu, np. w oczyszczalniach ścieków oraz w środowisku wodnym (Zhou i in., 2019).

W przeciwieństwie do omawianych wcześniej ILs, trwale obdarzonych ładunkiem, TPs leków zazwyczaj ulegają zjonizowaniu w wyniku dysocjacji grup funkcyjnych o charakterze kwasowym lub przyłączenia protonu do grupy zasadowej (np. dysocjacja badanego w niniejszej pracy 2-hydroksyibuprofenu przedstawiona na **Rysunku 2**). Jonogennymi grupami funkcyjnymi często występującymi w cząsteczkach farmaceutyków lub ich produktów przemian są ugrupowania aminowe, fenolowe czy sulfonowe.



Rysunek 2. Reakcja dysocjacji 2-hydroksyibuprofenu

Szczegółowy przegląd najnowszych doniesień związanych z obecnością produktów transformacji leków w środowisku, ich toksycznością oraz potencjalną biokoncentracją został przedstawiony w kolejnej pracy przeglądowej [**P3**].

Należy podkreślić, iż mimo że większość dotychczas przeprowadzonych analiz środowiskowych skupiała się na wykrywaniu leków w niezmienionej formie, w ostatnich latach zaczęły pojawiać się doniesienia sugerujące, że ich TPs mogą osiągać podobne lub nawet wyższe poziomy stężeń w środowisku (Kołecka i in., 2019, 2020). Analizy wód gruntowych w Barcelonie (Lopez-Serna i in., 2013) wykazały obecność licznych TPs jak np. metabolity enalaprilu i propranololu w ilości kilkukrotnie wyższej od związków macierzystych. Również w pracach de Oliveira Klein i in. (2021) oraz Langford i in. (2010) wykazano, że środowiskowe stężenia TPs gemcytabiny i karbamazepiny były wyższe niż substancji macierzystych. Zgodnie z pracą przeglądową Zhou i in. (2019), w europejskich wodach powierzchniowych zidentyfikowano 66 TPs farmaceutyków. Zaś wyniki badań Petrie i in. (2015) sugerują, że aż 20% nowo wykrytych zanieczyszczeń w wodach Wielkiej Brytanii to produkty metabolizmu.

Dane literaturowe dotyczące (eko)toksyczności tych indywiduów chemicznych skupiają się zaledwie na kilku najbardziej powszechnych TPs, takich jak norfluoksetyna, O-desmetyltramadol lub pochodne sulfonamidów. Jak wynika z informacji przedstawionych na **Rysunku 1** w publikacji [**P3**], w większości przypadków TPs leków wykazują niższą lub podobną toksyczność w porównaniu do form macierzystych. Jednak w przypadku niesteroidowych leków przeciwzapalnych, antybiotyków i leków przeciwpadaczkowych, odnotowano toksyczność wyższą w porównaniu do ich form natywnych (Cazzaniga i in., 2020; Bergheim i in., 2012; Toolaram i in., 2017).

Jeszcze bardziej ograniczony jest stan wiedzy na temat potencjalnej biokoncentracji TPs. Chociaż istnieją doniesienia literaturowe potwierdzające obecność niektórych metabolitów leków (np. antybiotyków, leków przeciwpadaczkowych) w tkankach organizmów wodnych, trudno jest ocenić ich spójność ze względu na niewielką ilość danych. W związku z tym w niniejszej pracy doktorskiej badania nad procesem biokoncentracji związków obdarzonych ładunkiem rozszerzono także o wybrane związki z tej grupy.

2.3. Analityka ILs i TPs leków

Pierwsze na świecie metody analityczne stosowane do oznaczania ILs zostały opracowane w Katedrze Analizy Środowiska Uniwersytetu Gdańskiego. Opierają się one przede wszystkim na wykorzystaniu techniki wysokosprawnej chromatografii cieczowej (ang. *High Performance Liquid Chromatography*, HPLC) (Stepnowski i in., 2003;

Stepnowski, 2006). Wynika to z faktu, że większość ILs to związki stosunkowo dobrze rozpuszczalne w wodzie. Z kolei ich bardzo niska lotność uniemożliwia zastosowanie techniki chromatografii gazowej. W przypadku próbek o dość prostym składzie matrycy, po skutecznym rozdzieleniu chromatograficznym, możliwe jest zastosowanie detektora spektrofotometrycznego z matrycą fotodiodową (ang. *Diode Array Detector*, DAD) (Zhou i in., 2017; Shao i in., 2018). Alternatywnym rozwiązaniem jest zastosowanie detektora konduktometrycznego (Markowska i Stepnowski, 2008; Stepnowski i Markowska, 2008; 409 Liu i in., 2021) stosowanego w technice chromatografii jonowej (ang. *Ion Chromatography,* IC). Jednakże również w tym przypadku istnieją istotne ograniczenia, wynikające przede wszystkim z niskiej specyficzności tego rodzaju detekcji, a także ograniczonej czułości względem złożonych jonów organicznych, w których ładunek elektryczny jest silnie ekranowany. W związku z tym najczęściej wykorzystywaną techniką analityczną oznaczania ILs w próbkach o złożonym składzie matrycy jest chromatografia cieczowa sprzężona z spektrometrią mas (ang. *Liquid Chromatography – Mass Spectrometry,* LC-MS), czy tandemową spektrometrią mas (LC-MS/MS) (Stepnowski i in., 2003; Liu i Yu, 2016; Pati i Arnold, 2020; Ruan i in., 2014).

Podobnie, jak w przypadku ILs, LC-MS czy LC-MS/MS to także najczęściej wykorzystywane techniki analityczne do oznaczania TPs leków (Hansen i in., 2009; Celiz i in., 2010), szczególnie w próbkach środowiskowych, choć niejednokrotnie borykają się one z wyzwaniami, takimi jak efekty matrycowe (Jones i in., 2001). Z kolei technika GC-MS (ang. *Gas Chromatography - Mass Spectrometry*), ze względu na niższe granice wykrywalności, może stanowić wartościową alternatywę, zwłaszcza w przypadku związków niepolarnych i lotnych (Ardrey, 2003).

3. Cele pracy

Zasadniczym celem niniejszej pracy doktorskiej było określenie potencjału do biokoncentracji związków obdarzonych ładunkiem na przykładzie dwóch grup analitów tj. cieczy jonowych (ILs) i produktów transformacji (TPs) leków wykorzystując metody *in vitro* oraz *in vivo*. Do szczegółowych celów należało:

1. Opracowanie i walidacja metod oznaczania końcowego wybranych kationów ILs z wykorzystaniem wysokosprawnej chromatografii cieczowej [**P4**];
2. Ocena powinowactwa ILs [**P4**] oraz TPs leków [**P5**] do lipidów membranowych metodą *in vitro* z wykorzystaniem SSLMs;
3. Opracowanie i walidacja nowej metodyki oznaczania kationów alkiloimidazoliowych ILs w tkankach małży z wykorzystaniem techniki wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas [**P6**];
4. Ocena biokoncentracji wybranych kationów alkiloimidazoliowych metodą *in vivo* z wykorzystaniem małży *Mytilus trossulus* [**P7**].

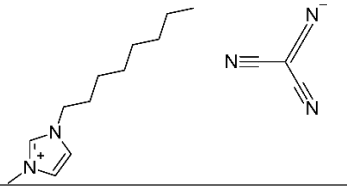
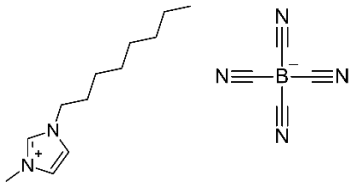
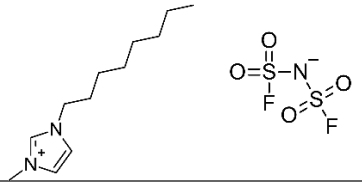
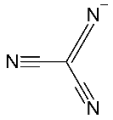
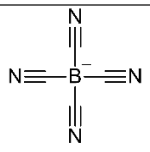
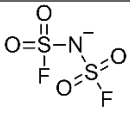
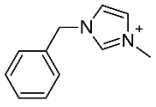
4. Część eksperymentalna

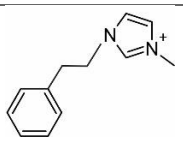
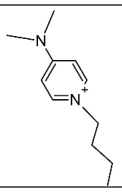
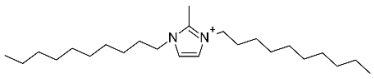
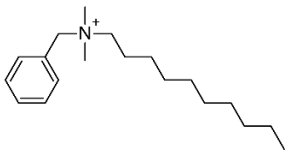
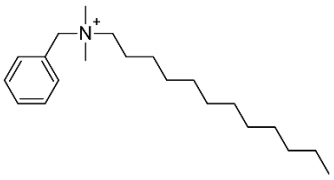
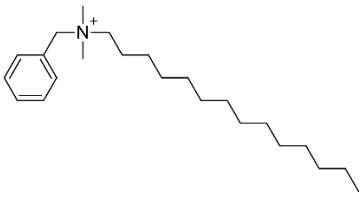
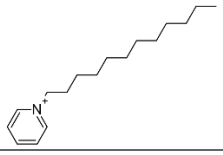
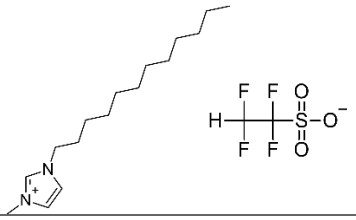
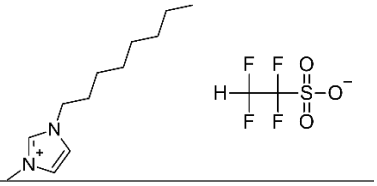
4.1. Wykaz wytypowanych do badań analitów

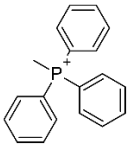
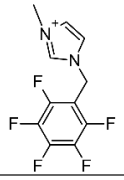
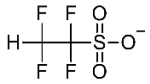
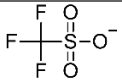
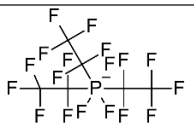
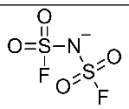
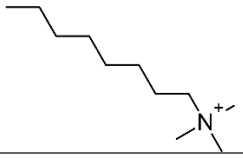
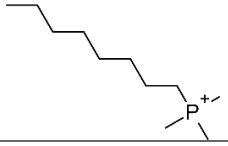
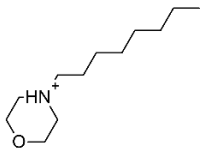
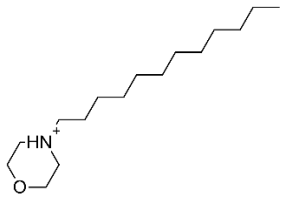
4.1.1. Ciecze jonowe (ILs)

Wszystkie ILs wykorzystane w ramach niniejszej pracy doktorskiej zostały zestawione w **Tabeli 1**. Zestaw ten obejmuje 27 ILs o zróżnicowanej budowie chemicznej, w tym 14 kationów organicznych z prostym przeciwjonem nieorganicznym, 8 anionów organicznych z prostym przeciwjonem nieorganicznym oraz 5 kombinacji kationów i anionów organicznych.

Tabela 1. Wykaz cieczy jonowych wykorzystanych w niniejszej pracy doktorskiej

L.p	Akronim	Pełna nazwa (numer CAS)	Wzór strukturalny
1	[IM18][C(CN)$_3$]	tricyjanometan 1-metylo-3-oktyloimidazoliowy (1203710-60-4)	
2	[IM18][B(CN)$_4$]	tetracyjanoboran 1-metylo-3-oktyloimidazoliowy (178631-04-4)	
3	[IM18][TF$_2$N]	bis(trifluorometanosulfon)imidek 1-metylo-3-oktyloimidazoliowy (178631-04-4)	
4	[Na][C(CN)$_3$]	tricyjanometan sodu (90076-67-8)	
5	[Na][B(CN)$_4$]	tetracyjanoboran sodu (261356-49-4)	
6	[Na][TF$_2$N]	bis(trifluorometanosulfonylo)imidek sodu (90076-67-8)	
7	[IM1-1Ph][Cl]	chlorek 1-benzylo-3-metyloimidazoliowy (36443-80-8)	

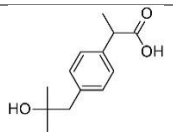
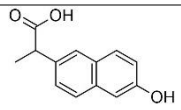
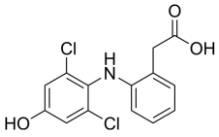
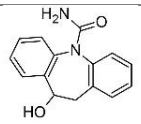
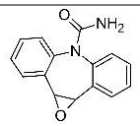
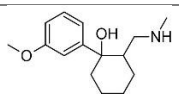
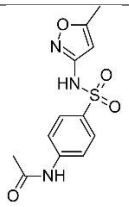
8	[IM1-2Ph][Cl]	chlorek 1-metylo-3-fenyloetyloimodazoliowy (brak numeru CAS)	
9	[Py4-4NMe2][Cl]	chlorek 4-amino-1-butylopirydyniowy (brak numeru CAS)	
10	[IM-10-10-2Me][Cl]	chlorek 1,3-didecylo-metylimidazoliowy (70862-65-6)	
11	[N11-10-Ph][Cl]	chlorek benzylodimetylodecyloamoniowy (965-32-2)	
12	[N11-12-Ph][Cl]	chlorek benzylodimetylododecyloamoniowy (139-07-1)	
13	[N11-14-Ph][Cl]	chlorek benzylodimetylotetradecyloamoniowy (139-08-2)	
14	[Py12][Br]	bromek dodecylopirydyniowy (104-73-4)	
15	[IM1-12] $[H(C_2F_4)SO_3]$	tetrafluoroetanosulfonian 1-metylo-3-dodecyloimidazoliowy (brak numeru CAS)	
16	[IM18][$H(C_2F_4)SO_3$]	tetrafluoroetanosulfonian 1-metylo-3-oktyloimidazoliowy (brak numeru CAS)	

17	[P1-3Ph][Cl]	chlorek metylotrifenylofosforanowy (1779-49-3)	
18	[IM1-1PhF$_5$][Cl]	chlorek 1-metylo-3-(2,3,4,5,6-pentafluorobenzylo)imidazoliowy (brak numeru CAS)	
19	[Na][PF$_6$]	heksafluorofosforan sodu (21324-39-0)	
20	[K][H(C$_2$F$_4$)SO$_3$]	tetrafluoroetanosulfonian potasu (123215-04-3)	
21	[Na][CF$_3$SO$_3$]	trifluorometanosulfonian sodu (2926-30-9)	
22	[K][(C$_2$F$_5$)$_3$PF$_3$]	tris(pentafluoroetylo)trifluorofosforan potasu (23215-04-3)	
23	[K][(FSO$_2$)$_2$N]	bis(fluorosulfonylo)imidek potasu (14984-76-0)	
24	[N1118][Cl]	chlorek oktylotrimetyloamoniowy (2083-68-3)	
25	[P1118][Br]	bromek oktylotrimetylofosforanowy (brak numeru CAS)	
26	[Mor8][Cl]	chlorek 1-metylo-3-oktylomorfoliniowy (brak numeru CAS)	
27	[Mor12][Cl]	chlorek 1-metylo-3-dodecylomorfoliniowy (brak numeru CAS)	

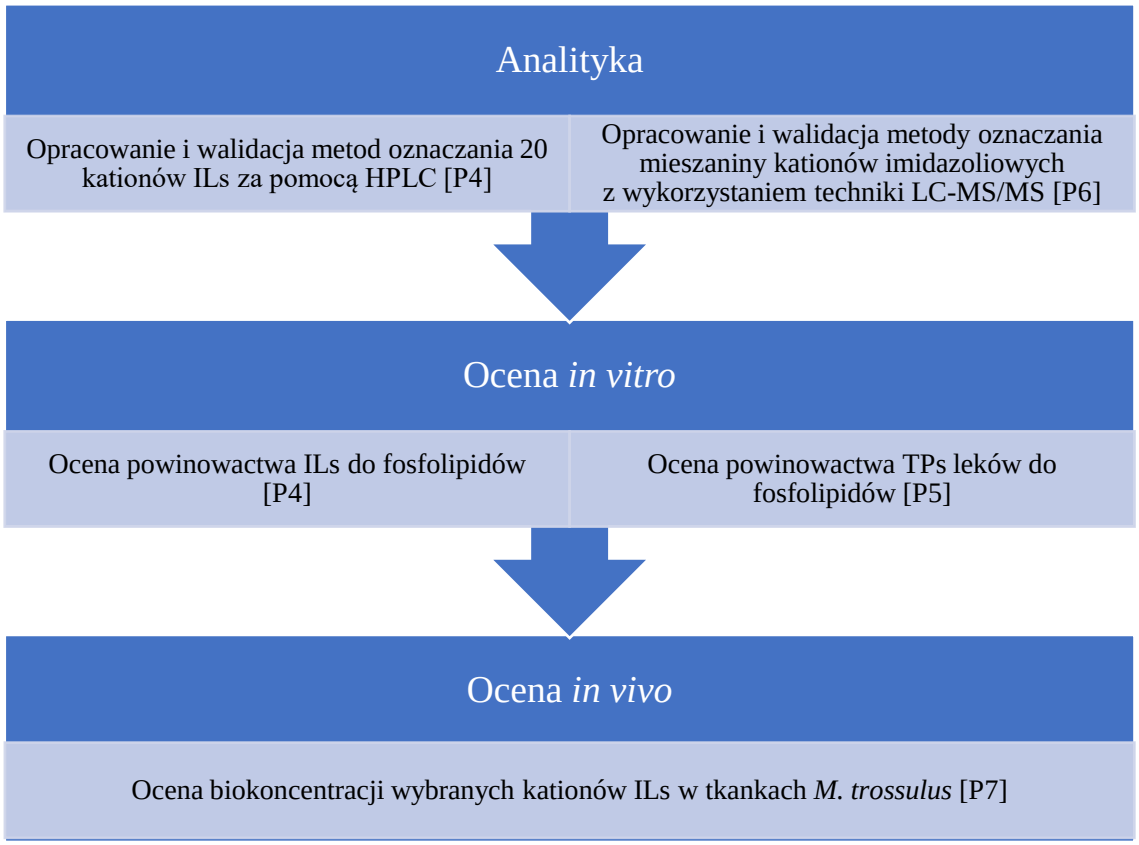
4.1.2. Produkty transformacji leków (TPs)

Szczegółowe zestawienie TPs wykorzystanych w niniejszej pracy zostało przedstawione w **Tabeli 2.**

Tabela 2. Wykaz TPs leków wykorzystanych w niniejszej pracy (wartości pK_a na podstawie Tzvetkov i in., 2011; Rossini i in., 2016)

L.p.	Akronim	Pelna nazwa (numer CAS)	pK_a	Wzór strukturalny
1	2-OH-IBU	2-hydroksyibuprofen	4,12	
2	*des*-NPX	O-desmetylnaproksen	4,87	
3	4-OH-DIC	4-hydroksydiklofenak	4,17	
4	10-OH-CBZ	10,11-dihydro-10-hydroksykarbamazepina	12,8	
5	CBZ-Ep	epoksyd karbamazepiny	16	
6	*O*-DMTRA	O-desmetyltramadol	9,13	
7	*ac*-SMX	N-acetylosulfametoksazol	5	

4.2. Schemat przeprowadzonych badań



Rysunek 3. Schemat badań przeprowadzonych w ramach pracy doktorskiej

4.3. Opracowanie i walidacja metod oznaczania końcowego wybranych kationów ILs z wykorzystaniem techniki HPLC-DAD

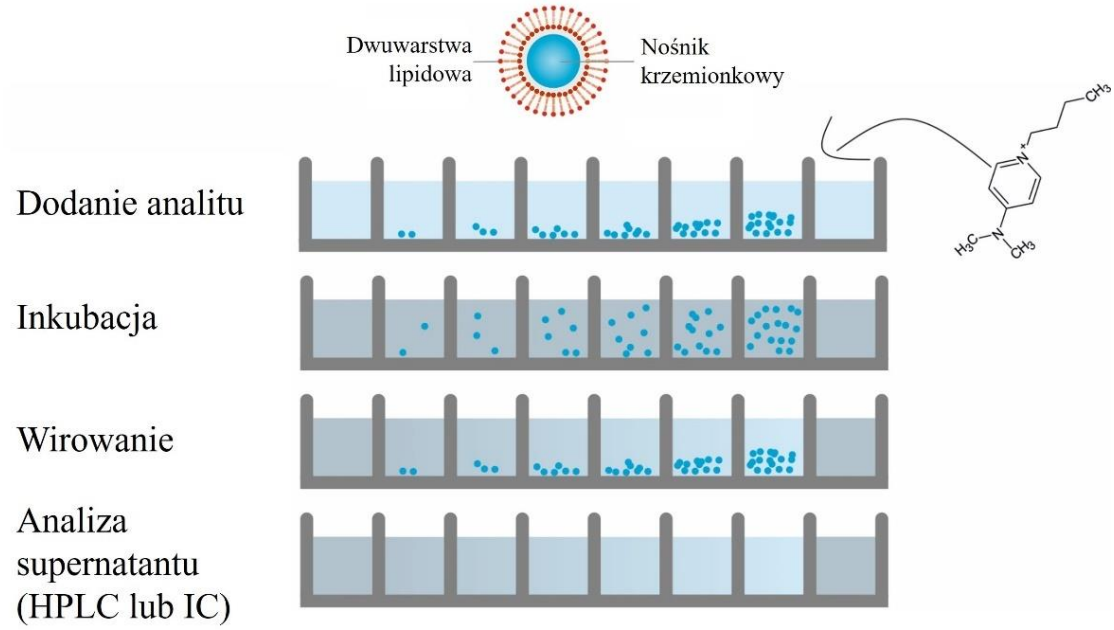
W pierwszym etapie badań opracowano i zwalidowano metody oznaczania końcowego wszystkich badanych kationów ILs zawierających w swej budowie ugrupowania chromoforowe, umożliwiające detekcję spektrofotometryczną (Shimadzu Nexera XR A). Szczegóły dotyczące dobranych warunków opracowanych metod analitycznych, w tym wykorzystane wypełnienia chromatograficzne, skład faz ruchomych, a także parametry walidacyjne podsumowano w informacjach uzupełniających do publikacji [**P4**] (**Tabela S1** oraz **S2**). Tak opracowane i w pełni zwalidowane metody oznaczania końcowego zostały wykorzystane jako narzędzia analityczne w ocenie potencjału do biokoncentracji ILs w badaniach metodami *in vitro* [**P4**].

Z kolei wszystkie aniony, a także kationy bez ugrupowań chromoforowych w swej strukturze chemicznej analizowano z wykorzystaniem techniki chromatografii jonowej

z detektorem konduktometrycznym (940 Professional IC Vario, Szwajcaria), wykorzystując metody uprzednio opracowane i zwalidowane przez Panią mgr Dorotę Kowalską, z którą byłem współwykonawcą w projekcie nr NCN2016/23/G/ST5/04245. Ich podstawowe parametry zostały przedstawione w publikacji **[P4]** (**Tabela S1** oraz **S2**). W przypadku analizy wytypowanych do badań TPs leków, skorzystano z metod uprzednio opracowanych i zwalidowanych przez zespół Katedry Analizy Środowiska (Toński i in., 2019).

4.4. Badanie powinowactwa ILs [P4] i TPs leków [P5] do lipidów membranowych z wykorzystaniem SSLMs

Dokładny opis przeprowadzonych badań przedstawiono w publikacjach [**P4-P5**]. Do badań wytypowano łącznie 27 ILs (**Tabela 1**), zróżnicowanych strukturalnie, tak aby można było ocenić wpływ budowy chemicznej na proces oddziaływania z fosofolipidami i finalnej biokoncentracji, przede wszystkim takich elementów jak rodzaj kationu lub anionu, długość łańcucha bocznego, czy obecność dodatkowych pierścieni aromatycznych w strukturze kationu [**P4**]. Podobna przesłanka wzięta była pod uwagę przy doborze 7 TPs, których zróżnicowanie strukturalne pozwoliło na ocenę zmienności struktury na badane procesy [**P5**]. Powinowactwo wszystkich badanych związków chemicznych do fosfolipidów tworzących błony komórkowe określano przy użyciu testów mikropłytkowych TRANSIL XL Intestinal Absorption Kit (Sovicell GmbH, Niemcy) zgodnie z procedurą przedstawioną na **Rysunku 4**. każdy z testowanych związków chemicznych przygotowanych w buforze PBS dodawano do 6 studzienek zawierających SSLMs (różne stężenia fosfolipidów) oraz dwóch studzienek kontrolnych (zawierających tylko bufor PBS). Następnie próbki inkubowano, wytrząsając je przez 12 minut w temperaturze pokojowej. Po inkubacji, próbki odwirowywano, po czym uzyskany roztwór znad osadu przenoszono do szklanych naczynek chromatograficznych i poddawano analizie chromatograficznej. W przypadku ILs wykorzystano metody opisane w **Tabeli S1** w publikacji [**P4**]. Z kolei stężenia końcowe TPs leków zostały oznaczone z wykorzystaniem metod przedstawionych w **Tabeli 1** w publikacji [**P5**]. Na podstawie oznaczonych stężeń ILs i TPs leków w analizowanej frakcji niezwiązanej wyznaczano izotermy i obliczano wartości logarytmicznego współczynnika podziału membrana-woda (logMA).



Rysunek 4. Schemat przedstawiający układ testowy TRANSIL

Otrzymane dane eksperymentalne zostały także porównane z wynikami uzyskanymi za pomocą modelu COSMOmic (ang. *COnductor like Screening MOdel for MICells*), który wykorzystując obliczenia kwantowo-mechaniczne został opracowany do przewidywania oddziaływania związków chemicznych z błonami biologicznymi (Klamt i in., 2008). Modelowanie zostało wykonane przez dr Stephana Beila z Uniwersytetu Technicznego w Dreźnie, z którym byłem współwykonawcą w projekcie nr NCN2016/23/G/ST5/04245. Uzyskane wyniki zostały w dalszej kolejności porównane statystycznie z wynikami uzyskanymi metodą *in vitro* we współpracy z dr hab. Agnieszką Gajewicz-Skrętną z Katedry Chemii i Radiochemii Środowiska Wydziału Chemii Uniwersytetu Gdańskiego [**P4**].

4.5. Opracowanie i walidacja nowej metodyki oznaczania kationów imidazoliowych ILs w tkankach małży [P6]

Koncepcja jak i dokładny opis badań przeprowadzonych w celu opracowania metodyki oznaczania kationów imidazoliowych ILs w tkankach małży zostały przedstawione na **Rysunku 1** w publikacji [**P6**]. W pierwszych dwóch etapach, które miały na celu dobranie odpowiedniego złoża w technice ekstrakcji do fazy stałej (ang. *Solid-Phase Extraction*, SPE) wykorzystywanej do izolacji analitów i oczyszczania uzyskiwanych ekstraktów oraz dobranie najbardziej wydajnej techniki ekstrakcji ILs

z tkanek małży wytypowano jeden związek modelowy: $[IM18]^+$. Podczas wyboru złoża w technice SPE do analizy instrumentalnej wykorzystano technikę HPLC-DAD, ze względu na uproszczony skład matrycy, którą na tym etapie stanowiła sztuczna woda morska. Jednak w kolejnych etapach próbki analizowano przy wykorzystaniu techniki LC-MS/MS, co wiązało się z koniecznością uprzedniego przeprowadzenia procesu dobrania warunków jednoczesnej analizy wybranych kationów ILs, jak i walidacji instrumentalnej, co zostało szczegółowo opisane w kolejnym rozdziale (**Rozdział 4.5.1**). Po wybraniu najbardziej wydajnej techniki ekstrakcji związku modelowego z tkanek małży, dalsze etapy, obejmujące wybór ostatecznych warunków metody ekstrakcji, przeprowadzono przy użyciu docelowej mieszaniny cieczy jonowych opartych o homologiczny szereg czterech kationów alkiloimidazoliowych: ($[IM16]^+$, $[IM18]^+$, $[IM1\text{-}10]^+$, $[IM1\text{-}12]^+$) [**P6**]. Ostatecznie, zaproponowana nowa procedura oznaczania mieszaniny wybranych kationów ILs z tkanek małży została poddana pełnej walidacji i w kolejnym etapie wykorzystana w toku badań nad oceną ich biokoncentracji metodą *in vivo*.

4.5.1 Opracowanie metody oznaczania końcowego wybranych kationów ILs z wykorzystaniem techniki LC-MS/MS

Analizy instrumentalne prowadzono przy użyciu systemu UHPLC składającego się z pompy wysokociśnieniowej, automatycznego podajnika próbek i termostatu, sprzężonego ze spektrometrem mas (LCMS-8050) wyposażonego w analizator typu potrójny kwadrupol (QqQ) oraz źródło jonizacji na drodze elektrorozpraszania (ESI) (Shimadzu, Japonia). Do rozdzielenia chromatograficznego badanych związków wykorzystano kolumnę Kinetex (2,6 μm, XB-C18, 100 Å, 100 × 3 mm) firmy Phenomenex (Kanada). Składnik A fazy ruchomej stanowił acetonitryl, natomiast składnik B 10 mM wodny roztwór octanu amonu. W toku analiz pracowano w trybie jonów dodatnich, a dla każdego analitu monitorowano dwa charakterystyczne przejścia fragmentacyjne (tryb monitorowania wybranych reakcji fragmentacji, ang. *Multiple Reaction Monitoring*, MRM). Opracowana metoda analityczna została w pełni zwalidowana poprzez wyznaczenie takich parametrów jak: precyzja (CV, [%]), poprawność (R, [%]), liniowość, a także instrumentalna granica wykrywalności (ang. *Instrumental Detection Limit*, IDL) i instrumentalna granica oznaczalności (ang.

Instrumental Quantification Limit, IQL), zgodnie z informacjami przedstawionymi w pracy [**P6] (Rozdziały 2.8** i **2.9)**.

4.5.2 Opracowanie procedury oznaczania wybranych kationów imidazoliowych ILs w tkankach małży

Małże *M. trossulus* wykorzystane na etapie opracowywania metodyki analitycznej pobrano z Zatoki Gdańskiej w ramach rejsu statkiem naukowo-badawczym Instytutu Oceanologii PAN *r/v* „Oceania". Organizmy pobrano wiosną 2021 r. i natychmiast zamrożono (w temperaturze -20 °C), a następnie, po przetransportowaniu do laboratorium, poddano liofilizacji. Suche tkanki homogenizowano w moździerzu porcelanowym do uzyskania jednolitej masy, po czym przechowywano je w lodówce.

Opracowanie optymalnych warunków procedury ekstrakcji imidazoliowych kationów ILs obejmowało kilka etapów, które szczegółowo zostały opisane w publikacji **[P6],** tj.:

a) Dobranie rodzaju złoża i warunków ekstrakcji do fazy stałej (SPE) jako techniki służącej do izolacji i wzbogacania analitów z próbek wód i ekstraktów z małży uzyskanych w teście *in vivo*.

SPE wykonano przy użyciu systemu Baker SPE 12G (Avantor Performance Materials, Polska). Wstępnego doboru odpowiedniego sorbentu dokonano dla wody dejonizowanej z dodatkiem wzorcowej cieczy jonowej [IM18][$C(CN)_3$]. W trakcie badań sprawdzono odzysk analitów z sorbentów wykorzystujących oddziaływania jonowe (Oasis WCX, Strata X-CW, Discovery SCX) oraz hydrofilowo-hydrofobowe (Strata X i Oasis HLB). Na tej podstawie ostatecznie w pracy wykorzystano złoże typu Oasis HLB (6 ml, 200 mg; Waters, USA). Na tym etapie badań wykorzystano uprzednio opracowaną metodę oznaczania kationu $[IM18]^+$ z wykorzystaniem techniki HPLC-DAD.

b) Wytypowanie najbardziej efektywnej techniki ekstrakcji modelowego kationu imidazoliowego $[IM18]^+$ z tkanek małży.

Na tym etapie sprawdzono wydajność trzech różnych technik ekstrakcji takich jak: przyspieszona ekstrakcja za pomocą rozpuszczalnika (ang. *Accelerated Solvent Extraction*, ASE), ekstrakcja rozpuszczalnikiem za pomocą homogenizatora (HOMO)

oraz ekstrakcja za pomocą rozpuszczalnika wspomagana promieniowaniem mikrofalowym (ang. *Microvawe Assisted Extraction*, MAE) dla wybranego modelowego kationu tj. $[IM18]^+$. Szczegóły dotyczące testowanych warunków ekstrakcji przedstawiono w **Tabeli 1 [P6]**. Należy podkreślić, iż uzyskane ekstrakty poddawano oczyszczeniu i zatężeniu (jeśli była taka konieczność) z wykorzystaniem uprzednio opracowanej procedury SPE (jak opisano to powyżej). Na tym etapie analizy końcowe otrzymanych próbek prowadzono z wykorzystaniem uprzednio opracowanej metody oznaczania końcowego kationów imidazoliowych ILs techniką LC-MS/MS w trybie MRM (**Rozdział 4.5.1**).

c) Dobranie odpowiednich warunków ekstrakcji mieszaniny czterech kationów imidazoliowych ILs z tkanek małży z wykorzystaniem techniki MAE-SPE-LC-MS/MS.

Na podstawie wyników uzyskanych dla modelowego kationu, do ekstrakcji imidazoliowych kationów ILs z tkanek małży wytypowana została technika MAE. Dlatego też w ramach opracowania wydajnej metody ekstrakcji czterech cieczy jonowych, różniących się właściwościami fizyko-chemicznymi testowane były różne warunki tego procesu, modyfikując temperaturę oraz moc promieniowania, co zostało przedstawione w **Tabeli 2 [P6]**. Uzyskane ekstrakty również oczyszczano z wykorzystaniem uprzednio opracowanej metody SPE. Na tym etapie próbki analizowano stosując wcześniej opracowaną metodę oznaczania końcowego kationów imidazoliowych ILs z wykorzystaniem techniki LC-MS/MS w trybie MRM (**Rozdział 4.5.1).** W toku badań oceniano wydajność testowanych procedur ekstrakcji na podstawie wyznaczonego odzysku bezwzględnego poszczególnych analitów oraz wartości efektów matrycowych, które zostały obliczone na podstawie wzorów przedstawionych w **Rozdziale 2.9.1. [P6**].

4.5.3 Walidacja opracowanej metodyki

Opracowana metodyka została poddana pełnej walidacji poprzez analizę próbek tkanek małży z dodatkiem mieszaniny wzorcowej czterech kationów ILs na sześciu poziomach stężeń (50, 100, 150, 200, 300, 500 ng/g suchej masy), w trzech powtórzeniach dla każdego stężenia ILs. Następnie obliczone zostały parametry walidacyjne zgodnie z informacjami przedstawionymi w **Rozdziale 2.8.1** [**P6**].

4.6. Badanie biokoncentracji wybranych kationów imidazoliowych w testach *in vivo* z wykorzystaniem małży *M. trossulus* [P7]

Koncepcja, jak i dokładny opis badań przeprowadzonych w celu oceny biokoncentracji wybranych kationów imidazoliowych zostały przedstawione i szczegółowo omówione w publikacji [**P7**]. W toku tych badań wykorzystane zostały osobniki *M. trossulus,* które złowiono z Zatoki Gdańskiej we wrześniu 2020 r., październiku 2021 r. i listopadzie 2021 r. podczas rejsów badawczych na pokładzie *r/v* „Oceania". Natychmiast po pobraniu wybrane osobniki o długości muszli od 2,7 do 4,5 cm przeniesiono na 24 godziny do zbiorników z napowietrzaną wodą morską, umożliwiając im filtracyjne oczyszczenie ustroju. Po dostarczeniu małży do laboratorium, dokładnie usunięto z ich muszli epifity i aklimatyzowano przez 14 dni w zbiornikach termostatowanych wypełnionych sztuczną wodą morską o odpowiednim zasoleniu i temperaturze, co zostało przedstawione w publikacji [**P7**].

Badania *in vivo* z wykorzystaniem małży *M. trossulus* zostały podzielone na dwie zasadnicze sekcje. W pierwszej części, w ramach wstępnych badań wykonane zostały testy toksyczności ostrej, co miało na celu określenie najwyższego stężenia niepowodującego śmiertelności badanych organizmów, które następnie zastosowano w testach biokoncentracji. W dalszej kolejności przystąpiono do testu głównego, tj. badania stopnia biokoncentracji wytypowanych cieczy jonowych w małżach. W tym celu do badań wybrano sole oparte o dwa kationy imidazoliowe: $[IM18]^+$ oraz $[IM1\text{-}12]^+$, które testowano w dwóch różnych zasoleniach tj. 8 oraz 16 PSU. Dodatkowo dla $[IM18]^+$ postanowiono sprawdzić wpływ anionu na biokoncentrację, poprzez ekspozycję małży zarówno na [IM18][Br] jak i [IM18][$C(CN)_3$].

W ramach przeprowadzonego testu właściwego biokoncentracji metodą *in vivo* wykorzystano cztery akwaria, które wypełnione były 6 L sztucznej wody morskiej w systemie termostatowanym (T = 10 °C) przy minimalnym oświetleniu. Do trzech zbiorników dodano roztwór testowanego związku do uzyskania końcowego stężenia 10 µg/L, natomiast czwarte akwarium służyło jako kontrola. W każdym ze zbiorników umieszczono po 50 osobników. W trakcie trwającego 21 dni eksperymentu zbiorniki były stale napowietrzane. Dodatkowo, co 3 dni wymieniano połowę objętości wody w akwariach, w celu zapewnienia stałego, wyjściowego poziomu stężenia testowanego związku. Aby ocenić biokoncentrację, co 72 godziny pobierano próbki wody oraz pięć osobników *M. trossulus*. Małże przenoszono do 1 L zbiorników i pozostawiano w czystej

wodzie morskiej (bez dodatku ILs) na 6 godzin w celu filtracyjnego oczyszczenia ich układu trawiennego. Ostatecznie preparowano tkanki miękkie organizmów, które wraz z próbkami wody przechowywano w temperaturze -20 °C do czasu analizy.

Wyizolowane tkanki małży liofilizowano, a następnie homogenizowano przy użyciu moździerza porcelanowego. Po odważeniu 0,5 g suchej masy, przygotowane tkanki ekstrahowano zgodnie z opracowaną wcześniej procedurą opisaną szczegółowo w publikacji [**P6]**.

Na podstawie analizy stężenia kationów ILs w tkankach małży oraz w wodzie wyznaczone zostały współczynniki BCF. Obliczenia zostały przeprowadzone zgodnie ze standardową metodą, której szczegóły przedstawiono w **Rozdziale 2.7** w publikacji [**P7**].

5. Wyniki i dyskusja

5.1. Charakterystyka opracowanych metod oznaczania końcowego ILs z wykorzystaniem techniki HPLC-DAD [P4]

Nowe metody oznaczania końcowego kationów ILs z wykorzystaniem techniki HPLC-DAD zostały opracowane i scharakteryzowane w celu oceny ich przydatności w analizach wykonywanych na kolejnych etapach niniejszej pracy. Szczegółowe informacje dotyczące tych metod zostały przedstawione w **Tabelach S1** oraz **S2** w publikacji [**P4**]. Opracowane metody zostały w pełni zwalidowane, uzyskując wartości precyzji i poprawności, mieszczące się w ogólnie przyjętych kryteriach, a także wysoką liniowość (R^2 > 0,99 dla wszystkich analitów). Granice oznaczalności opracowanych metod na poziomie 2,5 – 10 μM pozwoliły na zastosowanie ich jako narzędzi analitycznych w przeprowadzonych testach *in vitro* jak i na etapie opracowania metody ekstrakcji kationu imidazoliowego z wody z wykorzystaniem techniki SPE.

5.2. Ocena powinowactwa cieczy jonowych [P4] oraz TPs leków [P5] do lipidów membranowych z wykorzystaniem SSLMs

Badania przeprowadzone w ramach niniejszej pracy miały na celu dostarczenie nowych informacji, pozwalających na lepsze zrozumienie oddziaływań organicznych związków obdarzonych ładunkiem (takich jak ILs or TPs leków) z błonami komórkowymi, w kontekście ich potencjału do biokoncentracji. Szczegółowe zestawienie uzyskanych wyników zostało przedstawione w **Tabeli 3.**

Tabela 3. Powinowactwo wybranych kationów i anionów ILs **[P4]** oraz TPs leków **[P5]** do fosfolipidów wyznaczone w testach z SSLMs

	LogMA [L/kg]
Kationy	
[N11-10-Ph][Cl]	3,45 ± 0,06
[N11-12-Ph][Cl]	4,26 ± 0,10
[N11-14-Ph][Cl]	4,79 ± 0,06
[Mor8][Cl]	1,72 ± 0,15
[Mor12][Cl]	2,97 ± 0,14
[IM-10-10-2Me][Cl]	4,58 ± 0,06
[Py12][Br]	4,10 ± 0,06
[Py4-4NMe2][Cl]	<1,5
[N1118][Cl]	<1,5
[P1118][Br]	2,15 ± 0,05
Badanie wpływu anionu na kation	
[IM18][$C(CN)_3$]	kation: 4,23 ±0,06; anion: <1,5
[IM18][$B(CN)_4$]	kation: 2,45 ±0,05; anion: 1,98 ±0,07
[IM18][TF_2N]	kation: 2,44 ±0,06; anion: 2,47 ±0,45
[IM1-12][$H(C_2F_4)SO_3$]	4,14 ± 0,04
[IM18][$H(C_2F_4)SO_3$]	2,50 ± 0,04
Kationy z pierścieniami aromatycznymi	
[IM1-1Ph][Cl]	<1,5
[IM1-2Ph][Cl]	<1,5
[P1-3Ph][Cl]	1,64 ± 0,03
[IM1-1PhF_5][Cl]	1,18 ± 0,22
Aniony	
[Na][$C(CN)_3$]	<1,5
[Na][$B(CN)_4$]	1,87 ±0,07
[Na][TF_2N]	2,13 ±0,04
[Na][PF_6]	1,85 ± 0,17
[K][$H((C_2F_4)SO_3$]	<1,5
[Na][CF_3SO_3]	1,27 ± 0,07
[K][$(C_2F_5)_3PF_3$]	3,02 ± 0,10
[K][$(FSO_2)_2N$]	1,86 ± 0,23
TPs farmaceutyków	
2-hydroksyibuprofen	1,39 ± 0,13
O-desmetylnaproksen	<1,5
4-hydroksydiklofenak	2,17 ± 0,11
10,11-dihydro-10-hydroksykarbamazepina	1,79 ± 0,02
epoksyd karbamazepiny	1,62 ± 0,01
O-desmetyltramadol	1,59 ± 0,07
N-acetylosulfametoksazol	<1,5

Wyniki uzyskane dla trzech kationów amoniowych z łańcuchami bocznymi zawierającymi od 10 do 14 atomów węgla, a także kationów morfoliniowych i imidazoliowych z 8- i 12-węglowymi łańcuchami bocznymi, jednoznacznie wskazują na kluczowe znaczenie tej cechy strukturalnej w oddziaływaniu ILs z lipidami tworzącymi błony komórkowe. Chociaż w przypadku wszystkich testowanych rodzajów

kationów zaobserwowano wzrost wartości logMA wraz ze wzrostem liczby atomów węgla w łańcuchu bocznym, uzyskane wartości były dużo niższe w przypadku soli zawierających kationy alkilomorfoliniowe. W przypadku 12-węglowego łańcucha, wartość logMA dla kationu morfoliniowego wyniosła 2,97 ± 0,14 L/kg. Z kolei dla kationu amoniowego, pirydyniowego oraz imidazoliowego z analogicznymi łańcuchami alifatycznymi, wartość ta wyniosła odpowiednio 4,26 ± 0,10; 4,10 ± 0,06 oraz 4,14 ± 0,04 L/kg. Obserwacje te stanowią jednoznaczne potwierdzenie wcześniej stawianych hipotez na podstawie danych dotyczących ich toksyczności i biodegradowalności (Stolte i in., 2007; Beil i in., 2021), zgodnie z którymi ILs morfoliniowe wskazywane są jako bardziej przyjazna dla środowiska alternatywa w stosunku do ILs imidazoliowych i pirydyniowych. Z kolei zaobserwowana zależność wzrostu powinowactwa do lipidów membranowych, wraz ze wzrostem długości alkilowego łańcucha bocznego, znajduje potwierdzenie zarówno we wcześniejszych, wstępnych badaniach wykonanych w naszym zespole (Dołżonek i in., 2017), jak i danych literaturowych dotyczących podobnych strukturalnie związków, takich jak surfaktanty (Timmer i Droge, 2017; Droge, 2019).

Inną istotną cechą kationów ILs, której wpływ na powinowactwo do fosfolipidów poddano ocenie w trakcie prowadzonych badań, była obecność ugrupowań aromatycznych w strukturze. Słabsze powinowactwo kationów ILs, w których strukturach występują podstawniki arylowe, w porównaniu do kationów z alifatycznymi łańcuchami bocznymi, może wynikać z ich większej polarności oraz obszerniejszej zawady strukturalnej utrudniającej bezpośrednią interakcję z błoną. Uzyskane wyniki wskazują także, że ilość pierścieni aromatycznych nie wpływa znacząco na wzrost wartości logMA. W przypadku kationów imidazoliowych z jedną grupą fenylową oddziaływanie było na tyle słabe, że wyznaczenie logMA nie było możliwe. Z kolei w przypadku kationu fosfoniowego $[P1\text{-}3Ph]^+$ obecność trzech pierścieni aromatycznych spowodowała jedynie nieznaczny wzrost oddziaływania z fosfatydylocholiną (PC) (logMA = 1,64 ± 0,03), co może wynikać z większego udziału elektrostatycznego przyciągania czwartorzędowej grupy aminowej obecnej w PC i trzech układów aromatycznych obecnych w $[P1\text{-}3Ph]^+$.

Natomiast w przypadku anionów ILs zaobserwowano, że ich powinowactwo do fosfolipidów jest wyraźnie słabsze niż powinowactwo kationów. Najwyższą wartość logMA wyznaczono dla cieczy jonowej zawierającej anion $[(C_2F_5)_3PF_3]^-$ i wyniosła ona 3,02 ± 0,10 L/kg. Warto podkreślić, że związki chemiczne o zbliżonej budowie,

klasyfikowane jako substancje perfluoroalkilowe (ang. *Per- and Polyfluoroalkyl Substances*, PFAS), uznawane są powszechnie za poważne zagrożenie środowiskowe, ze względu na znikomą biodegradowalność i wysoki potencjał do biokoncentracji (Burkhard, 2021). Niniejsze badania potwierdzają, że również aniony ILs, posiadające w strukturze liczne atomy fluoru, powinny być traktowane ze szczególną ostrożnością. W przypadku pozostałych testowanych anionów ILs, wartość logMA przekroczyła wartość 2 L/kg tylko w przypadku $[Tf_2N]^-$ i wyniosła 2,13 ±0,04 L/kg. Sugeruje to, że o ile interakcje anionów z fosfolipidami są zazwyczaj ograniczone, ich oddziaływanie z kationami wymaga szczególnej uwagi w kontekście potencjalnej biokoncentracji. Co ważne, o ile powinowactwo samych anionów do fosfolipidów jest niskie, ich pośredni udział w oddziaływaniu ILs z błonami komórkowymi może być znaczący. W testach z wykorzystaniem kationu $[IM18]^+$ połączonego z czterema różnymi anionami zaobserwowano, że wartość logMA kationu, wynosząca od 2,44 ± 0,06 do 2,50 ± 0,04 L/kg w obecności anionów: $[B(CN)_4]^-$, $[Tf_2N]^-$ i $[H(C_2F_4)SO_3]^-$, wzrasta do 4,23 ± 0,06 L/kg, gdy jako przeciwjon obecny jest anion $[C(CN)_3]^-$. Obserwowany wpływ anionu $[C(CN)_3]^-$ może wynikać z trwałej asocjacji z kationem $[IM18]^+$, w wyniku czego niwelowane jest jego odpychanie przez dodatnio naładowaną cholinę w błonie lipidowej. Należy jednak podkreślić, że na obecnym etapie nie jest jasne, dlaczego spośród testowanych anionów, tego typu oddziaływanie z kationem dotyczy wyłącznie $[C(CN)_3]$. Wyniki eksperymentalne uzyskane w niniejszej pracy zostały porównane z wartościami logMA określonymi za pomocą modelu COSMO*mic*, które zostały wyznaczone we współpracy z dr. Stephanem Beilem i dr hab. Agnieszką Gajewicz-Skrętną. Wartości przewidywane przez model są w większości przypadków zbliżone do wyników eksperymentalnych, co potwierdza wysoki współczynnik korelacji ($R^2 = 0,82$) (**Rysunek 3, [P4]**). Co ważne, tylko w przypadku trzech spośród testowanych związków różnica między zlogarytmizowanymi wartościami eksperymentalnymi a modelowymi wynosi więcej niż 1. Głównymi punktami rozbieżności są: anion $[(FSO_2)_2N]^-$, kation $[IM18]^+$ w parze z anionem $[C(CN_3)]^-$ oraz kation $[IM10\text{-}10\text{-}2Me]^+$. Ze względu na ograniczoną liczbę danych, nie jest możliwe jednoznaczne wskazanie przyczyn obserwowanych różnic. Pomimo, że model COSMO*mic* jest przydatnym narzędziem do wstępnego szacowania powinowactwa do błon komórkowych, to dane uzyskane dla jonów bardzo zróżnicowanych strukturalnie, wskazują na konieczność jego dopracowania w celu wiarygodnego zastosowania dla związków jonowych.

Mając na uwadze dane uzyskane dla ILs, w ramach niniejszej pracy postanowiono również dokonać wstępnej oceny potencjału do biokoncentracji TPs leków, które w zależności od pH środowiska także mogą występować w formie zjonizowanej. Warto podkreślić, że wszystkie badane TPs, za wyjątkiem pochodnych karbamazepiny, ulegają dysocjacji w warunkach eksperymentalnych (pH = 7,4). Wartości pK_a dla wszystkich analitów zostały przedstawione w **Tabeli 2**.

Zasadniczo, wyznaczone wartości logMA dla TPs leków (**Tabela 3**) jednoznacznie potwierdzają, że powinowactwo tych związków do lipidów tworzących błony komórkowe jest znacznie słabsze niż w przypadku ILs. Spośród testowanych TPs, najwyższe powinowactwo zaobserwowano w przypadku 4-hydroksydiklofenaku, dla którego wartość logMA wyniosła 2,17 ± 0,11 (**Tabela 3**). Oznacza to jednak, że powinowactwo tego metabolitu do błon komórkowych może być nawet o dwa rzędy wielkości mniejsze niż związku wyjściowego. W pracy Balon i in. (1999) logMA wyznaczone dla diklofenaku przy użyciu liposomów wynosiło 4,3 L/kg. Pozostałe analizowane związki wykazywały bardzo niskie powinowactwo do fosfolipidów, poniżej 2 L/kg w skali logarytmicznej. Ponadto w przypadku O-desmetylonaproksenu i N-acetylosulfametoksazolu nie zaobserwowano żadnego wiązania z lipidami.

Wyniki testów przeprowadzonych w niniejszej pracy są zgodne z kluczowymi koncepcjami farmakokinetyki. W wyniku procesów metabolicznych leki ulegają zwykle przemianie do pochodnych o znacznie bardziej hydrofilowych właściwościach, co ostatecznie prowadzi do ich wydalania z organizmu (Hacker i in., 2009). Uzyskane dane wskazują, że dla wszystkich analizowanych metabolitów powinowactwo do fosfolipidów jest znacznie niższe niż w przypadku związków w formie niezmienionej. Należy jednak podkreślić, że powinowactwo do lipidów to tylko jedna ze składowych zjawiska biokoncentracji, a zatem związki te wciąż mogą zostać wchłonięte i dystrybuowane w organizmie w wyniku oddziaływania z innymi biomolekułami, zwłaszcza białkami (Ng i in., 2013).

5.3. Charakterystyka opracowanej metody analizy kationów imidazoliowych w tkankach małży z wykorzystaniem techniki MAE-SPE-LC-MS/MS [P6]

W toku przeprowadzonych badań opracowano i poddano walidacji metodę instrumentalną oznaczania mieszaniny czterech kationów alkiloimidazoliowych ($[IM16]^+$, $[IM18]^+$, $[IM1\text{-}10]^+$, $[IM1\text{-}12]^+$) z zastosowaniem techniki LC-MS/MS

w trybie MRM. Ze względu na różną długość alkilowych łańcuchów bocznych, analizowane kationy zostały dobrze rozdzielone w odwróconym układzie faz przy użyciu kolumny z wypełnieniem XB-C18 (2,6 μm; 100 Å, 100 × 3 mm) w czasie 6 minut (**Rysunek 2** w [**P6**]). Wyznaczone parametry walidacyjne opracowanej metody oznaczania wytypowanych kationów ILs takie jak: niskie granice oznaczalności (10-20 μg/L), wykrywalności (3,33-6,66 ng/mL) oraz precyzja (0,4-6,3 %), poprawność (90,8-130,3 %) i liniowość (r^2 > 0,999) potwierdziły możliwość wykorzystania jej do zaplanowanego celu (**Tabela 4,** [**P6**]). Zastosowanie tandemowej spektrometrii mas MS/MS do detekcji kationów alkiloimidazoliowych pozwoliło na oznaczenie tych związków w stężeniach o kilka rzędów wielkości niższych niż w przypadku innych technik, takich jak IC czy HPLC-UV/Vis, cechujących się wartościami IQL na poziomie mg/L (Zhang i in., 2019; Fan i in., 2018).

Podczas wstępnych badań zaobserwowano, że spośród przetestowanych rodzajów złóż wykorzystanych do ekstrakcji kationu $[IM18]^+$ z próbek wody morskiej za pomocą techniki SPE, złoże oparte o kopolimer diwinylobenzenu i winylopirolidonu Oasis HLB (6 ml, 200 mg; Waters, USA) cechowało się największą wydajnością. Wynikało to faktu, iż gwarantuje ono jednoczesne oddziaływania hydrofilowo-hydrofobowe z analitami.

W dalszej części zaplanowanych badań stwierdzono, że technika MAE jest najbardziej odpowiednią (spośród testowanych tj. ASE, HOMO i MAE) do ekstrakcji imidazoliowych kationów ILs z tkanek małży. Na podstawie uzyskanych wyników wykazano bowiem, że ASE jest najmniej efektywną techniką do zaplanowanego celu, ze względu na obserwowaną niską powtarzalność oraz niższe wartości odzysku bezwzględnego kationu $[IM18]^+$, nieprzekraczające 90% **(Rysunek 2 [P6]).** Natomiast zastosowanie techniki HOMO mimo, iż cechowało się zarówno wysokimi wartościami odzysku bezwzględnego $[IM18]^+$ (blisko 100%), jak i bardzo dobrą powtarzalnością, zostało wykluczone z dalszych badań z uwagi na znaczące ograniczenia modyfikacji zaproponowanej procedury ekstrakcji. Chcąc bowiem opracować efektywną metodę ekstrakcji kilku kationów ILs, różniących pod względem długości łańcucha alkilowego, a tym samym hydrofobowości, w dalszych badaniach uwzględniona została tylko technika MAE, gdyż daje one więcej możliwości dostosowywania warunków ekstrakcji (takich jak temperatura, moc promieniowania elektromagnetycznego, liczba cykli), mogących prowadzić do zwiększenia wydajności ekstrakcji mieszaniny ILs z tkanek małży.

W tym celu, jako punkt wyjścia do ekstrakcji mieszaniny kationów wybrano warunki, które najlepiej sprawdziły się w przypadku ekstrakcji samego $[IM18]^+$ (oznaczone MAE1). Jednak nie były one wystarczająco skuteczne w przypadku pozostałych analizowanych związków. W związku z tym wprowadzono modyfikacje tejże metody obejmujące: testowanie wpływu zwiększenia mocy promieniowania elektromagnetycznego z 400 do 800 W (MAE3), temperatury ze 100 °C do 120 °C (MAE4) oraz kombinacji tych dwóch czynników (MAE5), które jednak nie przyniosły pożądanego efektu tj. zwiększonego odzysku bezwzględnego dla wszystkich analitów. Ostatecznie postanowiono dodatkowo zwiększyć udział MeOH w mieszaninie ekstrakcyjnej z 50 % do odpowiednio 70 % i 90 % dla MAE6 i MAE7, w celu zwiększenia odzysku najbardziej niepolarnego analitu, czyli $[IM1\text{-}12]^+$. Ponadto, wprowadzono modyfikację w procedurze SPE, zastępując płukanie złoża 5% MEOH wodą dejonizowaną, aby zapobiec ewentualnej elucji najbardziej polarnego kationu $[IM16]^+$, którą podejrzewano za przyczynę bardzo niskich odzysków tego analitu. Obie te metody pozwoliły na osiągnięcie wysokich (>70 %) odzysków bezwzględnych wszystkich testowanych kationów ILs przy obserwowaniu niskich efektów matrycowych, mieszących się w zakresie od -0,05 % ($[IM1\text{-}10]^+$ w MAE6) do -38,40 % ($[IM1\text{-}12]^+$ w MAE6). W ten sposób potwierdzono, że opracowane metody pozwalają na skuteczną ekstrakcję kationów ILs z tkanek małży, jednocześnie zapewniając, że współekstrahowane substancje mają niewielki wpływ na jonizację badanych związków lub zostały wystarczająco skutecznie usunięte na etapie oczyszczania. Do dalszego postępowania wybrano więc metodę MAE7, czyli ekstrakcję 90 % roztworem MeOH trwającą 20 minut, przy temperaturze 120 °C oraz mocy 800 W.

Ostatecznie tak opracowana metodyka oznaczania czterech kationów alkiloimidazoliowych z tkanek małży została poddana procesowi walidacji. Wszystkie wyznaczone parametry zestawiono w **Tabeli 4** w publikacji **[P6]**. Opracowana metodyka cechuje się bardzo dobrą liniowością **(Rysunki S1-S8, [P6]**), granicami wykrywalności (16,67 do 50 ng/g suchej masy) oraz oznaczalności (50 do 150 ng/g suchej masy) dla wszystkich badanych związków. Wartości te są także zbliżone do danych literaturowych dla podobnych strukturalnie związków chemicznych. Przykładowo, w pracy Kierkegaard i in. (2021), wartości te uzyskane dla analizy czwartorzędowych kationów amoniowych w tkankach ryb oscylowały w granicach od kilku do kilkuset ng/g.

Jak wskazują autorzy innych prac (Pati i in., 2020; Lu i in., 2015), wydajna ekstrakcja kationów ILs o różnej długości łańcuchów bocznych może być trudnym

wyzwaniem analitycznym. Wynika to z faktu, że na etapie przygotowania próbek może dochodzić do utraty analitów o skrajnych właściwościach – najbardziej hydrofilowych lub hydrofobowych, co także obserwowano w toku przeprowadzonych badań. Mimo to, wykorzystanie techniki MAE oraz odpowiednie dobranie warunków tej ekstrakcji jak i warunków ekstrakcji do fazy stałej pozwoliło na zaproponowanie nowej, wydajnej i powtarzalnej procedury oznaczania wybranych kationów ILs z tkanek małży.

Mając na uwadze dostępne w literaturze rozwiązania analityczne dotyczące oznaczania kationów imidazoliowych w matrycach środowiskowych (**Tabela 2**, [**P2**]) należy zaznaczyć, iż wszystkie opierają się o wykorzystanie prostej ekstrakcji rozpuszczalnikowej wspomaganej ultradźwiękami. Co więcej, istnieje zaledwie jedna praca odnosząca się do oznaczania wybranych ILs w próbkach biologicznych (Nichthauser i in., 2009), w której to wartości odzysków badanych analitów mieściły się w zakresie nieco niższym niż w zaproponowanej procedurze, tj. od 70,2 % do 83,5 %. Pozostałe prace dotyczą w głównej mierze oznaczania ILs w glebach i osadach, dlatego niemożliwe jest ich bezpośrednie porównanie z wynikami uzyskanymi w niniejszej pracy doktorskiej. Potwierdza to jednak, że przeprowadzona ocena przydatności trzech wspomaganych technik do ekstrakcji ILs z próbek biologicznych, stanowi istotny wkład w wiedzę na temat analizy kationów imidazoliowych w próbkach środowiskowych.

Tym samym, opracowana i w pełni zwalidowana procedura oznaczania czterech kationów imidazoliowych w tkankach bezkręgowców morskich umożliwia oznaczenie tych związków nawet w śladowych ilościach i stanowi solidną podstawę dla przyszłych badań. Biorąc pod uwagę, że kationy imidazoliowe są wykrywane w środowisku, a ich produkcja wciąż wzrasta, związki te mogą wymagać uwzględnienia w przyszłych programach monitoringowych. Dlatego opracowano metodykę umożliwiającą oznaczanie tych substancji w tkankach małży, czyli organizmów powszechnie wykorzystywanych w biomonitoringu środowiska. Co więcej, metodyka ta umożliwiła przeprowadzenie testów biokoncentracji *in vivo* zaplanowanych w ramach niniejszej pracy doktorskiej.

5.4. Ocena biokoncentracji kationów imidazoliowyh w testach *in vivo* z wykorzystaniem małży *M. trossulus* [P7]

Małże z rodziny Mytilidae są dobrymi bioindykatorami i łatwo pobierają związki chemiczne obecne w wodzie z uwagi na odżywianie poprzez filtrację, a także są organizmami łatwymi w hodowli. Dlatego też są powszechnie wykorzystywane w badaniach środowiskowych dotyczących biomonitoringu, a także testach laboratoryjnych związanych z oceną biokoncentracji i toksyczności. Dodatkowymi cechami małży Mytilidae, które decydują o ich skuteczności w tego typu badaniach są: osiadły tryb życia i wysoka odporność na zanieczyszczenia (Świacka i in., 2019). Ponadto, gatunki z rodziny Mytlidae są szeroko rozpowszechnione na całym świecie, co z kolei umożliwia porównywanie otrzymanych wyników badań prowadzonych na organizmach pobranych w różnych częściach świata (Świacka i in., 2019). Stąd też w testach *in vivo* oceny biokoncentracji ILs wykorzystany został bałtycki przedstawiciel Mytilidae - *M. trossulus*.

We wstępnych eksperymentach, mających na celu wyznaczenie toksyczności ostrej badanych związków, wartość LC_{50}, czyli stężenia powodującego śmierć 50% osobników, (ang. *Lethal Concentration*) (21 d) wyniosła 0,68 mg/L dla [IM1-12][Br] i 11,66 mg/L dla [IM18][$C(CN)_3$]. Dlatego, na podstawie otrzymanych wyników zdecydowano, że we wszystkich testach biokoncentracji cieczy jonowych wykorzystane zostanie stężenie 10 μg/L. Jest to wartość o ponad 3 rzędy wielkości niższa od LC_{50} dla [IM18][$C(CN)_3$] i o prawie 2 rzędy wielkości niższa od LC_{50} dla [IM1-12][Br].

Wartości BCF dla $[IM1\text{-}12]^+$ wyznaczone w niniejszej pracy (**Tabela 1 [P7]**) wskazują, że zgodnie z wytycznymi przyjętymi w ramach REACH (EC 1907/2006) (Lombardo i in., 2014), kation ten należy uznać za związek o wysokiej zdolności do bioakumulacji (BCF = 21901 ± 3439 L/kg; logBCF = 4,34 ± 0,07 L/kg, 8 PSU). W jedynej dotychczas opublikowanej pracy dotyczącej biokoncentracji ILs *in vivo*, testom poddano kation imidazoliowy z krótkim, butylowym łańcuchem bocznym, który wykazał bardzo niską biokoncentrację (BCF < 10 L/kg) w tkankach *M. trossulus*. Uzyskane, w ramach niniejszej pracy, wyniki wskazują, że również łańcuch 8-węglowy nie zwiększa znacząco biokoncentracji imidazoliowego kationu. Wyznaczona wartość BCF dla $[IM18]^+$ wyniosła bowiem 57 ± 11 L/kg (**Tabela 4)**. W wyniku przeprowadzonych testów *in vivo* nie potwierdzono wpływu anionu $[C(CN)_3]^+$ na biokoncentrację $[IM18]^+$ (**Tabela 1 [P7]**), odmiennie do wyników uzyskanych

w testach *in vitro* z wykorzystaniem SSLM. Można to wytłumaczyć złożonością organizmu żywego. Niemniej jednak, zaobserwowana zgodność pomiędzy wynikami *in vitro* i *in vivo*, w odniesieniu do wzrastającej biokoncentracji wraz ze wzrostem długości łańcucha węglowodorowego, sugeruje, że testy *in vitro* oceny stopnia wiązania z lipidami membranowymi z powodzeniem mogą być stosowane w badaniach przesiewowych dla dużych grup związków chemicznych. Pomimo, że w badaniach *in vitro* z zastosowaniem SSLM niemożliwe jest doskonałe odzwierciedlenie wszystkich procesów zachodzących w ustroju, z uwagi na to, że testy te bazują na dobrze zdefiniowanych układach, stanowią przydatne narzędzie, gdy celem badania jest wyjaśnienie mechanizmów oddziaływania, określenie zależności stopnia wiązania od struktury związku chemicznego czy też jego budowy przestrzennej.

Dotychczasowe, dostępne w literaturze wyniki wskazywały, że dopiero kationy z alkilowym łańcuchem zawierającym powyżej 15 atomów węgla mogą ulegać nagromadzaniu w tkankach organizmów żywych (Droge i in., 2021). Również w pracy Kierkegaard i in. (2021), w testach z wykorzystaniem pstrąga tęczowego stwierdzono, że obdarzone permanentnym ładunkiem czwartorzędowe kationy amoniowe o 10- i 14-węglowych łańcuchach bocznych nie ulegają biokoncentracji. Dopiero w przypadku analogicznych trzeciorzędowych amin zaobserwowano znaczną biokoncentrację kwalifikującą te związki jako ulegające bioakumulacji wg REACH (BCF > 2000 L/kg). Dlatego też, badania przeprowadzone w ramach niniejszej pracy doktorskiej po raz pierwszy wskazują, że kationy ILs (w tym przypadku imidazoliowe), o krótszych niż 15 atomów węgla, łańcuchach, mogą ulegać znacznej biokoncentracji w tkankach organizmów wodnych.

W niniejszej pracy analizowano także wpływ zasolenia na zjawisko biokoncentracji, który był zróżnicowany w zależności od badanego związku. Dla $[IM18]^+$ zaobserwowano wzrost biokoncentracji wraz ze wzrostem zasolenia, natomiast odwrotny efekt zaobserwowano dla $[IM1–12]^+$, gdzie wartość BCF była niemal dwukrotnie mniejsza w przypadku dwukrotnie większego zasolenia (16 PSU) (**Tabela 1 [P7]**). Zaobserwowane różnice w biokoncentracji badanych cieczy jonowych w różnych zasoleniach mogą wynikać z wielu czynników. Zasolenie może wpływać zarówno na biodostępność związków chemicznych, jak i na fizjologię organizmów (np. efektywność procesów filtracyjnych czy osmotycznych). Obie wspomniane właściwości są kluczowe w procesach absorpcji i ewentualnej biokoncentracji (Jeon

i in., 2010). Biorąc jednak pod uwagę, uzyskane wyniki należy podkreślić, że pełne wyjaśnienie tego zjawiska wymaga dodatkowych badań.

6. Podsumowanie

Zaplanowane cele badawcze zostały zrealizowane, a opracowane nowe narzędzia analityczne oraz uzyskane wyniki testów *in vitro* oraz *in vivo*, stanowią istotny wkład w lepsze zrozumienie losu środowiskowego związków chemicznych obdarzonych ładunkiem, na przykładzie wytypowanych do badań cieczy jonowych (ILs) oraz produktów transformacji leków (TPs).

Wyniki uzyskane w testach *in vitro* pozwoliły na wskazanie ważnych cech strukturalnych, które mogą w największym stopniu odpowiadać za powinowactwo ILs do fosfolipidów, a co za tym idzie ich potencjalną biokoncentrację. Przeprowadzone badania jednoznacznie potwierdzają, że w przypadku ILs, szczególną uwagę w kontekście potencjału do biokoncentracji należy zwrócić na kationy z długimi łańcuchami alkilowymi, ale również aniony zawierające układy perfluorowane. Co więcej, zaprezentowane dane sugerują, że duże znaczenie w oddziaływaniu z fosfolipidami mogą mieć także interakcje pomiędzy kationami i anionami ILs. Porównanie wyników eksperymentalnych z istniejącym modelem COSMO*mic* potwierdziło, że narzędzie to, już na obecnym etapie, może być przydatne we wstępnym wskazywaniu związków chemicznych o dużym potencjale do biokoncentracji. Natomiast testy *in vitro* z wykorzystaniem TPs leków potwierdzają wcześniejsze założenia, zgodnie z którymi związki te mają niewielkie powinowactwo do błon komórkowych, co może wynikać z faktu, że w procesach metabolicznych zyskują one dodatkowe grupy funkcyjne o charakterze polarnym znacząco ograniczającymi potencjał do biokoncentracji. Nie zmienia to jednak faktu, iż w przypadku ich ewentualnej biokoncentracji, mogą one wciąż oddziaływać z białkami.

Opracowana nowa metodyka oznaczania mieszaniny kationów imidazoliowych ILs opierająca się o wykorzystanie technik łączonych MAE-SPE-LC-MS/MS pozwoliła na wydajną ekstrakcję i oznaczenie ich śladowych ilości w tkankach małży. Metodyka ta nie tylko umożliwiła przeprowadzenie zaplanowanych testów *in vivo*, ale może być także niezwykle pomocna w przyszłych badaniach biomonitoringowych.

Na podstawie przeprowadzonych po raz pierwszy badań *in vivo* z wykorzystaniem gatunku modelowego *M. trossulus* potwierdzono, że kationy imidazoliowe o względnie długich łańcuchach bocznych ulegają biokoncentracji w tkankach bezkręgowców morskich, a tym samym mogą stanowić poważne zagrożenie dla środowiska morskiego. Wykazano także, że zasolenie może być istotnym czynnikiem wpływającym

na potencjalną biokoncentrację związków chemicznych obdarzonych ładunkiem, jednak jego pełne zrozumienie wymaga bardziej szczegółowych badań.

Niemniej jednak, mając na uwadze wszystkie trzy rodzaje badań (*in vitro*, *in vivo* oraz *in silico)* potencjału do biokoncentracji związków obdarzonych ładunkiem, należy stwierdzić, że prezentują one wysoką zgodność w kwestii przewidywania generalnych tendencji. Wszystkie bowiem wskazują, że długość łańcucha alkilowego w kationie ILs jest decydującą cechą w przypadku jego potencjalnej biokoncentracji. Należy jednak pamiętać, że wyniki testów *in vivo* mogą być specyficzne dla badanego gatunku i wykazywać dużą zmienność pomiędzy organizmami. Tymczasem badania *in vitro* i *in silico* opierają się na oddziaływaniu z fosfolipidami, czyli molekułami stanowiącymi uniwersalną składową niemal wszystkich grup taksonomicznych. Ostatecznie należy podkreślić, że wysoka zgodność pomiędzy wynikami eksperymentów *in vitro* i *in vivo* dowodzi, że nowoczesne testy *in vitro* mogą być skutecznie wykorzystane w przesiewowych badaniach dla szerokiej gamy substancji chemicznych. Wychodzą one zatem naprzeciw obecnie bardzo pożądanym trendom związanym z zasadą 3R (ang. *Reduce, Reuse, Recycle*) i poszukiwaniem bardziej przyjaznych dla środowiska rozwiązań, w tym ograniczeniu liczby testów na zwierzętach. Tego typu badania *in vitro* mogą także stanowić użyteczne narzędzie w badaniach modelowych do wyjaśniania mechanizmów oddziaływania, ustalania zależności między powinowactwem do fosfolipidów a strukturą chemiczną związku lub jego konfiguracją przestrzenną.

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8. Prace opublikowane wchodzące w skład rozprawy doktorskiej [P1-P7]

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Review

In vitro methods for predicting the bioconcentration of xenobiotics in aquatic organisms

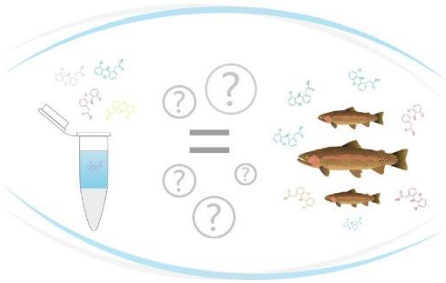
Jakub Maculewicz [a,*], Klaudia Świacka [b], Dorota Kowalska [a], Piotr Stepnowski [a], Stefan Stolte [c], Joanna Dołżonek [a]

[a] Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland
[b] Department of Experimental Ecology of Marine Organisms, Institute of Oceanography, University of Gdansk, Av. Pilsudskiego 46, 81-378 Gdynia, Poland
[c] Faculty of Environmental Sciences, Department of Hydrosciences, Institute of Water Chemistry, Technische Universität Dresden, Bergstrasse 66, 01069 Dresden, Germany

HIGHLIGHTS

- *In vitro* methods for estimating the potential for bioconcentration were reviewed.
- Reliable assessment of the potential for bioconcentration is still a challenge.
- More accurate models should replace the octanol-water partition coefficient.
- New methods are more reliable especially for ionic compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

The accumulation of anthropogenic chemical substances in aquatic organisms is an immensely important issue from the point of view of environmental protection. In the context of the increasing number and variety of compounds that may potentially enter the environment, there is a need for efficient and reliable solutions to assess the risks. However, the classic approach of testing with fish or other animals is not sufficient. Due to very high costs, significant time and labour intensity, as well as ethical concerns, *in vivo* methods need to be replaced by new laboratory-based tools. So far, many models have been developed to estimate the bioconcentration potential of chemicals. However, most of them are not sufficiently reliable and their predictions are based on limited input data, often obtained with doubtful quality. The octanol-water partition coefficient is still often used as the main laboratory tool for estimating bioconcentration. However, according to current knowledge, this method can lead to very unreliable results, both for neutral species and, above all, for ionic compounds. It is therefore essential to start using new, more advanced and credible solutions on a large scale. Over the last years, many *in vitro* methods have been newly developed or improved, allowing for a much more adequate estimation of the bioconcentration potential. Therefore, the aim of this work was to review the most recent laboratory methods for assessing the bioconcentration potential and to evaluate their applicability in further research.

Contents

* Corresponding author.
E-mail address: jakub.maculewicz@phdstud.ug.edu.pl (J. Maculewicz).

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1. Introduction

Water resources around the world are under enormous pressure due to climate change, ocean acidification, as well as excessive consumption in households and various economic sectors (Werritty 2002; Kliskey et al., 2019; Rayner, 2019; Wan et al., 2019). Another emerging problem is the release of various chemicals with potential harm to both the environment and people.

To fully understand how chemicals enter the body, what happens to them inside and how they are excreted, it is worthwhile to trace the processes of uptake, distribution, metabolism and excretion, the most important components of which are presented in Fig. 1. In the case of aquatic animals, there are three main ways by which chemicals can enter the body: gills, skin and gastrointestinal tract (Mackay et al., 2018; Wassenaar et al., 2020). Once they enter the body, the chemicals are distributed among the various tissues. The distribution process depends on the properties of the absorbed compounds, which can have a strong affinity to different biomolecules - blood proteins, structural proteins, storage lipids or membrane lipids - and therefore they accumulate primarily in organs which are rich in these biomolecules (Da Cuña et al., 2020; Hou et al., 2017; Grech et al., 2016). The next stage is metabolism, which leads to the transformation of absorbed compounds into various metabolites. Metabolic processes take place particularly intensively in the cells of such organs as the liver, gills and kidneys (Gomez et al., 2010). As a result, usually compounds are formed which are easier to remove from the body, although there are also known situations in which the products of metabolism turn out to be more harmful and more accumulative than the original compounds (la Farre et al., 2008). The last phase of interaction of chemicals with the body is excretion. In this case, the main routes are similar to absorption - elimination takes place primarily through the gills and by fecal egestion (Arnot and Gobas, 2006). Compounds that have entered the body can be excreted as derivatives formed during metabolic processes, but also as parent compounds. In addition, a pseudo-elimination process, involving a reduction in the concentration of chemical compounds by dilution in growing tissues, should be considered (Segner, 2015). All the processes described above are responsible for the bioaccumulation phenomenon, which represents the deposition of environmental chemicals in the tissues of organisms. Bioaccumulation includes bioconcentration, which involves the absorption of chemicals from the environment solely through respirators and dermal surfaces, excluding the digestive tract, as well as biomagnification, a process strictly linked to trophic transfer, in which the concentration usually increases at subsequent levels of the food chain, reaching a maximum value for the highest consumers (Yarsan and Yipel, 2013; Barron, 2003). All of these processes can be described mathematically using the appropriate factors - BCF (bioconcentration factor), BAF (bioaccumulation factor) and BMF (biomagnification factor). The BCF is calculated as the ratio of the chemical concentration in the organism and the chemical concentration in the surrounding medium at a steady state (Arnot and Gobas, 2006). In the case of the BAF, the possibility of taking chemical compounds through the digestive tract is also considered. The BMF, in turn, is an indicator that only concerns dietary intake and is expressed as the ratio of the chemical concentration in the organism and the chemical concentration in its diet (Arnot and Gobas, 2006).

One of the key regulations concerning the production and import of chemical substances into European Union countries is REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals). Under this regulation it is required to register all the chemicals above a production volume of 1 t per year in order to prevent the risk of these substances entering into the environment (Kemmlein et al., 2009). Moreover, if the production volume of a chemical exceeds 10 t per year, a risk assessment report must be prepared (chemical safety report), including information on the possible bioaccumulative properties of the compound and its persistence in the environment (Kemmlein et al., 2009; Hansen and Blainey, 2006; Williams et al., 2009). There are currently almost 23,000 compounds registered under the programme, although preregistration has even covered >100,000 chemicals. Moreover, many compounds whose presence in the environment has already been identified, are covered by the monitoring programme. For the monitoring of surface waters in the European Union, the Water Framework Directive (WFD) was established. The main objective of the WFD is to determine the quality of water resources and to implement standards enabling the sustainable management of those resources (Borja et al., 2010; Kaika, 2003; Kallis and Butler, 2001). Among the groups of compounds monitored under the WFD the following can be distinguished: polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides and polychlorinated biphenyls (PCBs), alkylphenols, short-chain chlorinated paraffins (SCCPs), tributyltin compounds, polybrominated diphenyl ethers (PBDEs), perfluorinated compounds (PFCs), as well as pharmaceuticals, such as nonsteroidal anti-inflammatory drugs and steroidal estrogenes (Rubirola et al., 2019; Könemann et al., 2018; Coquery et al., 2005; Lepom et al., 2009). The compounds monitored according to the WFD are hazardous substances characterized by high toxicity to organisms (*e.g.* disturbing reproductive processes or metabolism, deteriorating the condition, causing histopathological changes in tissues). Additionally, their high ability to bioaccumulate has been demonstrated. Such chemicals pose an enormous risk to human and aquatic life (Rhind, 2009; Karouna-Renier et al., 2007). For example, studies carried out by Karouna-Renier et al. (2007) have shown the presence of dioxins, furans and DL-PCBs in blue crabs (*Callinectes sapidus*) and oysters (*Crassostrea virginica*) from bays and bayous in the Pensacola, FL area. The recorded concentration values of these compounds were well above the acceptable daily human consumption threshold, posing a serious risk to potential consumers.

Therefore, it is clear that the assessment of the risks associated with the presence of anthropogenic chemicals in ecosystems is a key issue raised by environmental scientists. The bioconcentration potential constitutes an important factor for estimating the effects of the release of

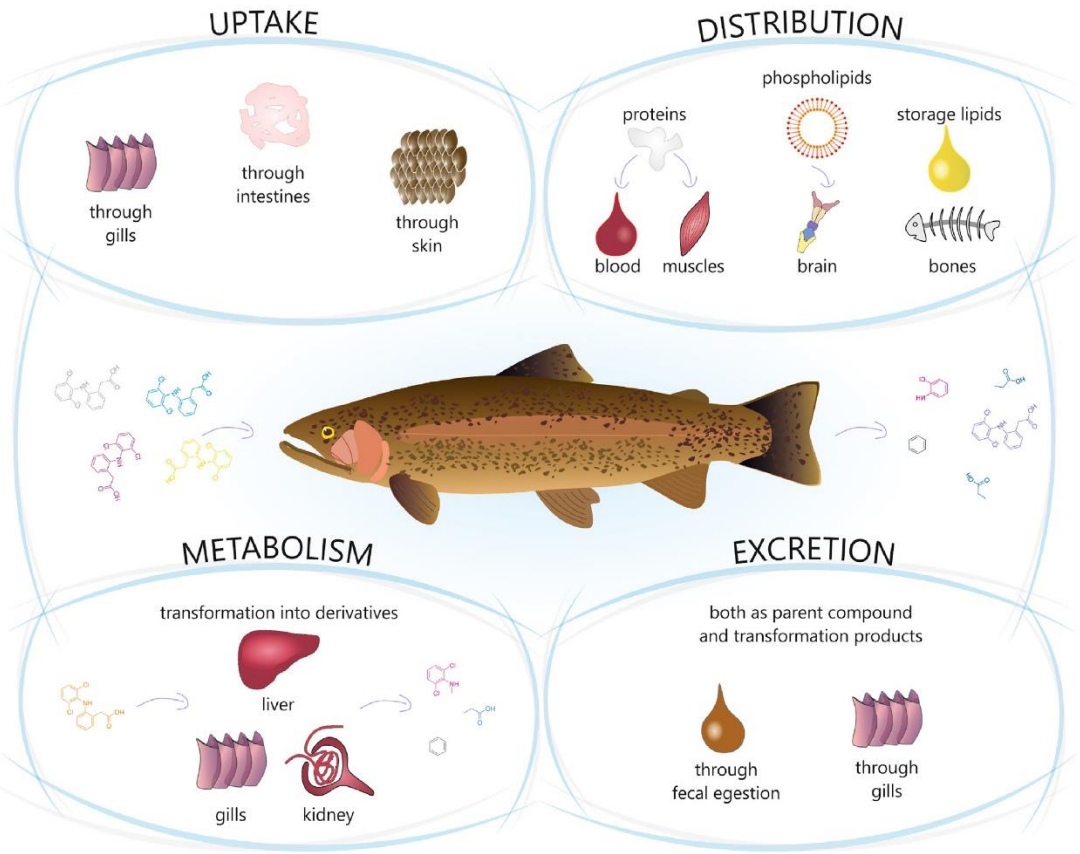

Fig. 1. Scheme explaining the processes of uptake, distribution, metabolism and excretion.

chemicals into the environment. It allows the determination of the extent to which the compound will accumulate in living organisms and is the sum of passive uptake from the environment, metabolism and excretion (Nichols et al., 2009; Treu et al., 2015).

According to the legal regulations (*e.g.* OECD Test Guideline 305), the standard method for estimating the bioconcentration of chemicals in aquatic organisms is based on experiments using fish. Testing with fish is very time-consuming and financially demanding. Given the already huge and still growing number of compounds that require a pollution risk assessment, the capacity of this method is many times too low to provide adequate data for all these chemicals (Weisbrod et al., 2009). Another important aspect to be taken into account is the ethical issue related to the testing of harmful compounds on living organisms, especially vertebrates. It is therefore extremely important that *in vivo* tests, which raise many doubts, are replaced by new laboratory-based *in vitro* methods and *in silico* models that would enable a much cheaper and faster determination of the bioconcentration potential, with equally high reliability.

So far, many *in silico* models have been developed to estimate the accumulation of chemical pollutants in aquatic organisms. However, most of them are based on the octanol-water partition coefficient (referred to as K_{ow} in Environmental Sciences and P in Pharmaceutical Sciences), a simple tool that determines the partitioning between the hydrophobic phase and the hydrophilic phase, which means that they take into account only the hydrophobicity-based absorption of the compounds. In the case of ionizable substances, the log D parameter can also be determined, which consists of measurements at different pH values, in which both ionic and non-ionic forms of the compound are present (Fu et al., 2009). Nevertheless, these coefficients assume that lipids (membrane and storage treated as one compartment), whose sorption properties are similar to octanol, make up the dominant fraction in organisms responsible for the accumulation of chemical compounds. However, it is difficult to expect that such a simplified model could adequately reproduce the behavior of complex biological structures. Although predictions obtained with this method may give an approximate view of the behavior of compounds in organisms, it is often underestimated, especially in the case of numerous ionic compounds, which may show a much greater bioconcentration potential than expected (Armitage et al., 2013; Bittermann et al., 2018; Dołżonek et al., 2017; Treu et al., 2015; Endo et al., 2013). It should be remembered that both storage lipids, *i.e.* triglycerides, and membrane lipids, *i.e.* phospholipids are vastly different, both in structure and functionality, therefore treating them as a single phase may lead to false estimations. The absorption of charged species in cell membranes is highly supported by electrostatic interactions with zwitterionic head groups of phospholipids. Hence estimations based solely on the $\log K_{ow}$, neglecting potential interactions that may occur between such compounds and charged phospholipids, might give highly inaccurate results in the case of ionic compounds. Moreover, it is clear that interactions of ionic compounds with biological membranes depend on both the location of the charge in the molecule, but also the entire structure of the compound and its steric properties (Armitage et al., 2013). In addition,

anionic species show a greater affinity to choline, while cations interact primarily with phosphate groups (Escher et al., 2000a, 2000b; Armitage et al., 2013). In general, it is certain that partitioning of ionic compounds is much more complex than that of neutral compounds, so they should be given special attention when estimating bioconcentration (Henneberger and Goss, 2019).

Moreover, it should be emphasized that lipids are not the only component of the body that can be responsible for the accumulation of xenobiotics. Numerous chemical compounds may also show a significant affinity to proteins, both structural, building muscles and blood proteins (*e.g.* perfluorinated alkyl acids, benzoic acids, pesticides, pharmaceuticals) (Henneberger et al., 2016a, 2016b; Endo et al., 2012, 2013; Endo and Goss, 2011; Ng and Hungerbühler, 2013). Therefore, it is highly relevant to take into account much more complex dependencies and properties than lipophilicity when estimating the accumulation of chemical compounds. Among simple indicators of bioconcentration, molecular size and mass should also be distinguished. According to one theoretical concept, there is a limit value of these two parameters above which chemical compounds are not able to pass through biological membranes. However, literature data indicate that such a method of estimating the bioconcentration potential does not always work, and comparison with BCF values shows discrepancies for many compounds (Lillicrap et al., 2016; Arnot et al., 2010).

Although this article focuses primarily on tests to determine the absorption of chemicals in aquatic organisms, it should be remembered that this is not the only process responsible for the final amount of accumulated substances. Biotransformation tests are an important supplement to the data obtained from the analyses of partitioning into membranes, proteins or triglycerides (Rehberger et al., 2018). These experiments make it possible to correct existing predictions, taking into account the metabolic processes occurring in organisms. Currently, fractions obtained from the liver, which is the main organ responsible for detoxification, are most often involved in this type of tests (Jeon and Hollender, 2019; Greaves et al., 2016; Saunders et al., 2019; Nakpheng et al., 2017). It is clear that the identification of metabolic processes makes it possible to estimate the extent of bioconcentration more accurately. The determination of the absorption in tissues alone can provide information about the bioconcentration potential in its worst case scenario (Bittermann et al., 2018). On the other hand, exploring the metabolic pathways allows an estimation of which part of the absorbed compound will be able to undergo transformations and eventually be excreted from the body.

The main aim of this work was to present an overview of *in vitro* methods for estimating the phenomenon of bioconcentration. We have analyzed the latest scientific achievements in laboratory-based studies on bioconcentration, with particular attention to tests estimating the absorption of xenobiotics. We have considered both methods that can be useful in quickly and accurately assessing the overall bioconcentration process, as well as those that can be used to better understand the specific mechanisms responsible for the interaction of contaminants with individual components of the body.

2. *In vitro* assays for the estimation of bioconcentration

2.1. Artificial membrane assays

A wide range of *in vitro* methods based on artificial biological membranes have already been used in research on physiological processes in biomedical and pharmacological sciences, as well as in analyses of environmental hazards (Armitage et al., 2013; Nichols et al., 2007, 2009; Van Der Heijden and Jonker, 2011). The implementation of these solutions, based on various artificial structures made up of phospholipids, is a more advanced alternative to octanol-based testing. Due to greater physiological similarity to real biological structures, the analysis of the partitioning to artificial membranes and their permeability allows for a better estimation of the bioconcentration potential of chemical compounds, especially polar and charged ones. Therefore, in this subchapter we present

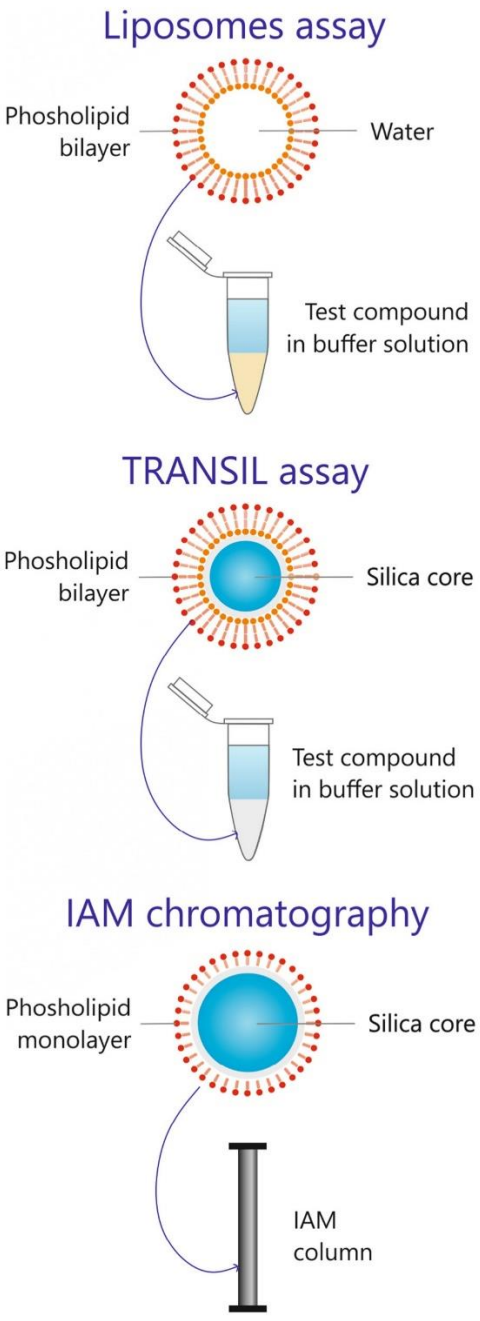

Fig. 2. Scheme illustrating the main characteristics of *in vitro* assays using an artificial membrane made of phospholipids.

the main types of *in vitro* experiments using artificial lipid membranes (Fig. 2).

2.1.1. Liposomes

The most common model of a synthetic membrane, which largely mimics the true properties of biological membranes, includes liposomes (Alves et al., 2013; Balon et al., 1999; Decker et al., 2012; Escher and Schwarzenbach, 1996). Liposomes are vesicles made of phospholipids that form bilayers. They are obtained in a simple way, based on the tendency of phospholipids to form multilamellar vesicles (MLV) in aqueous solutions. By applying sonication, multiple freeze/thaw cycles or extrusion by special membrane filters, multilamellar vesicles can be used to obtain single-bilayer liposomes of a desired size (SLV – small unilamellar vesicles; LUV – large unilamellar vesicles) (Ribeiro et al., 2010). Although the preparation of liposomes is very simple, experiments determining the affinity of compounds to artificial membranes are more complex.

Several methods, at least, have been developed to determine the partition coefficient in liposomes, with many different advantages and disadvantages (Van Der Heijden and Jonker, 2009). So far, equilibrium dialysis is the most often used, classic technique for the determination of membrane partitioning (Escher and Schwarzenbach, 1996; Escher et al., 2000a; Kwon et al., 2007a; Ottiger and Wunderli-Allenspach, 1997). However, a wide variety of methods have been used for this purpose, i.a. solid-phase microextraction (Van Der Heijden and Jonker, 2009), isothermal titration calorimetry (Patel et al., 2010), ultrafiltration (Austin et al., 1998), second-derivative spectrophotometry (Takegami et al., 2015), the polymer depletion method (Endo et al., 2013) and zeta-potential measurements (Freire et al., 2011). In addition to choosing the right laboratory procedure, the composition of phospholipids from which the liposomes are made is also important, as different ratios of particular fatty acids in biological membranes may give different results (Modi and Anderson, 2013; Van Der Heijden and Jonker, 2011; Yamamoto and Liljestrand, 2004). It is also important whether cholesterol is included in the membrane, as it changes its fluidity and therefore influences the partitioning behavior of the tested compounds (Kučerka et al., 2010; Subramanian et al., 2016; Wennberg et al., 2012). Among the difficulties associated with experiments on liposomes, it should be stressed that they are characterized by relatively low stability in buffer. Therefore, to avoid their spontaneous destruction, it is recommended to perform experiments on freshly prepared liposomes. Moreover, experiments with liposomes require the careful selection of the appropriate methods and lipid components, depending on the purpose of the study. However, if properly designed, they can provide many physiologically adequate answers to the matter of the bioconcentration potential.

2.1.2. Solid-supported lipid membranes

Solid-supported lipid membranes (SSLMs) are widely used for determining the partitioning of chemical compounds into membranes, especially when searching for new pharmaceutical compounds (Golius et al., 2016; Morandi et al., 2016), but also in the case of environmental risk assessment (Dołżonek et al., 2017; Timmer and Droge, 2017; Droge, 2018). This tool is based on porous silica beads that have been coated with a non-covalently bound phosphatidylcholine bilayer (Loidl-Stahlhofen et al., 2001a). A big advantage of SSLMs, in comparison to equilibrium dialysis using liposomes, is the fact that it takes only a few minutes to reach equilibrium, which enables fast screening tests for many compounds, especially important with regard to generating data for future models. Although they do not entirely reflect the physiology of real membranes, they can to a high degree imitate the processes involved in the absorption of compounds. The lipids placed on the solid support retain their fluidity, which allows a good simulation of the surface of biological membranes (Loidl-Stahlhofen et al., 2001b). According to studies carried out by Loidl-Stahlhofen et al. (2001a), this method shows a strong correlation with experiments on liposomes, as demonstrated for many pharmaceuticals.

However, due to limitations, SSLMs are mainly suitable for substances with a higher lipophilicity, reaching log $k_{lipw} > 1.5$. Additionally, although this method seems to be much easier and faster than liposome tests, its popularity in the context of environmental research is relatively low. The very limited amount of data prevents the establishment of appropriate correlations with *in vivo* results. This low popularity may be due to doubts regarding the potential interactions of tested compounds with the silica core of SSLMs. Although it is suggested that the presence of such interactions does not occur, this cannot be so easily excluded for all compounds. An additional limitation is the fact that these tests are only available in very limited variants regarding the membrane composition. Meanwhile, working with liposomes gives much more freedom, allowing for the free composition of membranes with different lipids and their mixtures, and additionally enabling the presence of cholesterol to be taken into account. Therefore, while SSLMs are a valuable tool for large-scale screening, they have limitations that make them unlikely to replace the more labor-intensive experiments with liposomes, but rather to complement them.

2.1.3. IAM chromatography

An interesting method, which was initially used mainly in biomedical sciences for determining the passing of new drug candidates through biological membranes, is immobilized artificial membrane (IAM) chromatography (Ong et al., 1995; Reichel and Begeley, 1998; Yang et al., 1997). It appears that this method can also be used more extensively in environmental research for the effective determination of parameters related to the accumulation of pollutants by organisms (Tsopelas et al., 2018, 2017a; Droge et al., 2017; Timmer and Droge, 2017). Since retention on IAM columns is caused by both hydrophobic and electrostatic interactions, it represents a border case between passive diffusion and binding (Tsopelas et al., 2017). Furthermore, important advantage of this approach is that it combines the amphiphilic properties of natural biological membranes with fast and precise chromatographic measurements.

The stationary phases of IAM columns are made of phospholipids, which are immobilized on a propylamine-silica skeleton. Currently, there are three products among the commercially available chromatographic columns of this type: IAM.PC, IAM.PC.MG and IAM.PC.DD2. Although these columns differ slightly in the design and purpose recommended by the manufacturer, research has shown that IAM.PC.MG and IAM.PC.DD2 columns have a similar retention mechanism and measurements made with them correlate with each other to a large extent (Tsopelas et al., 2018).

The use of IAM chromatography enables the determination of several different parameters related to the interaction between the compound and the membrane. The first is the simple retention factor of the compound, $k_{(IAM)}$, which is proportional to the partition coefficient. This coefficient is determined using a buffer, usually phosphate, to mimic physiological conditions. In turn, a pure aqueous phase can be used to obtain $k_{w(IAM)}$ values, while for compounds with a high affinity, the determination can be made with an organic modifier, and then, through linear extrapolation, the $k_{w(IAM)}$ value can be obtained. An alternative to these coefficients is the chromatographic hydrophobicity index, $CHI_{(IAM)}$, which refers to the percentage of acetonitrile needed for the equal partitioning of the compound between the mobile and stationary phase (Tsopelas et al., 2018; Valkó, 2004).

According to the latest research (Tsopelas et al., 2018), retention on IAM columns may reflect not only partitioning and lipophilicity, but also electrostatic interactions. This was mainly observed in the case of alkaline compounds, which is related to the attraction of protonated molecules by phosphate anions present at the hydrophobic nucleus of phospholipids (Tsopelas et al., 2017a; Vrakas et al., 2006). Moreover, it appears that the IAM retention index can highly reflect the permeability of physiological barriers such as the intestinal barrier or the blood-brain barrier (Tsopelas et al., 2016). For example, Yoon et al. (2004) has created a model that allows for correlating intestinal absorption with

isocratic retention factors ($k_{(IAM)}$) and molecular weight. By also taking into account electrostatic interactions, unlike many other models, the log $k_{w(IAM)}$ factor can reliably determine the partitioning of ionic compounds, as demonstrated by Stepnowski and Storoniak (2005). Of course, the application of IAM chromatography also has some disadvantages, and the results obtained with this method, in some cases, are inconsistent with the results obtained with liposomes. This may be caused by the lower lipid density in the IAM method (Tsopelas et al., 2016). However, considering the speed of measurements and their repeatability, IAM chromatography is an interesting alternative in the studies of the affinity of compounds to biological membranes.

2.1.4. PAMPA

Another method originally used to estimate the absorption of drugs by humans (Kansy et al., 1998; Wohnsland and Faller, 2001), but also adapted to bioconcentration studies in aquatic organisms, is the parallel artificial membrane permeability assay (PAMPA) (Nichols et al., 2009). Regarding aquatic organisms, this system was first used to simulate fish gill absorption by Kwon et al. (2007b). According to the diffusion mass transfer model, this test assumes that the gills, which consist of multiple layers of lipid membranes and water, are the main uptake route. As shown in Fig. 3, the PAMPA system uses a polymeric filter impregnated with a mixture of solvent, usually dodecane, and phospholipids, placed between two aqueous phases. This specially designed experimental set imitates processes in the gills and allows access to aqueous phases on both sides of the membrane, which are equivalent to the external and internal environment of fish. Therefore, both the uptake and elimination rates in fish can be determined. Changes in the concentration of the analyzed compound between the acceptor and donor phases are determined, and then the permeability of the membrane is evaluated.

However, due to the retention of hydrophobic compounds on the membrane, measurements with such compounds required modification. Originally, measurements with an additional solvent (acetonitrile) or additional elimination rate analyses were proposed (Kwon and Escher, 2008). However, such solutions proved to be ineffective because they required the additional extrapolation of data or significantly extended the analysis time, which may have increased the risk of biodegradation or reduced membrane stability. Kwon and Escher (2008) designed a new version of PAMPA, enabling the effective analysis of hydrophobic compounds through the use of polydimethylsiloxane disks (PDMS) as dosing and receiving systems. In the PDMS-PAMPA system, on one side of the membrane (also made of PDMS, with a thickness of 0.125 mm) there is a dosing disk, preloaded with the tested compound, while on the other side there is a clean acceptor disk. Using the data obtained by this method, it is possible to estimate the rate of passive elimination in fish (Kwon and Escher, 2008). However, the results obtained with the PAMPA systems may be underestimated for compounds that are metabolized by organisms. On the other hand, they still make it possible to quickly identify substances for which the passive elimination process itself indicates a low bioconcentration potential. For other compounds, however, the data need to be supplemented with information on metabolism, so the most accurate results can be expected when combined with *in vitro* metabolism tests.

2.2. Protein binding assays

The second important component of living organisms, which may be largely responsible for the absorption of environmental pollutants, is proteins (Escher et al., 2011; Henneberger et al., 2016a, 2016b; Ng and Hungerbühler, 2013). Their role in the bioconcentration process may be particularly significant, especially in the case of ionic compounds (Henneberger et al., 2016b). Among proteins, two important classes should be distinguished. The first group of these includes plasma proteins, especially albumin, which is known for its organic anion binding properties, but also alpha-1-acid glycoprotein (AGP), which can bind cations and neutral compounds (Kremer et al., 1988), as well as many other proteins present in the plasma of marine organisms. The second class potentially responsible for bioconcentration includes structural proteins, *i.e.* proteins building muscles, but also keratin, collagen, and others. In the following chapter, we describe the most relevant methods for estimating the contribution of protein binding to the bioconcentration process of contaminants in the aquatic environment.

The general assumptions of most tests determining the affinity of chemical compounds to proteins are based on measurements of the unbound fraction. The most popular method for determining the binding of xenobiotics to proteins is equilibrium dialysis (Oemisch et al., 2014). It is based on the separation of two chambers in a test vessel, between which there is a special dialysis membrane with pores small enough to retain proteins. The protein solution is then placed in one of the chambers and the buffer in the other. After adding a known concentration of the tested compound, diffusion occurs through the membrane until equilibrium is established, which usually takes 24–48 h. Finally, the remaining concentration of the compound in the protein-free chamber is analyzed with HPLC or LC-MS and the degree of binding is calculated from these results (Ng and Hungerbühler, 2013).

The second most popular technique used to analyze the binding of chemical compounds to proteins is ultrafiltration (Fung et al., 2003; Singh and Mehta, 2006). In this method, a solution containing proteins and a solution of the tested compound are first mixed together. After a specified time to bind the test substance to the protein, the mixture is placed in dedicated ultrafiltration tubes. These tubes are equipped with membranes that allow the aqueous solution to be separated from the protein molecules during centrifugation. After the separation of these fractions, it is possible to determine the degree of binding to the protein. The measurements can be done either indirectly by analyzing the filtered solution or directly by measuring the content of the ligand in the separated protein molecules. Of course, due to the simpler

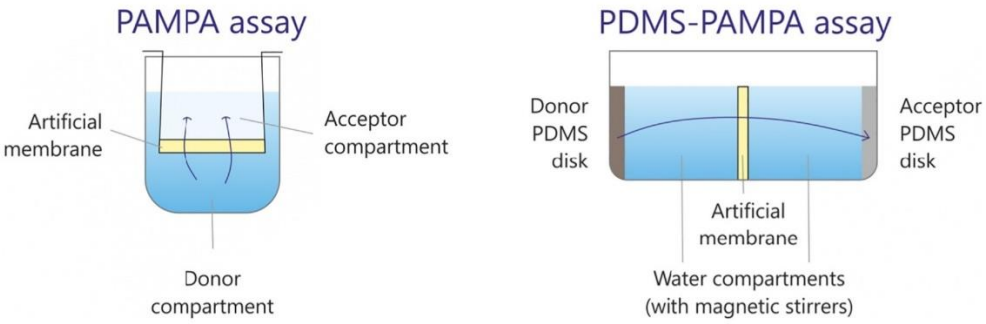

Fig. 3. Scheme illustrating the design of the PAMPA and PAMPA-PDMS systems.

procedure, the analysis of the unbound fraction is more frequently used. It is worth mentioning, however, that in the case of ultrafiltration, the occurrence of non-specific binding (NSB) with the membranes or filtrate collection tubes may be a significant problem, limiting the use of this method. However, there are some ways to reduce these interactions. According to the procedure proposed by Lee et al. (2003), pretreating the membrane with surfactants effectively protects against NSB problems. In turn, Taylor and Harker (2006) presented another modification of the method, which allows NSB to be eliminated by adding plasma retentate to the filtrate collection tubes before sampling for analysis (Fig. 4).

In addition, the use of solid-supported biomolecules (Loidl-Stahlhofen et al., 2001b) is also possible for protein binding testing. In these analyses, different protein fractions (*e.g.* human blood albumin, acid glycoprotein and others) that have been immobilized on silica beads are used. This method, similarly as in the case of lipids, allows the time of analysis to be significantly shortened and the efficiency increased (Hartmann et al., 2006). Reaching equilibrium in samples takes even a few minutes, allowing for easy and fast measurements on a large scale. The proteins bound to the silica beads allow simple separation of the unbound fraction by centrifugation and the collection of the supernatant, without involving membrane filters. However, it should be taken into account that in this case, as with SSLMs, potential interactions with the silica core may also be of concern.

Immobilized protein molecules are also used as a stationary phase of chromatographic columns. In the literature, there are at least a few different methods to immobilize proteins on a chromatographic bed, and ready-to-use columns are also commercially available (Kim et al., 2004; Mallik et al., 2005; Millot et al., 2001). According to Singh and Mehta (2006), plasma protein binding values obtained by the HSA-HPLC method show a very good correlation with the results obtained in earlier studies with different methods, including ultrafiltration. Moreover, the use of chromatographic columns with immobilized protein allows the analysis of substances that showed strong non-specific interactions with the membrane, excluding the use of ultrafiltration. However, not all compounds can be analyzed with this method, because some of them have such a strong binding to the protein that they cannot be eluted. This can be overcome by incorporating organic modifiers into the mobile phase and then extrapolating the results. Nevertheless, both methods have advantages and disadvantages that make their universal use rather difficult (Singh and Mehta, 2006).

2.3. Affinity to storage lipids

The second class of lipids which may also be responsible for the accumulation of environmental contaminants are storage lipids - mainly triacylglycerols. Due to the fact that their functioning in organisms and their interactions with potential xenobiotics are significantly less complex than in the case of proteins and phospholipids, the estimation of their potential contribution to bioconcentration is easier, although it also raises doubts and produces large variations between measurements in some cases (Endo and Goss, 2011). Generally, the affinity to storage lipids is estimated by the K_{tw} (triolein-water partition coefficient), which is determined analogically to the K_{ow} (Chiou, 1985). Therefore, these experiments are based on mixing an aqueous solution of the tested compounds and a mixture of storage lipids in a single tube (Fig. 5). The samples are incubated, usually at 37 °C or at room temperature, until equilibrium is established. After reaching equilibrium, it is possible to measure the concentration of the tested compounds and calculate the partition coefficient. The original "shake-flask" method used for this type of tests involves vigorous shaking of the mixture of water and lipids and then the separation of phases by centrifugation (Cheng

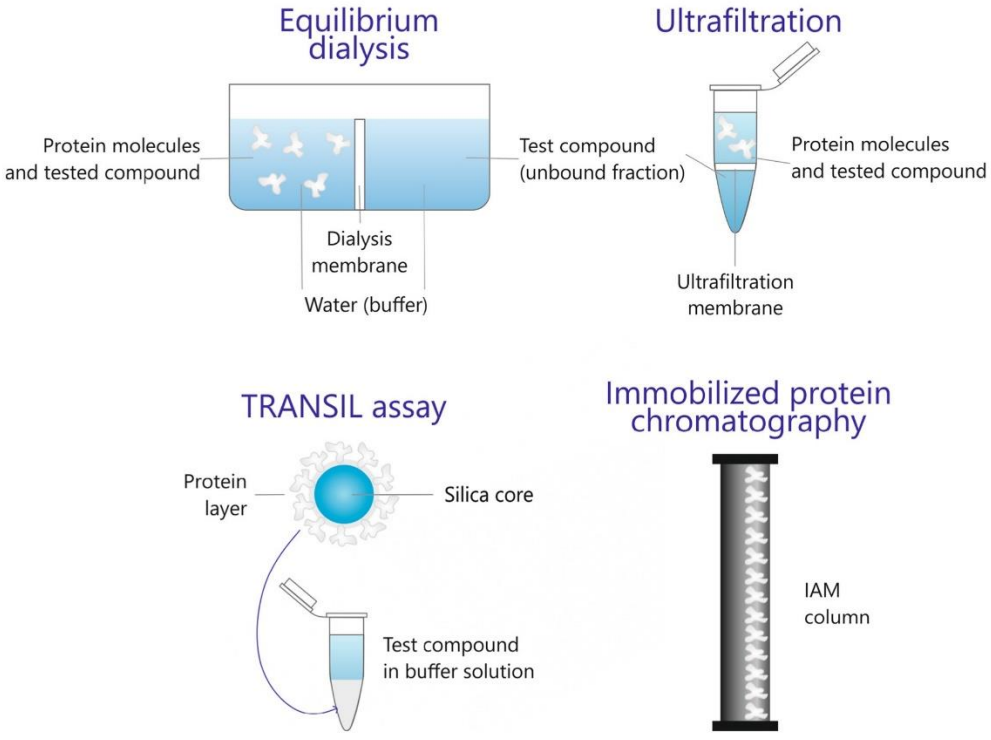

Fig. 4. Scheme illustrating the design of the *in vitro* assays using proteins.

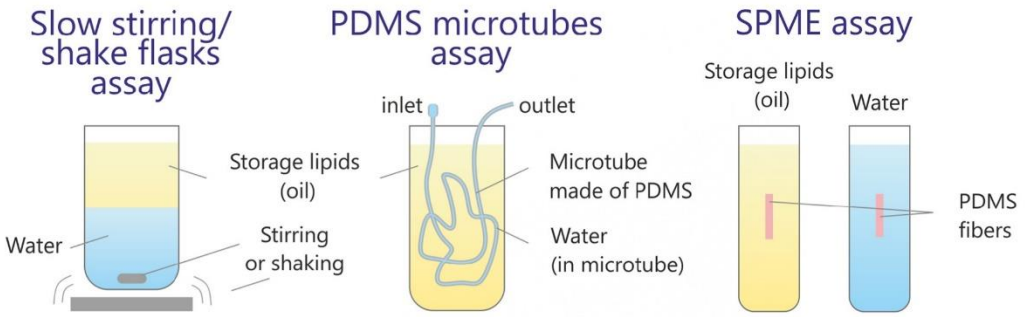

Fig. 5. Scheme illustrating the design of different oil-water partitioning systems.

et al., 2010; Chiou, 1985; Hung et al., 2014). However, for this method, there are doubts about the partition coefficient determined for highly hydrophobic compounds (K_{ow} > 5.0). In the case of such compounds, these tests require a large volume of the water phase, which can be additionally contaminated during the formation of emulsions by shaking. An alternative method is the "slow stirring" method, which was first introduced by De Bruijn et al. (1989). It is suggested that its use may be more effective, especially in the case of compounds with a higher affinity to the lipid phase (Jabusch and Swackhamer, 2005). Due to the very slow stirring, it is possible to prevent the formation of emulsions during the test, which also means that high partition coefficient values can be easily determined. However, it should be stressed that both of the above mentioned methods have a significant disadvantage, as it is impossible to analyze rapidly biodegradable compounds. Importantly, this problem can be eliminated by using biomimetic chromatography. In this technique, partition coefficients are determined on the basis of retention time, while quantitative analysis is not required. Therefore, potential degradation products appear as separate peaks without affecting the analysis result (Tsopelas et al., 2017).

An alternative way of determining the partitioning between the water and oil phase was invented by Mayer et al. (2009). In this new method, silicone microtubes made of PDMS are used, which in the first stage are placed in the donor phase - oil containing the examined compounds (Oemisch et al., 2013). Once the equilibrium between the donor phase and the PDMS is reached, the tube is filled with water and then the equilibrium between the water and oil phase is determined using the PDMS as a carrier. One of the major advantages of this method is that the water phase and the oil phase are completely separated from each other, which prevents undesirable effects such as dissolving oil in water or *vice versa*.

The affinity to storage lipids can also be determined by experiments either using headspace measurements or solid-phase microextraction (SPME) using fibers with PDMS (Geisler et al., 2012, 2011) or polyacrylate (PA) coating (Oemisch et al., 2013). In the case of both methods, lipids and water are first placed in separate tubes to which tested compounds are added. Once the equilibrium is reached, either direct measurements are performed or, as in the case of SPME, concentrations are assessed after extraction from the solid phase.

3. *In vitro* and *in vivo* – the environmental relevance of laboratory data

It is commonly assumed that the main sorption matrices in organisms are membrane lipids, storage lipids, muscle proteins (structural proteins), blood proteins and water (Bittermann et al., 2018; Endo and Goss, 2011; Goss et al., 2018). In pharmacological studies, it is becoming increasingly popular to take into account the individual components of the body in order to better determine the absorption and distribution of the compound in the organism (Endo et al., 2011). A similar concept is also increasingly being used in environmental research. The introduction of more advanced models will benefit both the prediction of bioconcentration and the identification of bioconcentration mechanisms. At present, however, there are too few data on individual biocomponents, so such predictions can only apply to a limited, narrow group of compounds (Endo et al., 2011). Therefore, it is essential to extend the existing knowledge with data obtained with the help of reliable *in vitro* methods, such as those presented in this paper.

Among the compounds that may accumulate in aquatic organisms, apart from the "classical" pollutants, a wide group of newly emerging compounds with different properties related to bioconcentration should be distinguished. For example, pharmaceuticals and their metabolites are an important group of unregulated, increasingly frequent environmental contaminants (aus der Beek et al., 2016; Ebele et al., 2017; Puckowski et al., 2016; Świacka et al., 2019). These compounds are continuously released into the environment *via* various routes. There are a lot of data on their wide distribution in the environment, although they report low concentrations in surface water, groundwater and drinking water. However, even such low concentrations can cause toxic effects on fauna and flora and damage the endocrine system of living organisms. In addition, pharmaceuticals are referred to as "pseudo-persistent" pollutants because their usually high transformation/purification rates in the environment are compensated by a steady inflow to water bodies, leading to the chronic exposure of organisms (Tsopelas et al., 2017a).

Important group of potential pollutants are ionic liquids, the production and use of which are growing at a very fast rate (Thuy Pham et al., 2010). These next-generation chemicals are becoming popular in various industries as harmless and safe alternatives. Their rapidly increasing use is recorded in sectors related to biotechnology, electrochemistry, chemical engineering and the pharmaceutical industry (Thuy Pham et al., 2010). Although it is believed that ionic liquids are environmentally friendly compounds, in recent years there have been more and more frequent reports contradicting this thesis. It appears that some of them may exhibit significant toxicity to aquatic organisms (Docherty and Kulpa, 2005; Matzke et al., 2007; Stolte et al., 2012, 2007; Thuy Pham et al., 2010; Zhao et al., 2007). Furthermore, there are serious doubts about their bioconcentration potential, since the results obtained so far with the octanol/water partition coefficient may have been significantly underestimated for these compounds (Dołżonek et al., 2017). It is therefore essential to anticipate how these compounds can impact the environment before their use reaches a large scale. Moreover, among the chemical compounds of an ionic character entering the aquatic environment, such groups as surfactants, pesticides, as well as poly- and perfluorinated acids should be distinguished.

The data on the bioconcentration of selected compounds obtained by various methods are presented in Table 1. It is undeniable that both the octanol water partition coefficient and the other methods give

Table 1
Comparison of data on bioconcentration obtained with different methods.

Tested compound	*In vitro*				*In vivo*
	log K_{ow} [L/kg]	Membrane partitioning (log K_{lipw}) [L/kg]	Protein binding [L/kg]	Storage lipids absorption (log K_{tw}) [L/kg]	log BCF [L/kg]
Chlorpyrifos	4.96[1]	na	3.83[a][1]; 4.46[b][1]	na	3.46 [31]
Methoxychlor	5.08[1]	na	4.20[a][1]; 4.94[b][1]	na	na
4-n-nonylphenol	5.76[1]	na	4.80[a][1]; 5.60[b][1]	na	3.08 [32]
Pyrene	4.88[1]	5.60–6.10[8]	3.77[a][1]; 4.92[b][1]	na	5.43–6.63[9–12]
BDE 28	5.94[2]	6.29[3]	5.12[a][3]	na	na
BDE 47	6.81[2]	6.86[3]	5.81[a][3]	na	na
BDE 100	7.24[2]	7.26[3]	5.16[a][3]	na	na
2,2′,5-Trichloro biphenyl	5.48[4]	5.27[4]	na	5.63[4]	na
2,4,4′-Trichloro biphenyl	5.87[4]	5.70[4]	na	6.12[4]	na
2,3′,4,4′,5-Pentachloro biphenyl	6.83[4]	6.56[4]	na	7.09[4]	na
2,2′,3,3′,4,4′,5-Heptachloro biphenyl	7.49[4]	7.44[4]	na	7.92[4]	na
l-Ampicillin	−1.70[5]	2.58[5]	na	na	na
[TEA][Amp]	−1.17[5]	2.96[5]	na	na	na
[N_{1112OH}][Amp]	−0.85[5]	3.05[5]	na	na	na
[C_{16}Pyr][Amp]	−0.52[5]	3.67[5]	na	na	na
[$P_{6,6,6,14}$][Amp]	−0.51[5]	3.68[5]	na	na	na
$[(CF_3SO_2)_2N]^-$	1.50[d][6]	2.50[7]	na	na	na
$[B(CN)_4]^-$	1.86[d][6]	2.33[7]	na	na	na
$[(FSO_2)_2N]^-$	1.51[d][7]	2.03[7]	na	na	na
$[(C_2F_5)_3PF_3]^-$	3.17[d][7]	3.67[7]	na	na	na
Phenanthrene	4.46[15]	4.90–5.42[8]	4.15[a][25]	na	4.24–5.54[9–12]
Benzo[*a*]pyrene	5.98[14]	7.13–7.87[8]	na	na	5.96–7.46[9–12]
Anthracene	4.54[16]	5.20–5.55[8]	na	na	4.44–5.70[9–13]
Fluoranthene	5.12[17]	5.52–5.96[8]	4.28[a][25]	na	5.37–5.90[10,13]
Acetylsalicylic acid	[e]1.1/−2.0[18]	[e]2.4/2.1[18]	na	na	na
Acyclovir	[e]−1.8/−2.0[18]	[e]1.7/2.0[18]	na	na	na
Allopurinol	[e]0.2/−0.9[18]	[e]2.5/2.7[18]	na	na	na
Amiloride	[e]0.1/−0.7[18]	[e]1.8/1.6[18]	na	na	na
Atenolol	[e]0.5/−1.5[18]	[e]2.2/1.0[18]	na	na	≤−0.57[30]
Diclofenac	[e]4.2/0.3[18]	[e]4.3/2.9[18]	2.47[c][24]	na	0.47–3.45[26]
Fluoxetine	[e]4.5/1.5[18]	[e]3.0/2.2[18]	2.04[c][24]	na	0.60–2.19[27]
Ibuprofen	[e]4.0/−0.1[18]	[e]3.8/2.1[18]	3.91[a][23]; 1.48[c][24]	na	1–2.73[28]
Proplanolol	[e]3.4/0.5[18]	[e]3.2/2.5[18]	1.43[a][23]; 1.48[c][24]	na	1–2.26[29]
26DNP	1.22[19]	2.03[20]	na	na	na
34DNP	1.79[19]	3.17[20]	na	na	na
DNPC	1.88[19]	2.34[20]	na	na	na
Dino2terb	3.54[19]	4.10[20]	na	na	na
Dino4terb	3.36[19]	3.81[20]	na	na	na
Dichlorobenzene	3.38[21]	na	3.03[a][25]	3.51[21]	3.51–3.80[22]
Trichlorobenzene	4.14[21]	na	3.60[a][25]	4.19[21]	4.15–4.47[22]
Tetrachlorobenzene	4.60[21]	na	4.21[a][25]	4.68[21]	4.80–5.13[22]
Pentachlorobenzene	5.20[21]	na	na	5.27[21]	5.19–5.36[22]
Benzoic acid	1.87[33]	na	2.23[a][23]	na	na
2-Chlorobenzoic acid	1.98[34]	na	1.84[a][23]	na	na
3-Chlorobenzoic acid	2.68[34]	na	3.22[a][23]; 0.72[c][24]	na	na
4-Chlorobenzoic acid	2.65[34]	na	3.21[a][23]; 0.72[c][24]	na	na
3,4-Dichlorobenzoic acid	3.25[35]	na	4.06[a][23]; 1.31 [c][24]	na	na
2,6-Dichlorobenzoic acid	na	na	1.65[a][23]	na	na
4-Fluorobenzoic acid	na	na	2.84[a][23]	na	na
4-Nitrobenzoic acid	na	na	2.66[a][23]	na	na
4-Bromobenzoic acid	na	na	3.48[a][23]; 0.91[c][24]	na	na
4-Methylbenzoic acid	2.36[34]	na	2.67[a][23]	na	na
2-Methylbenzoic acid	2.18[34]	na	1.99[a][23]	na	na
4-Ethylbenzoic acid	na	na	3.03[a][23]	na	na
4-Butylbenzoic acid	3.97[35]	na	3.73[a][23]; 1.39[c][24]	na	na
4-Hexylbenzoic acid	na	na	4.23[a][23]; 2.17[c][24]	na	na
2,4,6-Trimethylbenzoic acid	na	na	2.26[a][23]	na	na
2-Cyclohexylbenzoic acid	na	na	3.55[a][23]; 1.14[c][24]	na	na
2-Naphthoic acid	na	na	4.36[a][23]; 1.14[c][24]	na	na
2-Naphthaleneacetic acid	na	na	4.77[a][23]; 1.09[c][24]	na	na
1-naphthoic acid	na	na	2.81[a][23]; 0.87[c][24]	na	na
1-Naphthaleneacetic acid	na	na	3.43[a][23]; 0.98[c][24]	na	na
4-Fluoro-1-naphthoic acid	na	na	3.46[a][23]; 1.00[c][24]	na	na
1-Bromo-2-naphthoic acid	na	na	4.02[a][23]; 1.19[c][24]	na	na
6-Bromo-2-naphthoic acid	na	na	1.89[c][24]	na	na
2-Methoxy-1-naphthoic acid	na	na	2.16[a][23]; 0.70[c][24]	na	na
2-Ethoxy-1-naphthoic acid	na	na	2.66[a][23]; 0.74[c][24]	na	na
3-Methoxy-2-naphthoic acid	na	na	2.86[a][23]; 0.85[c][24]	na	na
2-Phenoxyacetic acid	na	na	2.53[a][23]	na	na
2,4-Dichlorophenoxyacetic acid	na	na	3,28[a][23]	na	na
4-(2,4-Dichlorophenoxy)butyric acid	na	na	4.12[a][23]; 1.53[c][24]	na	na

(continued on next page)

Table 1 (*continued*)

Tested compound	*In vitro*				*In vivo*
	log K_{ow} [L/kg]	Membrane partitioning (log K_{lipw}) [L/kg]	Protein binding [L/kg]	Storage lipids absorption (log K_{tw}) [L/kg]	log BCF [L/kg]
2,4,5-Trichlorophenoxyacetic acid	na	na	3.83[a][23]; 1.61[c][24]	na	na
Mecoprop	3.13[35]	na	3.74[a][23] 1.17[c][24]	na	na
Ketoprofen	3.12[35]	na	3.31[a][23]; 1.21[c][24]	na	na
Fenoprofen	3.1[35]	na	3.92[a][23]; 1.62	na	na
α,4-Dimethylphenylacetic acid	na	na	3.00[a][23]	na	na
n-Phenylanthranilic acid	na	na	1.86[c][24]	na	na
Flufenamic acid	5.25[35]	na	2.40[c][24]	na	na
Mefenamic acid	5.12[35]	na	2.40[c][24]	na	na
2,3,5,6-Tetrachlorophenol	na	na	2.63[c][24];	na	na
Pentachlorophenol	na	na	5.27[a][23]; 3.36[c][24]	na	na
Bromoxynil	2.70[35]	na	5.18[a][23]; 1.74[c][24]	na	na
Coumachlor	na	na	3.41[a][23]; 2.21 [c][24]	na	na
Coumafuryl	na	na	2.84[a][23]; 1.14[c][24]	na	na
Mefenamic acid	5.12[35]	na	4.36[a][23]	na	na
Sulcotrione	na	na	1.72[a][23]	na	na
4-Ethylbenzenesulfonate	na	na	3.17[a][23]	na	na
4-n-octylbenzenesulfonate	na	na	4.84[a][23]; 2.81[c][24]	na	na
2,4,6-Trimethylbenzenesulfonate	na	na	4.23[a][23]; 0.72 [c][24]	na	na
4-Bromobenzenesulfonate	na	na	3.32[a][23]; 0.86[c][24]	na	na
Naphthalene-2-sulfonate	na	na	4.71[a][23]; 1.03[c][24]	na	na
Alprenolol	3.10[35]	na	1.02[a][23]; 1.17[c][24]	na	na
Imipramine	4.80[35]	na	1.58[a][23]; 1.83[c][24]	na	na
Verapamil	3.79[35]	na	0.97[a][23]; 1.54[c][24]	na	na
Difenzoquat	<−1.00[36]	na	0.92[c][24]	na	na
Benzyltributylammonium	na	na	0.89[c][24]	na	na
Benzyldimethyloctylammonium	na	na	1.44[c][24]	na	na
Tetraphenylphosphonium	na	na	1.80[c][24]	na	na

na – not available.

Definitions to the acronyms: BDE 28: 2,4,4′-tribromodiphenyl ether, BDE 47: 2,2′,4,4′-tetrabromodiphenyl ether, BDE 100: 2,2′,4,4′,6-pentabro-modiphenyl ether, [TEA][Amp]: Tetraethylamonium ampicillin, [N_{1112OH}][Amp]: Choline ampicillin, [C_{16}Pyr][Amp]: Cetylpyridinium ampicillin, [$P_{6,6,6,14}$][Amp]: Trihexyltetradecylphosphonium ampicillin, [$(CF_3SO_2)_2N$]$^-$: bis(trifluoromethylsulfonyl)imide, [B(CN)$_4$]$^-$: tetracyanoborate, [(FSO2)2 N]$^-$: bis(fluorosulfonyl)imide, [(C2F5)3PF3]$^-$: tris(perfluoroalkyl) trifluorophosphate, 26DNP: 2,6-dinitrophenol, 34DNP: 3,4-dinitrophenol, DNPC: 4-methyl-2,6-dinitrophenol, Dino2terb: 2-tert-Butyl-4,6-dinitrophenol, Dino4terb: 4-tert-Butyl-2,6-dinitrophenol Dino4terb.

References: [1] Escher et al., 2011; [2] Braekevelt et al., 2003; [3] Endo et al., 2013; [4] Quinn et al., 2014; [5] Florindo et al., 2013; [6] Cho et al., 2014; [7] Dołżonek et al., 2017; [8] Van Der Heijden and Jonker, 2009; [9] Landrum, 1988; [10] Muijs and Jonker, 2009; [11] Petersen and Kristensen, 1998; [12] Van Hattum and Cid Montanes, 1999; [13] Boese et al., 1999; [14] Paterson et al., 1990; [15] Chefetz, 2003; [16] Onken and Traina, 1997; [17] Brannon et al., 1995; [18] Balon et al., 1999; [19] Escher and Schwarzenbach, 1996; [20] Escher et al., 2000b; [21] Chiou, 1985; [22] Oliver and Niimi, 1983; [23] Henneberger et al., 2016a; [24] Henneberger et al., 2016b; [25] Endo et al., 2012; [26] Świacka et al., 2019; [27] Silva et al., 2016; [28] Contardo-Jara et al., 2011; [29] Ericson et al., 2010; [30] Steinbach et al., 2014; [31] EURAS bioconcentration factor (BCF) gold standard database; [32] Ekelund et al., 1990; [33] Qiao et al., 2008; [34] Ming et al., 2009; [35] PubChem [36] Pohanish, 2017

[a] Partitioning to bovine serum albumin
[b] Partitioning to blood protein
[c] Partitioning to muscle protein
[d] Ha – anionic hydrophobicity
[e] Neutral form/ionic form

very similar results in the case of non-polar and nonionic compounds. This should not be surprising, as the main factor responsible for the bioconcentration of such compounds is the hydrophobic effect. On the other hand, the presented results clearly show how large the discrepancies can be between the estimations obtained with the octanol-water partition coefficient and those obtained with the use of more advanced models. This is particularly important for ionic compounds, as can be seen in several pharmaceuticals. While in their neutral form they show a similar affinity to octanol and membrane lipids, their ionized forms behave very differently, not partitioning at all to octanol, but to a large extent to liposomes. For example, ionized forms of diclofenac and ibuprofen, one of the most commonly used non-steroidal anti-inflammatory drugs, reach a log K_{ow} close to 0, while a log K_{lipw} as high as 2.9 and 2.1, respectively. A similar situation was also observed in the case of ionic liquids based on amicillin - their affinity to octanol is negligible, which could suggest a lack of bioconcentration potential. It turns out, however, that the log K_{lipw} reaches values even above 3 for these compounds, which suggests a significant affinity to cell membranes. The table also presents data on the observed log BCF values in experiments with marine animals. However, due to the small amount of data and the large discrepancy in the BCF values between species, the applied concentrations and the exposure time, a direct comparison of *in vitro* and *in vivo* values is difficult and imprecise. Nonetheless, it should be stressed that a significant number of compounds, especially ionic ones, show a higher bioconcentration potential than previous estimates suggested.

In general, there is a growing consensus that non-lipid related phenomena may increase the intensity of bioconcentration of chemical compounds above the values predicted on the basis of the K_{ow} (Henneberger et al., 2016a, 2016b; Endo et al., 2012, 2013). However, current assessment criteria in screening studies often neglect compounds that are involved in such processes, and the risks associated with their application are not considered. Interestingly, some scientists suggested that although accumulation may occur through binding to proteins, this process may be sufficiently anticipated by the determination of the K_{ow} (Treu et al., 2015). This means, therefore, that from a total bioconcentration perspective, binding to proteins does not provide additional information beyond what can be obtained from the K_{ow}. However, such far-reaching conclusions appear to be insufficiently confirmed (Endo and Goss, 2011) and questionable, especially in the case of many inaccuracies related to the K_{ow} itself. In fact, current research supports the opposite thesis, that for ionic compounds, which account for a large proportion of the pollutants, binding to proteins may be the main route of bioconcentration (Endo and Goss, 2011; Goss et al., 2018), Therefore, there is no doubt that testing with proteins requires much more

attention. Furthermore, it should be stressed that information on protein binding is valuable in the framework of improving the knowledge of different mechanisms of absorption.

On the other hand, some studies have suggested that there is a limit value for the K_{ow} above which there is no further increase or even decrease in the K_{lipw}. An attempt was made to explain this by the large amount of free energy needed to partition to the membrane and, as a result, the non-linear increase in this parameter, as opposed to the K_{ow}. Current research suggests, however, that the supposed limit value may be largely explained by experimental artifacts, *e.g.* the lack of equilibrium during an experiment or the overestimation of the concentration in the aqueous phase. These doubts make it necessary to treat the literature data on the K_{lipw} with caution. Among the factors influencing the K_{lipw}, the fluidity of the membrane should be taken into account. When heated above the transition temperature (T_c), the membrane changes from a gel phase with low fluidity to a liquid crystalline phase with high fluidity. Under natural conditions, biological membranes are generally in a liquid crystalline state, due to the high content of unsaturated chains. Cooling below T_c causes a drastic, often multiple, decrease in the K_{lipw} value. Also, at a constant temperature, membranes in a gel state show much lower values, even several tens of times lower than for liquid crystal membranes. Therefore, the results which are representative apply to membranes such as those in natural conditions, *i.e.* above T_c, in the liquid crystal state (Endo et al., 2013). Among the tests carried out at temperatures above T_c, the K_{lipw} values for different PC types are within a narrow range, and the differences between them are small. Nonetheless, if lipids other than PC are used, different membrane behavior is observed. The inclusion of cholesterol reduces the partitioning into membranes and this effect increases with increasing cholesterol content. Partition coefficients for liposomes containing 10–50% cholesterol can be up to 10 times lower than for liposomes without cholesterol (Endo et al., 2011). The cholesterol content affects the fluidity of the membrane, reducing it, which leads to lower K_{lipw} values. Thus, given how common cholesterol is in eukaryotic membranes, and how much it represents, its impact on the K_{lipw} may be significant and should be taken into account. Other types of membrane lipids may also affect the partition coefficient, but to a lesser extent than cholesterol. However, it should be emphasized that the K_{lipw} of ionic compounds is significantly modified by the presence of charged lipids in the membranes.

It should be noted that in the case of research on the accumulation of compounds in membrane lipids, the temperature at which the measurements are made is an important parameter. As mentioned earlier, in the case of experiments using membrane lipids, it is essential to perform tests above T_c when phospholipids are in a crystalline liquid state. On the other hand, the partition coefficients of storage lipids and protein binding are usually measured at 37 °C, as a reference to humans and other mammals, or around 20 °C for convenient experimentation. However, in order to determine the bioconcentration in fish and other aquatic organisms, it is necessary to conduct such experiments at lower temperatures. Given the variability of temperature in the environment, it is necessary to create a model determining the dependence of the bioconcentration potential in different biomolecules on the temperature.

Nevertheless, in the case of estimating bioconcentration, the least doubts are raised by storage lipids, which, due to their structure, have a simple absorption mechanism based on hydrophobic properties. In Geisler et al. (2012), it was shown that the values for all tested chemicals, including non-polar species, as well as H-bond donors and H-bond acceptors, usually differ by no >0.1 logarithmic unit between the different analyzed storage lipids. Moreover, no trend was observed between them. Therefore, it was concluded that all available data indicate that there are no significant differences between the different storage lipids in terms of the accumulation capacity. In addition, it has been shown that, in contrast to membrane lipids, the temperature is negligible in the case of storage lipids. Since the lipids used in Geisler et al. (2012) present a wide variety in terms of fat chain composition, it can be assumed that these conclusions may be true for all storage lipids.

3.1. Bioconcentration assessment - suggested steps to obtain reliable data

A credible estimation of the problem of bioconcentration remains a major challenge. Although numerous models exist, their accuracy remains a questionable issue, especially for ionic and ionogenic compounds, which account for a large part of the pollution already recorded in the environment or potentially present in the future. Therefore, we would like to propose a comprehensive approach for reviewing existing incomplete data and making much more accurate predictions, including for charged species (Fig. 6). As a first step, the data set on absorption in different components of organisms should be updated. Only a combination of data obtained with proteins (both blood and structural), membrane lipids and storage lipids will allow a realistic estimation of this phenomenon (Endo and Goss, 2011). In addition, this approach will allow for a preliminary determination of the internal tissue distribution.

Although accurate recognition of the absorption process is a key element in the context of bioconcentration, a full understanding of this phenomenon also requires an understanding of metabolic processes. Therefore, in the next step, data should be complemented by experiments to determine whether and to what extent given compounds undergo biotransformation. With this data set, it will be possible to confirm the reliability of previous estimations using *in vivo* studies. This will help to confirm the laboratory results by using living organisms. In addition, it will be possible at this stage to correct any inaccuracies that may arise. Finally, with a sufficient amount of reliable and

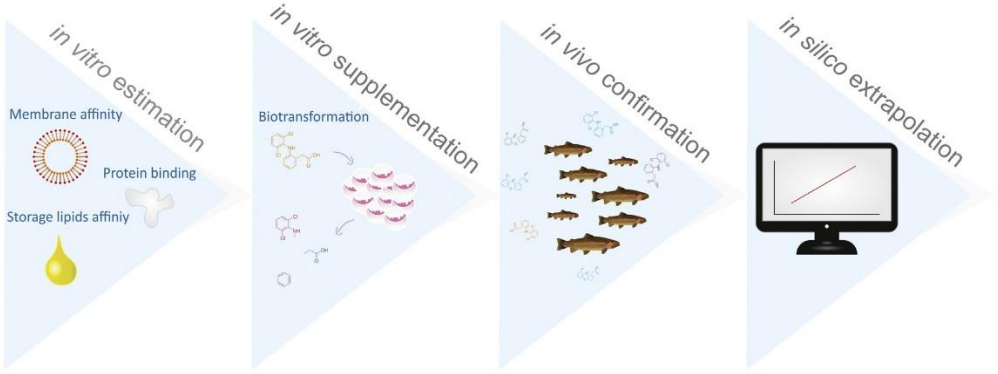

Fig. 6. Proposed research approach to obtaining reliable data predicting bioconcentration of chemical compounds.

confirmed data, it will be possible to create reliable models that will replace the existing ones that are not effective enough.

4. Conclusions

The accumulation of environmental pollutants in aquatic organisms is an issue that has received increasing attention in recent years. Although previous attempts to estimate this phenomenon have been ineffective, there are now numerous tools to adequately assess the bioconcentration potential without the use of animals. This is extremely important given the rapidly growing number of diverse compounds that could potentially enter the environment. Using the methods presented in this paper more widely, and replacing obsolete solutions such as the K_{ow} may allow for a better understanding of the processes involved in bioconcentration and the complete elimination or radical reduction of *in vivo* experiments. If a sufficient amount of reliable data can be obtained, in the future it will be possible to create models that, unlike the current ones, will accurately estimate the risks associated with pollution regarding ionic and ionogenic compounds.

Declaration of competing interest

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

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Review

Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments

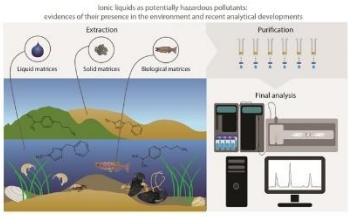
Jakub Maculewicz [a,*], Klaudia Świacka [b], Piotr Stepnowski [a], Joanna Dołżonek [a], Anna Białk-Bielińska [a]

[a] Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland
[b] Department of Marine Ecosystems Functioning, Institute of Oceanography, University of Gdansk, Av. Piłsudskiego 46, 81-378 Gdynia, Poland

HIGHLIGHTS

- ILs are beginning to be detected in different environmental matrices.
- The most commonly studied ILs are the ammonium compounds.
- The number of studies on the analysis of ILs in environmental matrices is limited.
- Reliable methods for the determination of ILs in environmental matrices are needed.

GRAPHICAL ABSTRACT



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ABSTRACT

Ionic liquids (ILs) are considered to be very promising group of chemicals and the number of their potential applications is growing rapidly. However, while these compounds were originally proposed as a green alternative to classical solvents, there are certain doubts as to whether this classification is correct. Although in recent years there have been first reports published proving the presence of some ILs in the environment and even in human blood, at this point the scale of this possible problem is not yet fully understood. However, there is no doubt that as the number of ILs applications increases, analytical capabilities for rapid detection of possible environmental contamination should be also considered. Therefore, in this review paper, recent evidences for the ILs environmental contamination as well as analytical achievements related to the extraction of ILs from various environmental matrices have been summarized and important gaps and future perspectives have been pointed out. Based on the presented data it might be concluded that there is the urgent need for further development towards risk assessment of these potential environmental contaminants.

1. Introduction

Since the beginning of the 21st century, ionic liquids (ILs) have gained immense popularity in the scientific community, providing a promising alterative to many classical approaches in chemistry. As defined, ILs are salts that reach a liquid state already at room temperatures (Beil et al., 2021; Guo et al., 2015). Their structure is usually based on a complex organic cation combined with an inorganic or organic anion (Guo et al., 2015). Among many different properties that have made ILs so popular is extremely low vapour pressure, which

* Corresponding author.
E-mail address: jakub.maculewicz@phdstud.ug.edu.pl (J. Maculewicz).

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places them as an excellent alternative for classical volatile solvents. Their important characteristics also include low viscosity, high thermal stability range, as well as non-flammability (Singh and Savoy, 2020). Thanks to their unique properties, which can additionally be modified and customised in a very simple way, they find many applications in many branches of modern chemistry. For example, ILs in the recent years have been proposed as: efficient biomass extractants (Tan et al., 2020), additives for hybrid electrolytes for lithium metal batteries (Yang et al., 2020), sustainable corrosion inhibitors (Verma et al., 2021), additives for lubricants (Oulego et al., 2018), CO_2 absorbents, (Aghaie et al., 2018), polymers used as sorbents in Solid-Phase Extraction (SPE) technique (Liu et al., 2021) or a medium for the preparation of biopolymers enabling gene or drug delivery into tissues (Chen et al., 2018).

ILs, since gaining popularity, have been promoted as green and safe compounds, although their potential environmental impact is still the subject of much scientific debate and raises numerous concerns (Cvjetko Bubalo et al., 2014; Amde et al., 2015; Kowalska et al., 2021). An important issue in this context is the toxicity of ILs, which, according to many reports, can be significant, both for microorganisms such as bacteria and algae and for aquatic invertebrates or fish (Bubalo et al., 2017; Thuy Pham et al., 2010). Importantly, although there is an extensive amount of data on the toxicity of ILs available in the scientific literature, detailed information on the exact mechanisms responsible for their effects on organisms is still lacking (Costa et al., 2017). According to recent reports, some ILs may also show a high potential for bioconcentration. Recent works have demonstrated that these compounds exhibit a high affinity for lipid membranes and blood proteins, which may indicate their potential bioconcentration in organisms (Dołżonek et al., 2017; Kowalska et al., 2022). Another important issue is the resistance of selected ILs to biodegradation as well as to hydrolysis, which in the context of possible environmental pollution (Amde et al., 2015) are an undesirable properties. A recent paper by Bystrzanowska et al. (2019) analysed a set of 319 ILs for their "greenness", taking into account various criteria, including biodegradability and toxicity. The analyses showed that tested ILs can be positioned between non-polar and polar classical solvents. However, due to the limited amount of data it is still impossible to prepare fully conclusive assessments related to the safety and greenness of ILs. Nonetheless, existing evidence of their presence in the environment indicate that ILs should not be consider as "potential" pollutants anymore. There is no doubt that, when talking about a real, already existing threat, ammonium compounds should be mentioned first. For several years up to now, these compounds have been regularly observed both in aqueous samples and in food products (e.g., Bertuzzi and Pietri, 2014; Hepperle et al., 2014; Friedle et al., 2012; Xiang et al., 2015; Ferrer and Furlong, 2002; Marc Wieland et al., 2019). Their widespread use, especially in surfactants and disinfectants, makes this contamination very difficult to avoid. For example, (Pati and Arnold, 2020) detected total concentrations of QACs in wastewater effluents at several μg/L. Similarly, Li and Brownawell (2009) detected these compounds in estuarine sediment, with concentrations reaching up to 74 μg/L.

Even more worryingly, according to recent reports, these compounds show high affinity for human blood proteins, which may result in their bioaccumulation in the body. The phenomenon, which has so far been mainly suggested by in vitro and *in silico* tests, was confirmed in the work of (Zheng et al., 2021), which detected the presence of 15 different QACs in human blood samples collected in China. The concentration of these compounds reached a maximum of 68.6 ng/mL. The samples were collected in 2018 and in 2020. The concentration of QACs in samples collected in 2020 was statistically significantly higher, which may indicate an increase in human exposure to these compounds. Also in the work of (Hrubec et al., 2021), QACs were detected in human blood. In a study involving 43 volunteers, these compounds were detected in up to 80 % of them, confirming the magnitude of this hitherto rarely addressed problem. Furthermore, this study shows for the first time that inflammatory markers, mitochondrial function, and cholesterol synthesis intermediaries are altered in a dose-dependent manner with QACs concentration. Such adverse effects on humans indicate that these compounds should be approached with a lot of caution. It is also worth remembering that, according to current research, QACs show many negative effects not only in humans, but also in diverse aquatic and soil organisms (Zhang et al., 2015). Importantly, as shown in the work of (Di Nica et al., 2017), QACs can exhibit synergistic toxic effects on organisms, that in some cases can be seen at potentially environmentally relevant concentrations. Moreover, their presence in the environment has been linked to an increase in antibiotic resistance in bacteria, which could be another major problem (Mulder et al., 2018). In light of these findings, there is no doubt that special attention should be given to QACs, the spread of which in the environment and hence human exposure to it can be a serious problem.

Importantly, it appears that not only ammonium cations are already present in the environment and even in human tissues. Recent reports indicate that this problem may also apply to imidazolium ILs, for which this was not yet expected. The work of Leitch et al. (2021) showed that 1-octyl-3-methyl imidazolium cation was present in 5/20 patients with autoimmune liver disease primary biliary cholangitis (PBC) and in 1/20 control patients. These results, which were also confirmed by studies on hepatocytes (Leitch et al., 2020), suggest that humans may be exposed to this compound, but also it may be a cause of PBC. Also in the work of Probert et al. (2018) it was shown that the same compound could be a present environmental cause of PBC. In a screening study of soil samples surrounding a landfill waste, it was shown that samples exhibiting hepatotoxicity contained high concentrations of 1-octyl-3-methyl imidazolium cation and this compound was responsible for the toxic effect. Furthermore, the study by Probert et al. (2018) suggests that also other imidazolium ILs with similar structures may show similar effects.

When referring to ILs, it should be remembered that they are not only complex organic cations, but also an enormous wealth of anions. In the context of the potential environmental pollutants analysis, however, the subject of anions remains poorly studied. This is undoubtedly a gap in the knowledge, especially in light of the reports by Neuwald et al. (2020), who encountered a perfluorinated ILs anion, namely tris(pentafluoroethyl)trifluorophosphate, during a screening search for polyfluoroalkyl contaminants in river water samples. Of the 20 different surface reservoirs sampled, this compound was detected in three connected rivers, suggesting rather local contamination. The maximum concentration of tris(pentafluoroethyl)trifluorophosphate in these samples was 3.4 μg/L. In this context, a completely new light is shed on the magnitude of ILs pollution risk by the recent work by Neuwald et al. (2021), which screened two river systems in Germany for 1310 potentially persistent and mobile contaminants. In this work, as many as 20 compounds belonging to ILs, both anions and cations, were detected in the rivers. Moreover, of the detected compounds, 7 were perfluorinated, confirming that these types of compounds also require special attention for ILs. Importantly, both the detection frequency and concentration (up to tens of μg/L) were significantly higher for anions than for cations, which may be related to the lower diversity of anions used and their higher persistence and mobility compared to cations. Although these are some of the first research works to detect ILs anions in the environment, they should be the start of a discussion as whether these compounds need to be included in larger-scale studies.

Interestingly, while there is still an increase in scientific interest in ILs, it is difficult to find such reflection in the number of papers focused on the analytical methods development for their analysis in environmental matrices. Although the number of studies on the use of ILs reaches thousands per year, those relating to their analysis in the context of potential contamination have so far been only a few tens (Fig. S1).

Moreover, although interest in the use of ILs is reaching enormous levels, this is not yet adequately reflected in the industrial production of these compounds. However, it is estimated that their practical application will only reach the expected level in the coming years (Kalb, 2020). Therefore, being aware of concerns regarding the potential

environmental fate of ILs (as new and potentially hazardous environmental pollutants) it must be remembered, that the real dimension of this problem is yet to come. Consequently, it is worth considering to what extent we are analytically prepared to monitor and detect possible contamination by ILs in diverse environmental matrices.

Therefore, the main objective of this paper was to summarise the achievements in the field of analysis of ILs (as potential environmental pollutants) in the environmental matrices as well as to summarize already published evidence for their presence in the different samples, proving that the presence of ILs in the environment is inevitable in the coming years. Although there has been numerous review papers published concerning the application of ILs, to our knowledge, this is the first such review considering the extraction, purification and instrumental analysis of ILs in the environmental samples. Due to the wide range of matrices in which contamination by ionic liquids can be expected, we have divided the collected data into three main groups: aqueous environmental matrices, solid environmental matrices such as soils or sediments, and biological matrices, i.e. animal and plant tissues including food products. Furthermore, this work aims to identify important knowledge gaps and the direction in which techniques for the analysis of ILs, as potential environmental contaminants, should be developed.

2. Material and methods

In order to acquire the literature data to prepare this review, a search for publications was conducted between November 2021 and February 2022. Publicly available databases of scientific articles were used, especially Google Scholar, Science Direct and Scopus. Various combinations of keywords directly relevant to the main topic were used to search these databases, including words 'ionic liquids' and 'extraction' or 'analysis' but also the names of individual groups of compounds (e.g., imidazolium, ammonium, phosphonium, pyridinium) or single compounds' names in combination with types of matrices, e.g. 'water, 'soil, 'food'. This resulted in formulation of three-level keywords based on the scheme "Search = group of compounds + type of matrix + extraction". The selection of articles for inclusion in this work was done by evaluating their relevance to the main topic. The preparation of novel extraction method for analysis of ILs was a key factor in the inclusion of data in this work. In total, 45 articles concerning presence of ILs in the environment and the extraction methods for their analysis were selected for use in this study. The data were complemented by 20 articles concerning general information regarding ILs.

As there are different approaches to classifying ILs, in some cases it can be difficult to clearly assess which compound should be included in this group. This situation is particularly common for ammonium cations used in surfactants, which are often classified as benzalkonium chlorides (BACs) or quaternary ammonium cations (QACs) and are not included in ILs. For the purpose of this work, it was decided to include all these compounds in the review. It was justified due to their structural similarity, which allows their joint evaluation in the context of extraction from different environmental matrices.

3. Extraction of ILs from aqueous environmental matrices

Based on the performed literature study it might be concluded that the number of studies concerning the analysis of ILs in aqueous environmental samples is dominant, which mainly results from the fact that extraction of compounds from water is in general much faster and less complicated than from solid or biological matrices. Available publications on this topic have been summarized in the Table 1. It should be also noted that almost all of the existing articles deal with individual cation groups. In addition, apart from the very abundantly analysed ammonium ILs, the remaining groups of ILs appear in only an individual papers.

Moreover, most of the available studies deal with the extraction of ILs from surface waters, both fresh and marine. However, a little attention is given to the extraction of these compounds from wastewater, where possible contamination would be expected in the first place. This, most probably, results from the fact that wastewater represents a much more complex matrix, so the extraction and analysis of ILs from such samples is much more complicated. Therefore, it is clear is that this subject needs more attention.

Among the presented and proposed approaches for the extraction of ILs from aqueous matrices (Table 1), the most commonly applied technique is classical SPE, which allows the concentration of analytes while removing some of the matrix interferents from the samples. For ILs cations, two types of sorbents are mainly used - strong cation exchangers (Van De Voorde et al., 2012; Liu et al., 2021; Zhang et al., 2019a,b) or hydrophobic sorbents (C18 or polymeric) (Fan and Yu, 2018; Stepnowski and Nichthauser, 2008; Ding and Liao, 2001), with quite satisfactory recoveries - usually higher than 50 %. The structure of ionic liquid cations, in which the charged core is usually surrounded by a hydrophobic chain (or chains), allows for a wide choice of sorbents based on both electrostatic and hydrophobic interactions.

When analyzing high salinity water samples, it may be advantageous to prefer sorbents based on hydrophobic interactions. Such sorbents do not exhibit retention of inorganic salts, allowing a large fraction of potential interferents to be quickly removed. On the other hand, for aqueous samples rich in non-ionic interferents (e.g., wastewater samples), it may be more advantageous to use cationites, which will adsorb these interferents to a lesser extent. Since ILs are permanently charged compounds, the pH of the samples analyzed does not directly affect their recovery. However, some SPE sorbents have ionizable functional groups where it may be beneficial to adjust the sample pH prior to sample loading. However, it must be mentioned that in all of the work discussed here, elution of ILs from the SPE cartridges was possible using pure MeOH, which allows for easy concentration of the samples after purification.

It should be also noted that some authors proposed an alternative to SPE by extracting ILs from water using the Solid-Phase Microextraction (SPME) technique with different coatings (Boyacı et al., 2014; Chen et al., 2012). This approach was characterized with much wider range of the calculated recoveries of ILs: from very low values of 13 % for the most lipophilic compounds up to very good recoveries of 96 % for less lipophilic ones. The reason for that was adsorption of analytes on laboratory instruments - pipette tips, extraction plate and sample bottle. As pointed out by the authors, the problems observed in the present method should not occur in classical approaches to sample preparation methods, such as LLE, in which regardless of secondary interactions of the bound/free part, all analytes are extracted from the sample and adsorption losses are washed off the walls. This in general highlights the fact, that this technique cannot be recommended for routinely applied for such analysis yet.

Nevertheless, it was observed that the most often applied technique for the analysis of ILs in the aqueous samples is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). These analyses are performed in the reversed phased by applying in many cases the C18 column and mobile phases consisting of water with the addition of ammonium acetate/formate and/or acetic/formic acid and organic modifiers such as acetonitrile (ACN) or methanol (MeOH). These methods are in general characterized by very low limits of detection (LOD) and quantification (LOQ) of few ng/L up to μg/L (Bassarab et al., 2011; Boyacı et al., 2014; Chen et al., 2012).

Very efficient approach for the determination of ILs (together with other suspected environmental pollutants) in the aqueous environmental samples was proposed in the study by Neuwald et al. (2021). It was based on the application of suspect screening based on the use of hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC), both coupled with high-resolution mass spectrometry (HRMS). The solution used in this work fits into the latest trends in analytical chemistry, and sheds new light on the analysis of

Table 1
Comparison of methods used for extraction, purification and analysis of ILs from aqueous environmental matrices.

Compounds analysed	Matrix	Concentration found in the environmental samples	Concentration and purification	Final analysis	Recovery [%]	LOQ/ LOD	Reference
Morpholinium cations	lake and river water	NA	SPE – UF-SCX	IC; A Shodex IC YK-421 column; mobile phase: 3 mM methane sulphonate acid and ACN	75 – 98	1.13 – 1.70 mg/L [LOQ]	Zhang et al. (2019a,b)
Ammonium cations	river water	NA	SPE – strong cation exchanger	IC; Column: Shodex IC YK-421; Mobile phase: 4 mM methanesulfonic acid (in water) / ACN	86–102	0.3 – 2.1 mg/ L [LOQ]	Liu et al. (2021)
Pyrolidinium cations	water	NA	SPE - C18	IC; Column: Baseline WCX; Mobile phase: 1 mM methanesulfonic acid (in water) / ACN	74–97	0.06 – 0.16 mg/L [LOQ]	Liu et al. (2020)
Piperidinium cations	river water	NA	SPE – C18	HILIC-IUV; Column: TSK-GEL Amide-80 HR; Mobile phase: 1 mM 1-ethyl-3-methyl imidazolium trifluoroacetate aqueous solution/ACN	80 – 98	0.73 – 1.20 mg/L [LOQ]	Fan et al. (2018)
Ammonium cations	river water	NA	SPE - C18	HILIC-IUV; Column: TSK-GEL Amide-80 HR; Mobile phase: 1 mM 1-ethyl-3-methyl imidazolium trifluoroacetate aqueous solution/ACN	61 – 96	0.31 – 0.84 mg/L [LOQ]	(Fan and Yu, 2018)
Ammonium and phosphonium cation	water	NA	SPE – UF-SCX	IC; A Shodex IC YK-421 column; mobile phase: 3 mM methane sulfonate acid and ACN	94–103	0.448 – 0.502 mg/L [LOD]	Liu and Yu (2016)
Ammonium cations	seawater	didecyldimethylammonium chloride 130–195 ng/L	SPE – Strata-X	LC-MS/MS; Column: C18 Xterra MS; Mobile phase: ACN with 1 % acetic acid; 50 mM ammonium acetate	83–115	300 ng/ L [LOQ]	Bassarab et al. (2011)
Ammonium cations	water	NA	Thin-film SPME with HLB particles	LC-MS/MS; Column: Gemini-NX C18; Mobile phase: A - 10 mM ammonium acetate in ACN/ water; B - 0.1 % formic acid in isopropyl alcohol	13–96	0.05 – 1 ng/mL [LOQ]	Boyacı et al. (2014)
Ammonium cations	water	NA	polyacrylate coated SPME fibres	LC-MS/MS; Column: Luna C18 (2); mobile phase: acidic 90/10 MeOH–water mixture	NA	100 ng/ L [LOQ]	Chen et al. (2012)
Ammonium cations	effluent from hospitals	mixture of benzalkonium chlorides 1.90 mg/L	SPE – C18	HPLC with fluorescence detector; Column: CN–NH_2; Mobile phase: chloroform – MeOH	87–94	50 ug/L [LOD]	Kümmerer et al. (1997)
Ammonium cations	water	C_{12} BAC 1.22 – 5.82 μg/L C_{14} BAC 2.38 – 36.6 μg/L	hollow fibre (HF) liquid-phase microextraction (LPME)	LC-MS; Column: HyPURITY ADVANCE; Mobile phase: A – water with 15 % ACN and 0.5 % anhydrous acetic acid; B – 0.5 % acetic acid in ACN	NA	900 ng/ L [LOD]	Hultgren et al. (2009)
Ammonium cations	wastewater	NA	SPE – C18,	LC-MS/MS; Column: C18; Mobile phase: ACN and 10 mM ammonium formate buffer,	71–78	3 ng/L [LOD]	Ferrer and Furlong (2001)
Ammonium cations	sewage and river water	C_{12} BAC 9.7 – 49 μg/L C_{14} BAC 0.14 – 36 μg/L C_{16} BAC 0.99 – 9.5 μg/L	SPE – Bond Elut Jr	LC-MS/MS; Column: Nova Pack C8; Mobile phase: MeOH and 50 mM ammonium formate buffer	54–75	3 ng/L [LOD]	Merino et al. (2003)
Ammonium cations	water	1.2 – 3.7 ng/L	Polymer-monolith microextraction with poly(MAA-co-EDMA) monolithic capillary column	LC-MS/MS; Column: homemade OSS column; Mobile phase: ACN/10 mM ammonium formate (80/20, pH 2.5).	84–115	50 – 80 ng/L [LOQ]	Peng et al. (2011)
Ammonium cations	urban stormwater	C_{12} and C_{14} BAC up to 105 mg/L	SPE – Strata X-CW	LC-MS/MS; Column: Aquity UPLC BEH C18; mobile phase: water and MeOH solution with 0.1 % formic acid in each solvent.	37–77	0.06 ng/L [LOQ]	Van De Voorde et al. (2012)
Pyridinium cations	river water	NA	SPE – UF-SCX	IC; column: Shodex IC YK-421; Mobile phase: 3 mM methane sulphonic acid; 8% ACN.	99–100	0.52 – 1.71 mg/L [LOQ]	Zhang et al. (2019)
Imidazolium and pyridinium cations	seawater and freshwater	NA	SPE – Chromabond® C18	HPLC-UV/VIS; Column: Gemini C6-Phenyl; Mobile phase: MeOH or ACN at concentrations from 1 % to 3 % mixed with 5 mM phosphate buffer (KH2PO4/ H3PO4) adjusted to pH 3.	70 – 102	0.02 – 0.1 mg/ L [LOQ]	Stepnowski and Nichthauser (2008)

(continued on next page)

Table 1 (*continued*)

Compounds analysed	Matrix	Concentration found in the environmental samples	Concentration and purification	Final analysis	Recovery [%]	LOQ/ LOD	Reference
Ammonium cations	river water and sewage effluent	C_{12} BAC to C_{18} BAC 0.3 – 18	SPE - C18	GC-MS/MS; Column: DB-5MS capillary	81 – 92	0.01 – 0.1 mg/ L [LOQ]	Ding and Liao (2001)
Per- and polyfluoroalkyl substances	river water	tris(pentafluoroethyl) trifluorophosphate 0.4 – 3400 ng/L	SPE – Oasis WAX	LC-HRMS/MS; Column: Acquity UPLC HSS T3; Mobile phase: 95 % water and 5 % MeOH with 5 mM ammonium formate as eluent A and 10% water and 90% MeOH with 5 mM ammonium formate as eluent B	83 – 125	NA	Neuwald et al. (2020)
Persistent and mobile chemicals; suspect screening	river water	More than 10 µg/L for PF_6 and BF_4	Multi-layer SPE; vacuum-assisted evaporative concentration; azeotropic evaporative concentration	Hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC), both coupled to HRMS	NA	NA	Neuwald et al. (2021)

such compounds, confirming for the first time that their presence in the environment is already a fact.

Nevertheless, as demonstrated in many works, the LC-MS technique using C-18 columns also enables efficient separation and analysis of up to several tens of ILs in a single run. Although ILs are characterized by low volatility, their permanently ionic nature makes the signals obtained in MS/MS analysis very intensive, allowing quantification of these compounds at trace amounts. Moreover, to enhance their signal intensity, standard mobile phase additives in LC-MS technique, such as ammonium acetate and acetic acid or ammonium formate and formic acid, are most commonly used.

However, alternative techniques for this purpose have been also proposed: ion chromatography (IC) (Zhang et al., 2019a,b; Liu et al., 2021; Liu et al., 2020), high-performance chromatography with UV-Vis detector (Stepnowski and Nichthauser, 2008) or fluorescence detector (Kümmerer et al., 1997) and gas chromatography with mass spectrometry (GC-MS) (Ding and Liao, 2001). Among them IC has been quite frequently proposed for the analysis of ILs in aqueous samples (Zhang et al., 2019a; b; Liu et al., 2020; 2021a; 2021b). Note, however, that this option may not be sufficient for the analysis of real environmental samples. It results from the fact that most ionic liquid cations, due to their structure - where the charge is surrounded by one or more long aliphatic chains, exhibit low conductivity, which makes analyses using conductometric detection much more difficult than in case of inorganic ions. Detection limits reported in the literature are in the mg/L range, which is an order of magnitude higher than the concentrations that would be expected during possible environmental contamination with ILs. Only for pyrolidinium cations in the study of Liu et al. (2020) relatively low LOQ (ranging from 60 to 160 µg/L) was achieved. In the case of ammonium, morpholinium, pyridinium or imidazolium cations, the LOQs of IC-based methods are so high that their application to environmental samples seems to have a rather limited potential.

An interesting alternative to classical determination was proposed by (Fan et al., 2018), who have applied indirect UV detection to determine ammonium cations that do not have chromophore systems. 1-ethyl-3--methylimidazolium trifluoroacetate at concentration of 1.0 mM was used as the mobile phase additive, allowing for the determination of analytes. However, even though this is an interesting alternative to IC with conductometric detection, due to its low sensitivity and detection specificity, its application for the analysis of ILs in the real environmental samples is also limited.

As it was already mentioned the analysis of imidazolium ILs in the environmental samples have been almost completely overlooked in the literature up to date. Despite the fact that these compounds constitute a sizeable, huge model group of classical ILs, and many of them have already been sold commercially, they have so far remained almost ignored as a potential environmental pollutants. (Stepnowski and Nichthauser, 2008) have proposed a method for the determination of a mixture of imidazolium and pyridinium cations, successfully using C18 cartridges to extract these compounds from fresh and salt water. This method involved detection by HPLC-UV/VIS reaching LOQ of tens of ng/L. It can be assumed that the improvement of this method by applying LC-MS/MS analysis would allow determination of trace concentrations of these compounds. There is no doubt, however, that the development of analytical methods related to the determination of imidazolium ILs in aqueous environmental samples are desired in the nearest future.

4. Extraction of ILs from solid environmental matrices

Only a few studies have been performed up to date that have considered development of the methods for the extraction of ILs from solid environmental matrices, i.e. soils, sediments and also sewage sludge. However, in contrast to aqueous samples available methods for the extraction of ILs from solid matrices are not so focused on the ammonium compounds, but also on imidazolium cations. The most important information on the available methods for the extraction of ILs from solid matrices is summarised in Table 2. Based on the data presented in the Table 2 seven out of the eight performed studies have applied simple ultrasound-assisted extraction (UAE). In most cases this approach resulted in high recoveries, above 70% (Zhou et al., 2017; Ruan et al., 2014; Li and Brownawell, 2009; Lara-Martíńán et al., 2010). Moreover, after the extraction usually purification step was included which was mainly based on the application of SPE based on ion exchangers.

Among the proposed methods for the analysis of ILs in solid matrices, particularly noteworthy is the one proposed by Lu et al. (2015), in which 23 imidazolium and ammonium cations with different side chains (from polar to hydrophobic) were successfully extracted from soil. For this purpose also the UAE was used and samples were concentrated and purified using SPE technique with weak cation exchange (X-CW) columns. Final analysis was performed using LC-MS/MS, which resulted in low LOQs (less than 10 ng/g for all analytes tested). A similar method involving a mixture of ILs, including imidazolium and ammonium, but also pyridinium and piperidinium cations, was proposed by Pati and Arnold (2020). However, they used sediment collected from the lake. Also in this work, sonication followed by clean-up with a weak cation exchanger (Oasis WCX) was used, achieving LOQs ranging from 12 to 118 ng/g. These methods (Pati and Arnold, 2020; Lu et al., 2015) can undoubtedly be considered a desirable direction in the field of ILs analytics.

In addition to the solid matrices associated with the natural environment, i.e. soils and sediments, municipal sewage sludge also requires special attention in terms of pollution. Due to its direct contact with

Table 2
Comparison of methods used for extraction, purification and analysis of ILs from solid environmental matrices.

Tested compounds	Matrix	Concentration found in the environmental samples	Extraction	Concentration and purification	Final analysis	Recovery [%]	LOD/ LOQ	Reference
Imidazolium, ammonium and phosphonium cations (23 analytes in total)	soil	NA	UAE in 20% 0,2 M NH_4 Cl 80 % ACN; pH= 3	SPE - Strata X-CW	LC-MS; Column: C18; Mobile phases: 0,1 % formic acid and ACN	69–107	0.5 – 9.5 ng/ g [LOQ]	(Liu and Yu, 2016)
Imidazolium catianos (C4 to C10)	soil	NA	UAE in 90 % MeOH with 10% saturated NH_4 Cl	none	HPLC/UV–VIS, Column: C18; Mobile phase: 25 mM phosphate buffer and ACN	90–108	< 1 ng [LOD]	Zhou et al. (2017)
Imidazolium cations (C4 to C12)	soil	NA	UAE in 90 % MeOH with 10% saturated NH_4Cl	syringe filter 0,22 µm	HPLC/UV–VIS, Column: C18; Mobile phase: 25 mM phosphate buffer and ACN	NA	NA	Shao et al. (2018)
Imidazolium, pyridinium, piperidinium and ammonium cations with different side chains	lake sediment	14 – 436 ng/g (ammonium cations)	UAE in 1 M HCl with 90% MeOH; before clean-up addition of citric buffer to get pH around 5–6.	SPE - Oasis WCX	LC-HRMS; Column: manually packed nanoflow column (approx. 75 mm × 200 mm) containing Cogent 4 Diamond Hydride material (4 mm particle size, MicroSolv Technology Corporation); Mobile phases: 0.1 % formic acid with 5 mM ammonium acetate; 0,1 % formic acid in ACN;	68–86	12 – 118 ng/g [LOQ]	Pati and Arnold (2020)
Imidazolium cations (C2 to C6); one pyridinium cation	soil	NA	UAE in 90 % MeOH with 10% of saturated NH_4Cl	none	HPLC/UV–VIS, Column: Luna SCX or Gemini C 6; Mobile phase: ACN and phosphate buffer	40–98	0.18 ng/g [LOQ]	Nichthauser et al. (2009)
Ammonium cations	municipal sewage sludge	0.04 – 343 ng/g	UAE in 10 mL of MeOH with 1 M hydrochloric acid at 60 °C; extraction repeated 3 times	SPE – tubes filled with 3 g of AG1-X2 anion exchange resin	LC-MS/MS; Column: ZORBAX RX-C8; Mobile phase: A – MeOH; B – water; C – 0.1 % acetic acid, 10 mM ammonium acetate in isopropanol	78–111	1.5 – 4 ng/g dw [LOQ]	Ruan et al. (2014)
Ammonium cations	estuarine sediments	0.1 – 74,000 ng/ g	UAE in 60 °C (1 h × 3) with 10 mL of acidic (1 M HCl) MeOH (30 mL total).	LLE (5 × washing with water; then extraction with 3 × 10 mL of chloroform); then SPE - AG 1 X2 resin	LC-ToF-MS; Column: Luna C18; Mobile phase: A - 20:80 ACN: water with 1% acetic acid; B - 95:5 ACN: water with 10 mM ammonium acetate; C - 2-propanol with 0.1 % formic acid.	98–118	0.1 – 2.6 ng/ g dw [LOQ]	Li and Brownawell (2009); Lara-Martín et al. (2010)
Ammonium cations	sediments near WWTPs	22 – 206 ng/kg	ASE; 60 % ACN 40% water; 10.340 kPa; 120 °C; 3 cycles	SPE - PLRP-s cartridges	LC-ESI/MS; Column: CP18; Mobile phase: A - ACN B - 10 mM ammonium formate buffer,	79–85	0.2 – 0.16 µg/kg [LOD]	Ferrer and Furlong (2002)
1-methyl-3-octylimidazolium chloride	soil	up to 94 mg/kg	UAE in 60 °C (10 min × 2) with MeOH	none	NA	NA	NA	Probert et al. (2018)

sewage, this is where the accumulation of pollutants can often be observed first, providing an alarm. The analysis of ILs in these matrices has only been discussed in one paper so far (Ruan et al., 2014), focusing exclusively on ammonium compounds. In this work, threefold extraction using UAE followed by SPE with a cation exchanger were employed, with LC-MS/MS analysis achieving LOQs of a few ng/g. In the case of municipal sewage, special attention for ammonium cations is warranted due to their common use in households, hence these compounds can be already present in wastewater. However, as applications for other ILs increase, they can also be expected to end up in wastewater, so they cannot be ignored in future analysis.

Other, currently applied in many analytical laboratories more effective techniques for the extraction of different analytes from solid matrices such as e.g. Accelerated Solid Extraction - ASE (or Pressurized Solvent Extraction - PLE) have been only proposed in one study (Ferrer and Furlong, 2002). Considering recoveries not exceeding 85%, however, this method was not more efficient than those proposed in the work using UAE.

Furthermore, it must be also highlighted that similarly to the analysis of ILs in aqueous samples, also in the case of solid matrices LC-MS/MS technique is most commonly applied, which allows to determine the ILs in such matrices at the levels of ng/g. (Fig. 1).

5. Extraction of ionic liquids from biological matrices

Although at first sight it would seem that there has been many papers

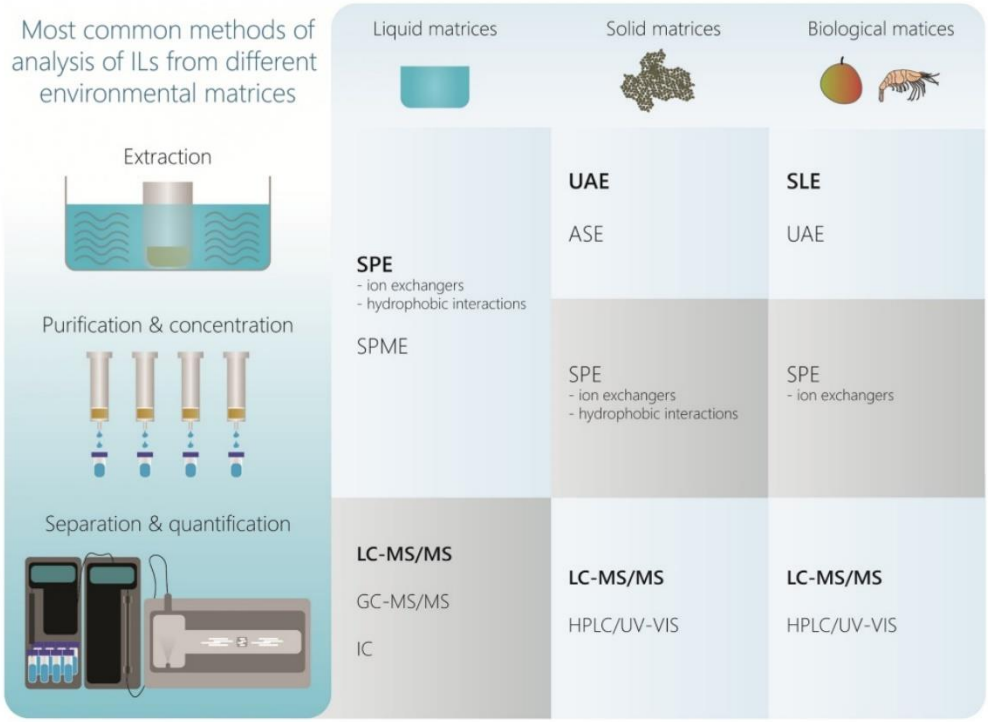

Fig. 1. Typical process of extraction, purification and analysis of ILs from environmental matrices.

published concerning the extraction and analysis of ILs in biological matrices (Table 3), this actually does not cover neither the wide range of different ILs nor different matrices. Apart from the work of Nichthauser et al. (2009), dealing with imidazolium cations and one pyridinium cation, all the available studies concern ammonium compounds exclusively. Additionally, almost all of these works are related to food, leaving the analysing of these contaminants in animal and plant tissues almost unstudied.

Among the proposed methods, simple procedures based on such extraction techniques as UAE and Solid-Liquid Extraction (SLE) have been mainly applied. These methods in most cases give satisfactory recoveries of 70–100 % (e.g., Xian et al., 2016; Miyauchi et al., 2005; Xiang et al., 2015; Cao et al., 2014). In works where SPE was used to purify samples, only sorbents based on ionic interactions are involved. Also the procedure originally proposed for the extraction and analysis of pesticides in fruits and vegetables - QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) was applied for the analysis of ILs from such food matrices. This method is simple and significantly faster than classical extraction and purification. It was successfully applied in the works of Arrebola-Liébanas et al. (2014) and Hepperle et al. (2014), in both obtaining recoveries at levels above 85%.

The only method concerning the extraction of ILs from animal tissues has recently been proposed by Kierkeegard (2020; 2021). In this work, experiments were conducted to evaluate the bioconcentration of ammonium and amine cations in fish. Solvent extraction was supported first by a bullet blender and in the next step by sonication. After purification of the samples by SPE with strong cationite and then they were analysed using LC-MS/MS. This procedure can be classified as an effective one - with the recoveries higher than 80 %.

Of the food samples in which the presence of ammonium cations has been analysed so far, products such as dairy products, fruit and vegetables or tea dominate. Interestingly, although most of food matrices are complex and rich in potential interferents, in some cases the proposed sample preparation processes are simplified. Usually, they involve simple solvent extraction (SLE or UAE), often omitting purification by SPE, achieving LOQs at levels ranging from < 1 to several hundred ng/g product (e.g., Yu et al., 2018; Van Bruijnsvoort et al., 2004; Friedle et al., 2012). However, it is important to note that analysis of samples from the most rich matrices without intensive purification can have a significant impact on the results obtained, especially considering the effect of interferents on analyte ionization and analytical reproducibility.

Similarly, to the analysis of ILs in solid environmental samples, LC-MS/MS was proposed as the final analysis method in almost all papers, which is not surprising given abundance of potential interferents in biological matrices.

6. Conclusions and future perspectives

Although ILs are researched on an extremely large scale, as well as increasingly implemented in commercial solutions, it is difficult to conclude that this interest is adequately reflected in research on the issues related to risk assessment and monitoring of these compounds as potential environmental hazards. The number of papers dealing with the analysis of ILs in environmental matrices is scarce. It appears that the scientific community is largely still believe that ILs are inherently 'green' and safe, and therefore there is no need to be particularly concerned about their potential environmental fate. However, some existing already facts contradict this, and there are increasing indications that ILs should be treated with great caution.

In the case of the analysis of aqueous and biological matrices, imidazolium ILs have so far been almost completely neglected. This gap

Table 3
Comparison of methods used for extraction, purification and analysis of ILs from biological matrices including food samples.

Tested compounds	Matrix	Concentration found in the environmental samples	Extraction	Concentration and purification	Final analysis	Recovery [%]	LOQ/LOD	Reference
Ammonium cations	human blood	0.453 – 68.6 ng/mL	UAE in 100% ACN	SPE - Oasis WCX;	LC-MS; Column: C18; Mobile phases: 0,1% FA in water; 0,1% FA in ACN	62–127	0.01 – 0.37 ng/mL [LOD]	Zheng et al., 2021
Ammonium and amine cations	fish tissues	NA	100% MeOH with 3 steel balls – shaking in bullet blender for 1 min, after that UAE – 1 hr in 50 °C	SPE - Oasis WCX	LC-MS; Column: C18; Mobile phases: 10 mM ammonium acetate in 5% MeOH; 10 mM ammonium acetate in 95% MeOH.	> 80	Several ng/g	Kierkegaard et al., (2020, 2021)
Imidazolium cations; one pyridinium cation	plants	NA	UAE (1.5 h) in 0,1 M solutions of mineral or organic acids	none	HPLC/UV–VIS; Column: Luna SCX or Gemini C-6; Mobile phase: ACN and phosphate buffer	70–84	0.11 – 0.47 μg/kg [LOQ]	Nichthauser et al. (2009)
Ammonium cations	diary products	31.9 – 122 μg/kg	SLE with ACN and vortexing	dSPE – 600 mL of extract added to 400 mg of PSA	LC-MS/MS; Column: ACQUITY UPLC HILIC; Mobile phase: 0.05% formic acid, ACN and 10 mM ammonium acetate	93–102	1.5 – 48 μg/kg [LOQ]	Xian et al. (2016)
Ammonium cations	food simulants	NA	none	none	FI/MS; no chromatography column; Mobile phase: 90% ACN; 10% 5 mM aqueous ammonium acetate buffer (pH 5.1).	61–117	5 ppb [LOQ]	Yu et al. (2018)
Ammonium cations	cheese and milk products	up to 287 μg/kg	SLE with ACN/ethyl acetate; shaking for 10 min at about 100 rpm	filtration through a 0.45 μm PVDF filter	LC MS/MS; Column: Synergi Fusion-RP 80 A; Mobile phase: 0.3% formic acid in ultrapure water (A)– MeOH (B) – 0.3% formic acid in ACN (C).	9–106	5 – 50 μg/kg [LOQ]	Slimani et al. (2017)
Ammonium cations	treated wood	0.5 – 3.2 mg/cm^3	UAE with 20 mL HCl–ethanol (3/100, v/v) for 2 h	SPE – Oasis MCX	LC-MS/MS; Column: Xtera C18; Mobile phase: ACN – 100 mM ammonium formate buffer, pH 3.5	92–101	240 μg/g [LOQ]	Miyauchi et al. (2005)
Ammonium cations	fruits and vegetables	up to 0.16 mg/kg	QuEChERS extraction		LC-MS/MS; Column: Synergi 4 μ Hydro-RP; Mobile phase: A - 5 mM ammonium formate in purified Water; B - 5 mM ammonium formate in MeOH	NA	NA	(Schüle et al., 2012)
Ammonium cations	food products	NA	salting-out solvent extraction with ACN–water (70 + 30) with 0.16 mL/L TFA (2 mM) and NaCl	none	LC-MS/MS; Column: XTerra RP8; mobile phase: ACN–water (60/40) with 0.16 mL/L TFA (2 mM).	95–99	NA	Van Bruijnsvoort et al. (2004)
Ammonium cations	oranges and cucumbers	15 – 210 μg/kg	QuEChERS extraction		LC-MS/MS; Column: ACQUITY UPLC bridged ethyl hybrid C18; Mobile phase: 0.01% formic acid in water (solvent A) and MeOH (solvent B).	85–97	2 – 4 μg/kg [LOQ]	Arrebola-Liébanas et al. (2014)
Ammonium cations	vegetables	23 – 180 μg/kg	UAE in 10 mL of 0.1% acidified MeOH (hydrochloric acid/MeOH, v/v); extraction repeated 3 times	SPE – neutral alumina oxide sorbent;	GC-MS/MS; Column: DB-5MS fused silica capillary	71–108	2.3 – 20 μg/kg [LOQ]	Xiang et al. (2015)
Ammonium cations	foodstuffs	NA	UAE with 90% MeOH. Then repeated UAE with 80% MeOH	SPE – WCX cartridges	LC-MS/MS; Column: CAPCELL PAK CR 1: 4; Mobile phase: 0.1% formic acid– 50 mM ammonium formate aqueous solution (A) and ACN (B).	77–103	10 μg/kg [LOQ]	Cao et al. (2014)
	food	10 – 340 μg/kg		none		76–112	NA	Friedle et al., 2012

(continued on next page)

Table 3 (*continued*)

Tested compounds	Matrix	Concentration found in the environmental samples	Extraction	Concentration and purification	Final analysis	Recovery [%]	LOQ/LOD	Reference
Ammonium cations			SLE in 70% ACN and 30% 0.1% formic acid		LC-MS/MS; Column: Kinetex XB-C18; Mobile phase: A – 0.1% formic acid in water; B – 0.1% formic acid in ACN			
Ammonium cations	fruits and vegetables	NA	SLE with shaking in 10 mL of ACN	QuEchERS extraction	LC-MS/MS; Column: Synergi Hydro RP; Mobile phase: A – 5 mM ammonium formate in purified water; B - 5 mM ammonium formate in meOH.	88–104	< 10 µg/kg [LOQ]	Hepperle et al. (2014)
Ammonium cations	milk	0.1 – 535 µg/kg	UAE in 5% formic acid in MeOH for 60 min (60 °C)	SPE – WCX cartridges	LC-MS/MS; Column: XBridge BEH HILIC; Mobile phase: A – ACN; B - aqueous 50 mM ammonium formate acidified to pH 3.2 with formic acid;	85–99	0.4 – 2 µg/kg [LOQ]	Bertuzzi and Pietri (2014)

needs to be filled very urgently especially in light of recent reports of the presence of one of the imidazolium ILs (1-octyl-3-methyl imidazolium) not only in the environment (Probert et al., 2018) but also in human blood (Leitch et al., 2021). These reports make it clear that threat posed by imidazolium ILs is no longer just potential and hypothetical - it already exists and requires action.

The case is different for ammonium ILs. As they are commonly used in surfactants and disinfectants, the problem of their presence in the environment has already been recognised. Nevertheless, even in the case of these compounds, some knowledge gaps can be observed. Although there are a large number of papers dealing with the analysis of these compounds in aqueous and biological matrices, in the case of solid environmental matrices there are only three papers, all dealing with sediments. There is no doubt that deposition of these compounds in soils can also be important and therefore techniques to extract ammonium ILs from soils are urgently needed.

Taking a closer look at the methods concerning the analysis of biological matrices, it should be emphasised that almost all solutions proposed so far concern the extraction of quaternary ammonium cations from food. Obviously, this is a priority topic because potential contaminants in food carry significant risks for humans. However, the environment should not be forgotten. An important issue with new contaminants is potential bioconcentration. It is therefore extremely important to verify that these compounds do not accumulate in the tissues of wild animals and plants. So far, however, only one paper describes the extraction of ILs from animal tissues (Kierkegaard et al., 2020, 2021), which confirms that this topic has not received enough attention.

In summary, examination of the literature in search of methods for the extraction and analysis of ILs from environmental matrices leads to the conclusion that the state of the knowledge is very limited. This is a serious issue, given that in recent years these compounds have been detected not only in the environment but also in humans. Therefore, it seems clear that it is necessary to expand the analytical methods allowing the analysis of ILs and to use it to assess whether recent reports of the presence of these compounds in the environment are just individual contaminants or a larger problem.

Declaration of Competing Interest

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

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Statement of Environmental Implication

According to recent reports, ionic liquids are already present in the environment. Moreover, it has been suggested that they may have harmful effects on organisms, including humans. This paper presents, for the first time, a broad summary of the existing literature on the analysis of ionic liquids in environmental matrices. In addition to identifying relevant data confirming the presence of these compounds in the environment, we discuss the range of analytical methods for their detection. By pointing out important shortcomings, we emphasise how necessary it is to increase interest in this topic.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129353.

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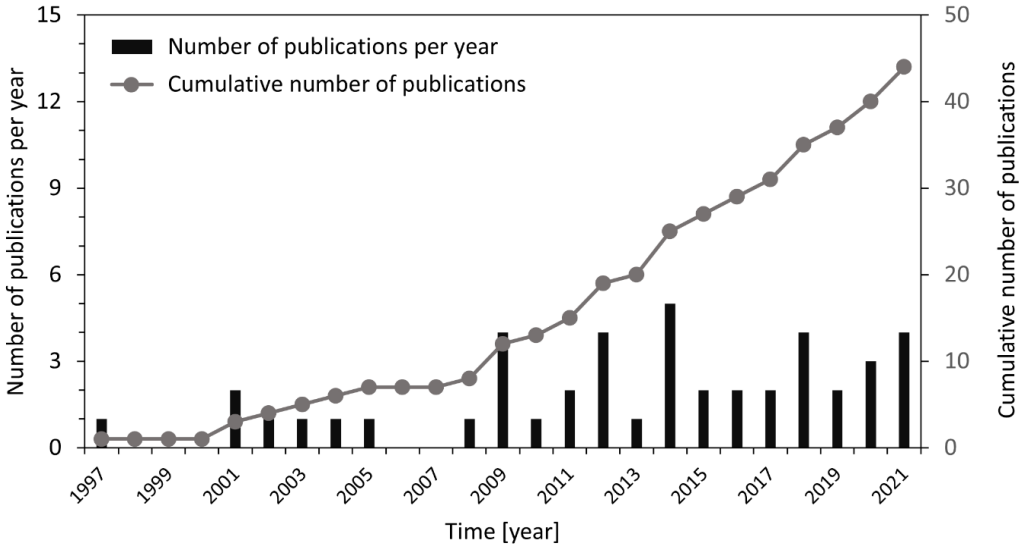

Figure S1. Number of publications regarding extraction methods for determination of ionic liquids.

Publikacja 3

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Review

Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential

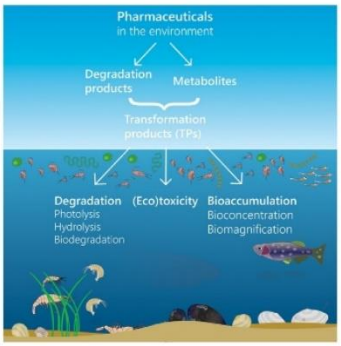
Jakub Maculewicz [a], Dorota Kowalska [a,*], Klaudia Świacka [b], Michał Toński [a], Piotr Stepnowski [a], Anna Białk-Bielińska [a], Joanna Dołżonek [a]

[a] Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland
[b] Department of Experimental Ecology of Marine Organisms, Institute of Oceanography, University of Gdańsk, Av. Pilsudskiego 46, 81-378 Gdynia, Poland

HIGHLIGHTS

- Stability, (eco)toxicity and bioaccumulation of pharmaceuticals' TPs was reviewed.
- Some TPs may be as harmful as their native forms.
- There is an evidence of their toxicity and potential to bioconcentration.
- Many of drugs TPs are suspected to be stable in the environment.
- Knowledge about drugs TPs is still scarce and definitely needs more research.

GRAPHICAL ABSTRACT



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ABSTRACT

Nowadays, a huge scientific attention is being paid to the chemicals of emerging concern, which may pose a significant risk to the human and whole ecosystems. Among them, residues of pharmaceuticals are a widely investigated group of chemicals. In recent years it has been repeatedly demonstrated that pharmaceuticals are present in the environment and that some of them can be toxic to organisms as well as accumulate in their tissues. However, even though the knowledge of the presence, fate and possible threats posed by the parent forms of pharmaceuticals is quite extensive, their transformation products (TPs) have been disregarded for long time. Since last few years, this aspect has gained more scientific attention and recently published papers proved their common presence in the environment. Also the interest in terms of their toxicity, bioconcentration and stability in the environment has increased. Therefore, the aim of our paper was to revise and assess the current state of knowledge on the fate and effects resulting from the presence of the pharmaceuticals' transformation drugs in the environment. This review discusses the metabolites of compounds belonging to six major pharmaceutical groups: SSRIs, anticancer drugs, antibiotics, antihistamines, NSAIDs and opioids, additionally discussing other individual compounds for which literature data exist. The data presented in this paper prove that some TPs may be as harmful as their native forms, however for many groups of drugs this data is still insufficient to assess the risk posed by their presence in the environment.

* Corresponding author.
E-mail address: dorota.kowalska@phdstud.ug.edu.pl (D. Kowalska).

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Contents

1. Introduction

Pharmaceuticals are a group of compounds of particular importance in the context of environmental risk assessment. In recent decades, the risks posed by these substances have begun to be recognised, resulting in a large number of studies aimed at better understanding of their impact on ecosystems. It is already known without any doubts that pharmaceuticals are present in the environment, and scientists are increasingly familiar with their modes of action on non-target organisms, being able to estimate the consequences of this phenomenon (Li et al., 2021; Swiacka et al., 2021). However, it seems that pharmaceuticals themselves are just the tip of the iceberg, under which another threat is hidden, in the form of their transformation products (TPs). TPs include both the metabolites excreted by humans as well as the products of biotic and abiotic transformations occurring in wastewater treatment plants and in the environment.

Although up to date most environmental analyses have focused on the detection of parent compounds of pharmaceuticals, there are some recent reports suggesting that TPs can reach similar or even higher concentration levels in the environment (Kolecka et al., 2019, 2020). Groundwater analyses performed in Barcelona by López-Serna et al. (2013) showed the presence of numerous TPs in the studied samples. Particularly noteworthy were enalapril and propranolol, whose metabolites enalaprilat and 4-hydroxypropranolol reached concentrations several times higher than the parent compounds, up to 12.5×10^{-6} and 21.4×10^{-6} mg/L, respectively. In a recent study of de Oliveira Klein et al. (2021) it was shown that concentration of metabolite of gemcitabine (20-deoxy-20,20-difluorouridine) reached up to 116×10^{-3} mg/L in the effluent from Brazilian hospital, significantly higher than the parent compound. In turn, in a study conducted by Langford and Thomas (2011) focused on the determination of pharmaceuticals in Oslo Fjord, carbamazepine-10,11-epoxide was detected in all surface water samples, with concentrations ranging from 14×10^{-6} to 77×10^{-6} mg/L, compared to 4×10^{-6} to 20×10^{-6} mg/L for the parent compound. Similar results were obtained by Martinez et al. (2016), who indicated that coastal water of France is polluted by major metabolites of carbamazepine, such as 10,11-dihydro-10,11-trans-dihydroxycarbamazepine and 10-hydroxy-10,11-dihydrocarbamazepine. Interestingly, analyses performed by Scheurell et al. (2009) confirmed the presence of five different metabolites of diclofenac in the river flowing through Karachi. Alygizakis et al. (2016) showed that norvenlafaxine is one of the most common compounds in the coastal waters of Greece, while its parent pharmaceutical – venlafaxine was not detected. On the other hand, Azuma et al. (2016) indicated that metabolites of acetaminophen – acetaminophen glucuronide and acetaminophen sulfate, reached the highest concentrations among compounds analysed in the effluent from hospital, and their levels were as high as 24×10^{-6} mg/L. Moreover, the presence of metabolites of omeprazole, a commonly used proton-pump inhibitor, was also detected in the environment by different research groups (Gracia-Lor et al., 2014; Boleda et al., 2013; Boix et al., 2015).

As summarized by Zhou et al. (2019), 66 TPs of pharmaceuticals have been already recorded in European surface waters. Consistently, according to analyses by Petrie et al. (2015), up to 20% of the newly recorded emerging contaminants in United Kingdom waters are products of metabolism. However, it should be also noted that even these assessments may be significantly underestimated. One of the main problems with the detection of TPs in the environment is the fact that analytical standards are not available for many of them, or their structures have not even been identified yet (Yin et al., 2017). Despite these limitations, the analyses to date give the confidence that TPs of pharmaceuticals are widely present in the environment, and that their concentrations may be equal to, or even higher than, the corresponding parent compounds. Consequently, this means that these compounds, whose input to surface waters is constant, can interact in complex ways with the environment, both participating in biotic/abiotic processes and affecting the organisms.

Therefore, the main aim of our review paper is to assess the state of knowledge on the environmental fate and effects of pharmaceuticals' TPs in terms of their stability and risks associated with their toxic effects on organisms as well as accumulation potential. Even though some summarizing papers have been recently published concerning the presence of TPs in the environment (Yin et al., 2017; Meng et al., 2021; Ibáñez et al., 2021) they are mainly focused either on their analysis,

Table 1
Environmental fate of TPs of pharmaceuticals.

Parent compound (PC)	Transformation product (TP)	Experimental/matrix	Results/conclusions						Ref.
Anticancer drugs									
Tamoxifen	4-Hydroxy-*N*-desmethyl-ramoxifen (endoxifen)	Preliminary stability test in HPLC water at 4 °C for 24 h. Extensive tests with raw wastewater from 1 day to 3 months in −20, 4 and 25 °C and pH 2 and 7. Samples kept in amber glass vials in the dark.	Preliminary - *+4 °C, 24 h in HPLC water*: negligible degradation. Extended −20 °C pH 2 High stability, around 20% degradation in 3 months. pH 7 High stability, only after 3 months over 20% degradation. 4 °C pH 2 High stability for the first month, finally over 40% degradation in 3 months.			- *+4 °C, 24 h in wastewater*: over 80% remaining. pH 7 High stability for 3 months (around 90%). 25 °C pH 2 Degradation between 40 and 50% during entire experiment. pH 7 Gradual degradation from over 20% at day 1 to nearly 60% after 3 months.		(Negreira et al., 2014)
	(Z)-4-hydroxytamoxifen		Preliminary - *+4 °C, 24 h in HPLC water*: completely stable. Extended −20 °C pH 2 Around 80% stability after 3 months. pH 7 Results vary, stability after 1–2 months 65–85%, over 50% after 3 months. 4 °C pH 2 More stable than at pH 7, high stability within 2 months, 30% degradation after 3 months.			- *+4 °C, 24 h in wastewater*: over 50% remaining. pH 7 30% degradation in 9 days, over 50% in 3 months. 25 °C pH 2 Stable in first 9 days, degradation between 20 and 40% within 3 months. pH 7 Over 40% degradation within 9 days, 80% degradation in 1 month.		
	5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide		Preliminary - *+4 °C, 24 h in HPLC water*: 78% degradation. Extended −20 °C pH 2 Faster degradation than at pH 7, less than 40% remained after 1 month. pH 7 50% degradation in 1 month, almost complete degradation in 3 months. 4 °C pH 2 Quick degradation, less than 10% remaining after 3 days.			- *+4 °C, 24 h in wastewater*: complete degradation. pH 7 Complete degradation after 1 day. 25 °C pH 2 Over 90% degradation in a day. pH 7 Complete degradation in 1 day.		
	Temozolomide								
	Paclitaxel	6(α)-Hydroxypaclitaxel	Preliminary - *+4 °C, 24 h in HPLC water*: no degradation. Extended −20 °C pH 2 Around 70% stability after 2–3 months. pH 7 High stability for 2 months, around 60% remaining 4 °C pH 2 High stability for 2 months; however, less than 30%			- *+4 °C, 24 h in wastewater*: no degradation. pH 7 High stability for 2 months; however, less than 25 °C pH 2 High stability within 9 days, less than 70% pH 7 20% degradation in 1 day, nearly 80% after		

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Table 1 (*continued*)

Parent compound (PC)	Transformation product (TP)	Experimental/matrix	Results/conclusions						Ref.
				after 3 months.	remaining after 3 months.	40% remaining after 3 months.	remaining after 1 month, 50% after 2 months and over 20% in 3 months.	1 month.	
Methotrexate	7-Hydroxy methotrexate		Preliminary - *+4 °C, 24 h in HPLC water and wastewater:* over 80% of the compound remaining. Extended						
			-20 °C pH 2 Faster degradation than at pH 7, completely stable for 1 month, around 30% degradation after 2 months and more than 50% degradation in 3 months.	pH 7 Around 90% stability after 2 months, over 60% stability after 3 months.	4 °C pH 2 Only 10% degradation during 2 months; over 40% degradation after 3 months.	pH 7 Results of the stability are varying between over 60% to less than 90%.	25 °C pH 2 Around 70% stability in first 9 days, less than 20% after 1 month. Complete degradation after 2 months.	pH 7 Faster degradation than at pH 2, almost 50% degradation in 9 days, nothing observed after 2 months.	
		Stability verification during ecotoxicological tests with *R. subcapitata* (22 °C, light-darkness cycles) and *L. minor* (25 °C, continuous illumination) – compound dissolved in water-based medium.	- Completely unstable during the tests.						(Bialk-Bielinska et al., 2017a, 2017b)
		Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2}$ > 1 year at 25 °C.						(Tonski et al., 2021)
Cyclophosphamide	Phosphoramide mustard	Elaborate investigation of degradation at various conditions. Hydrolysis stability at 0, 25 and 38 °C and pH range 1–12 in phosphate buffers; stability in 1 M Tris sulfate and Tris chloride buffers at pH 7.4 and 38 °C; the effect of chloride ion stabilization: 0–2 M chloride at pH 7.4 and 38 °C; other additional experiments.	- Hydrolysis follows apparent first-order kinetics, - Hydrolysis rates increase from pH 1.7 up to around pH 5, then it becomes more or less constant up to pH 12, - Exemplary half-lives: • 0 °C, pH 1.9: 213.0 h, • 0 °C, pH 7.4: 112.0 h, • 0 °C, pH 12: 120.0 h. • 25 °C, pH 1.7: 5.5 h, • 25 °C, pH 7.4: 1.6 h, • 25 °C, pH 12: 1.7 h. • 38 °C, pH 1.7: 62.5 min, • 38 °C, pH 7.4: 14.4 min, • 38 °C, pH 12: 13.3 min. - Hydrolysis occurred faster in Tris sulfate than in Tris chloride buffer, which indicates chloride ion stabilization effect - The chloride stabilization effect was confirmed, when half-life of fosforamide mustard increased with an increase of chloride ion concentration from 18 min (0 M) to 63 min (2 M).						(Watson et al., 1985)
Ifosfamide	2-Dechloroethylifosfamide	Hydrolysis kinetics at pH 1–13 and 70 °C.	- The degradation followed pseudo first-order kinetics, - Hydrolysis is pH-dependent, U-shape, with the highest rates at acidic conditons and						(Kaijser et al., 1996)

			lowest k_{obs} at plateau between pH 6 and 11. At strongly basic conditons the degradation rates increase, - Degradation occurs faster than for 3-dechloroethylifosfamide, - k_{OH}: 2.4×10^{-4} [mol/L/s]; k_0: 3.5×10^{-5}; k_H: 3.6, - E_a [kJ/mol] at pH 2: 13.0, pH 7: 84.8, pH 12: 51.3.	
		Hydrolysis kinetics at buffered water of pH 3.0–6.8. Investigation of degradation pathway.	pH 3 (25°C): $k_{obs} = 2.8 \times 10^{-2}$ [min] $t_{1/2} = 25$ min	pH 5.5 (20°C): $k_{obs} = 1.3 \times 10^{-4}$ [min] $t_{1/2} = 3.8$ days	pH 6.8 (20°C): $k_{obs} = 1.3 \times 10^{-5}$ [min] $t_{1/2} = 36$ days	(Gilard et al., 1999)
			- Several degradation products found in different conditions, some probably stable.			
	3-Dechloroethylifosfamide	Hydrolysis kinetics at pH 1–13 and 70 °C.	- The degradation followed pseudo first-order kinetics, - Hydrolysis is pH-dependent, V-shape, with high hydrolysis rates at acidic conditions, the lowest at pH 6, from where there is increase with more basic condtions, - k_{OH}: 1.0×10^{-2} [mol/L/s]; k_0: 2.8×10^{-5}; k_H: 4.0×10^{-1}. - E_a [kJ/mol] at pH 2: 216.0, pH 7: 110.0, pH 12: 47.0.			(Kaijser et al., 1996)
		Hydrolysis kinetics at buffered water of pH 3.0 and 5.5. Investigation of degradation pathway.	pH 3 (25°C): $k_{obs} = 4.1 \times 10^{-3}$ [min] $t_{1/2} = 2.8$ h	pH 5.5 (20°C): $k_{obs} = 1.0 \times 10^{-5}$ [min] $t_{1/2} = 49$ days		(Gilard et al., 1999)
			- Several degradation products found in different conditions, some probably stable.			
	2,3-Didechloroethylifosfamide		pH 5.4 (25°C): $k_{obs} = 2.1 \times 10^{-2}$ [min] $t_{1/2} = 33$ min	pH 6.1 (25°C): $k_{obs} = 7.1 \times 10^{-3}$ [min] $t_{1/2} = 1.6$ h	pH 6.8 (25°C): $k_{obs} = 1.7 \times 10^{-3}$ [min] $t_{1/2} = 6.8$ h	
			- Several degradation products found in different conditions, some probably stable.			
NSAiDs, analgesics, antipyretic and anti-inflammatory						
Ibuprofen	2-Hydroxy ibuprofen	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C.			(Tonski et al., 2019)
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals.	- Formed constantly until the total degradation of ibuprofen, then very quick disappearance from water within several days.			(Li et al., 2014)
		1 L of deionized water with 5 mg of form of PC was mixed with 10 mg/L of fresh sludge from a membrane bioreactor used to treat wastewater. Mixture kept in glass bottle for 28 days. External carbon source was added to investigate co-metabolism of parent compound, since no degradation occurred without it.	- Formed together with an isomer with unknown position of the hydroxyl-moiety; both isomers exhibited similar path of formation and degradation, - After a phase of increasing amount, rapid degradation occurred, correlated with a fast disappearance of parent compound, which indicates susceptibility to biodegradation.			(Quintana et al., 2005)
	Carboxy ibuprofen	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C.			(Tonski et al., 2019)

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Table 1 (*continued*)

Parent compound (PC)	Transformation product (TP)	Experimental/matrix	Results/conclusions	Ref.
		dark.		
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals.	- Formed constantly for a week, then disappearance from water within another week.	(Li et al., 2014)
Diclofenac	4-Hydroxy diclofenac	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 7 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C. - Mildly unstable at pH 4: ➢ at 70 °C $k_{obs} = 1.1 \times 10^{-2}$ [1/h], $t_{1/2} = 62.6$ [h]; ➢ at 50 °C $k_{obs} = 1.1 \times 10^{-3}$ [1/h], $t_{1/2} = 608.0$ [h]; at 20 °C no degradation observed in 30 days. - Mildly unstable at pH 9: ➢ at 70 °C $k_{obs} = 1.6 \times 10^{-2}$ [1/h], $t_{1/2} = 43.6$ [h]; ➢ at 50 °C $k_{obs} = 8.9 \times 10^{-4}$ [1/h], $t_{1/2} = 778.8$ [h]; at 20 °C no degradation observed in 30 days.	(Tonski et al., 2019)
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals.	- Formed in a small amount, - The concentration was increasing until day 25, after that there was significant loss of the compound in the water.	(Li et al., 2014)
5-Hydroxydiclofenac (diclofenac)	p-Benzoquinone imine of 5-hydroxydiclofenac	10 mL of sterile solution with p-benzoquinone imine of 5-hydroxydiclofenac incubated alone, with sterilized sediment or native sediment from a river (1 g wet weight). Incubation in the dark, at 27 °C for 60 days.	- Negligible degradation in only water solution (depletion rate 0.004 [1/d]) – no hydrolysis, - Significant loss observed with both native and sterile sediment, with depletion rates 0.051 [1/d] and 0.048 [1/d], respectively. It indicates sorption to the sediment/biofilm as a main route of elimination from water.	(Gröning et al., 2007)
Naproxen	*O*-desmethylnaproxen	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C.	(Tonski et al., 2019)
		1 L of deionized water with 5 mg of solid form of PC was mixed with 10 mg/L of fresh sludge from a membrane bioreactor used to treat wastewater. Mixture kept	- Formed in small amount, with fast initial increase, followed by rather stable and slow growth, correlated with slow degradation of naproxen; by the end of the experiment concentration started to decrease. Additional measurements showed, that it was entirely removed from water after 39 days, together with naproxen.	(Quintana et al., 2005)

		in glass bottle for 28 days. External carbon source was added to investigate co-metabolism of parent compound, since no degradation occurred without it.		
Ketoprofen	3-(Hydroxy-carboxymethyl)hydratopic acid	1 L of deionized water with 20 mg of solid form of PC was mixed with 10 mg/L of fresh sludge from a membrane bioreactor used to treat wastewater. Mixture kept in glass bottle for 28 days.	- Product of direct microbial transformation of ketoprofen, reached maximum concentration in 14 days, - After 14 days quick and full degradation within a week, - Possibly a by-product in a microbial transformation cycle of ketoprofen to 3-(keto-carboxymethyl)hydratopic acid, also found in this experiment, - In parallel experiment with addition of external carbon source, ketoprofen was very stable a no degradation products were observed.	
Ketoprofen or (3-(hydroxy-carboxymethyl) hydratopic acid)	3-(Keto-carboxymethyl)hydratopic acid		- Started to form after 10 days of the test, with increasing concentration until the end of experiment, - Probably a final product of macrobial transformation of ketoprofen through by-products, such as 3-((hydroxy-carboxymethyl)hydratopic acid, - Potentialy stable and persistent, - In parallel experiment with addition of external carbon source, ketoprofen was very stable a no degradation products were observed.	
Phenazone	1,5-Dimethyl-1,2-dehydro-3-pyrazolone	Batch tests with biologically active filter material (clay) from open bed filter used in drinking water purification. 5 g of clay mixed with 400 mL of water fortified with analytes (parent compounds or their metabolites), shaken for 168 h with sampling during test. Parallel test with inactivated material and material unpolluted with phenazone residues were performed.	- Formed during batch test with phenazone and filter material with phenazone residues through biodegradation in small amount, quickly degraded, - Quick and full degradation during batch test with DP and filter material with phenazone residues, - Authors claim, that degradation of DP was observed also with the inactivated material, suggesting abiotic transformation, but no supporting data is showed, - Despite observed full degradation in batch test, found in significant amount in purified water from big-scale water treatment facility using the same filter material – indication of too short contact time, which was confirmed with low velocity tests.	(Zuehlke et al., 2007)
Propyphenazone	4-(2-Methylethyl)-1,5-dimetyl-1,2-dehydro-3-pyrazolone		- Formed during batch test with propyphenazone and filter material with phenazone residues through biodegradation in small amount, quickly degraded, - Quick and full degradation during batch test with PDP and filter material with phenazone residues, - Authors claim, that degradation of PDP was observed also with the inactivated material, suggesting abiotic transformation, but no supporting data is showed, - Despite observed full degradation in batch test, found higher amount in purified water from big-scale water treatment facility using the same filter material than raw water– indication of too short contact time, which was confirmed with low velocity tests.	
Dimethylaminophenazone	1-Acetyl-1methyl-2-phenylhydrazide		- Formed during batch test with dimethylaminophenazone and filter material with phenazone residues, detected during entire experiment on constant level, - Complete degradation during batch test within 168 h, - Despite observed full degradation in batch test, found at low levels in purified water from big-scale water treatment facility using the same filter material – indication of too short contact time, which was confirmed with low velocity tests.	
	1-Acetyl-1-methyl-2-dimethyloxamoyl-2-phenylhydrazide		- Formed during batch test with dimethylaminophenazone and filter material with phenazone residues, detected during entire experiment on constant level, - Completely stable during batch test – high persistency, - Found in purified water from big-scale water treatment facility using the same filter material at the same level as in the raw water.	
Dimethylaminophenazone or metamizole	Acetoaminoantipyrine		- Formed in very small amount in a batch test with dimethylaminophenazone and disappeared by the end of the test, - Fully degraded in the batch test.	
	Formylaminoantipyrine		- Formed in very small amount in a batch test with dimethylaminophenazone and disappeared by the end of the test, - Fully degraded in the batch test.	
Metamizole (dipyrone)	4-Methylaminoantipyrine	Experiments in milliQ water, synthetic seawater	- Very fast degradation at any conditions; k [1/h]: 1.20–5.89, $t_{1/2}$ [h]: 0.12–0.58, - No hydrolysis observed in control sample,	(Gómez et al., 2008)

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Table 1 (*continued*)

Parent compound (PC)	Transformation product (TP)	Experimental/matrix	Results/conclusions	Ref.
		and synthetic freshwater. Concentrations of 10 and 50 mg/L, different irradiation intensity (250 and 756 W/m^2), different temp. (17 and 40 °C). Control sample in the dark. Additional sample of 10 mg/L of analyte and humic acid/nitrate.	- Fastest degradation in seawater, then fresh water, then milliQ water, - Higher temperature and irradiation increases degradation rates, - No indirect photolysis; addition of humic acids and nitrate did not affect degradation rates.	
	Acetoaminoantipyrine (4-acetyloaminoantipyrine)	Synthetic freshwater, 10 mg/L, irradiation 250 W/m^2, 25–30 °C.	- Rate constant *k* 0.025 [1/h], - $t_{1/2}$ 27.9 [h].	
	Formylaminoantipyrine (4--formylaminoantipyrine)		- Rate constant *k* 0.028 [1/h], - $t_{1/2}$ 24.7 [h].	
Antibiotics				
Metronidazole	Hydroxy metronidazole	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 4 and 7 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C. - Mildly unstable at pH 9: ➢ at 70 °C $k_{obs} = 2.5 \times 10^{-2}$ [1/h], $t_{1/2} = 27.3$ [h]; ➢ at 50 °C $k_{obs} = 3.4 \times 10^{-3}$ [1/h], $t_{1/2} = 203.4$ [h]; ➢ at 20 °C no degradation observed in 30 days.	(Tonski et al., 2019)
Sulfamethoxazole	N^4-acetylsulfamethoxazole	Spiked sewage sludge.	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C. - No N^4-acetylsulfamethoxazole detected in the sludge after spiking, while amount of sulfamethoxazole increased; it suggests biological transformation back to the parent compound.	(Göbel et al., 2005)

		Direct photolysis with Solar Simulator Setup with light from above at pH 3.2 and 8.4, 19 °C, 24 h. Additional setup with Rayonet to explore degradation products – much higher conc.	pH 3.2: $t_{1/2} = 0.111$ [h];
			pH 8.4: $t_{1/2} = 3.43$ [h];
			pH 9.2 (Rayonet): 6.14 [h].
				(Bonvin et al., 2013)

	4-Nitrososulfamethoxazole		pH 3.2: $t_{1/2} = 2.29$ [h];
			pH 8.4: $t_{1/2} = 3.87$ [h];
			pH 9.2 (Rayonet): 1.10 [h].	

	4-Nitrosulfamethoxazole		pH 3.2: $t_{1/2} = 11.3$ [h];
			pH 8.4: $t_{1/2} = 79.3$ [h];
			pH 9.2 (Rayonet): no degradation.	

	Sulfamethoxazole β-D-glucuronide		pH 3.2: $t_{1/2} = 0.042$ [h];
			pH 8.4: $t_{1/2} = 0.043$ [h];
			pH 9.2 (Rayonet): 0.045 [h].	

Chlortetracycline	4-Epi-chlortetracycline	Influence of light, oxygen, temperature (6 and 20 °C), pH (3–9) and matrix (milliQ water and soil interstitial water) on chemical stability.	- No significant light influence on degradation, - Faster degradation in soil interstitial water at all pH levels, especially acidic, - No influence of oxygen presence or absence during test, - Higher degradation rates at 20 °C than 6 °C, - Much higher stability at acidic pH.	(Søeborg et al., 2004)
	Anhydro-chlortetracycline		- Significant influence of light on disappearance, - Ambiguous data on matrix effect, in many cases more stable in natural water, - No influence of oxygen presence or absence during test, - Higher degradation rates at 20 °C than 6 °C, - Lowest stability at pH 5.6, higher at more acidic and alkaline conditions.	
	4-Epi-anhydro-chlortetracycline		- Significant influence of light on disappearance from 5.6 to alkaline conditions, - No significant matrix effects influence; however, more stable in natural water below pH 8.5, - No influence of oxygen presence or absence during test, - Higher degradation rates at 20 °C than 6 °C, - Much lower stability at high pH values.	

Sulfapyridine	N^4-acetylsulfapyridine	HPLC grade water and WWTP effluent spiked, incubated for 100 h in SunTest CPS instrument, which imitates real sunshine. 450 W/m^2, 200–800 nm.	➢ HPLC grade water: - k [1/h]: 2.8, - $t_{1/2}$ [h]: 0.25, - 99.9% removal after 5 h. ➢ WWTP effluent: - k [1/h]: 0.8, - $t_{1/2}$ [h]: 0.86, - 98.7% removal after 5 h.		(García-Galán et al., 2012a, 2012b)
Anticonvulsants					
Carbamazepine	10-Hydroxy carbamazepine 2-Hydroxy carbamazepine Carbamazepine-10,11-epoxide	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C. - Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C. - Stable at pH 7 and 9 in 50 °C for 5 days, $t_{1/2} > 1$ year at 25 °C. - Unstable at pH 4: ➢ at 70 °C $k_{obs} = 3.6 \times 10^{-1}$ [1/h], $t_{1/2} = 1.9$ [h]; ➢ at 50 °C $k_{obs} = 1.0 \times 10^{-1}$ [1/h], $t_{1/2} = 7.0$ [h]; ➢ at 20 °C $k_{obs} = 3.5 \times 10^{-3}$ [1/h], $t_{1/2} = 197.0$ [h]; Extrapolated: ➢ at 15 °C $k_{obs} = 2.2 \times 10^{-3}$ [1/h], $t_{1/2} = 315.1$ [h]; ➢ at 10 °C $k_{obs} = 1.2 \times 10^{-3}$ [1/h], $t_{1/2} = 563.2$ [h]; ➢ at 4 °C $k_{obs} = 9.7 \times 10^{-4}$ [1/h], $t_{1/2} = 714.5$ [h]. • $E_a = 78.8$ kJ/mol. - Formed in quite low concentrations from carbamazepine with increasing concentration throughout entire experiment, which indicates that it was stable in tested conditions and may be mobile in soil.	(Tonski et al., 2019)
		13 types of soil, 50 g of each put in a plastic flask and spiked with parent compound. Incubated at 20 °C in dark for max. 61 days.			(Koba et al., 2016)
		Experiments conducted in flume filled with water and sediments from a lake. Water was recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples of water from upper and lower levels as well as from pores in sediments were taken at time intervals. TPs formation was monitored. Test duration: 30 days.	- Formed from carbamazepine probably in the sediment water; however, found in similar concentrations in surface water, which indicates high mobility from sediments to surface water. - The concentration has been growing through entire test (30 days). Nevertheless, carbamazepine was degrading very slowly, so the growing concentration could be due to either high stability of carbamazepine epoxide, constant transformation or both.		(Li et al., 2015)
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals	- Started to be detected after a week of experiment, with increasing concentration until the termination of the test.		(Li et al., 2014)
		Plasma separated from human blood from patients treated with	- No degradation was observed at −20 °C and 4 °C for three months, - Around 50% degradation after 2 months and over 70% after 3 months was observed, probably due to microbial processes, which resulted from fermentation of samples at room		(Mendez-alvarez et al., 1986)

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Table 1 (*continued*)

Parent compound (PC)	Transformation product (TP)	Experimental/matrix	Results/conclusions	Ref.
		carbamazepine. Sample stored at −20 °C, 4 °C and room temperature for 3 months.	temperature.	
	10,11-dihydroxy carbamazepine	13 types of soil, 50 g of each put in a plastic flask and spiked with parent compound. Incubated at 20 °C in dark for max. 61 days.	- Formed from carbamazepine only in selected soils in low concentration; however it grew throughout entire test, which may indicate high stability of the compound.	(Koba et al., 2016)
Opioids				
Tramadol (beta-blocker)	*O*-desmethyltramadol	Pure, autoclaved water with pH adjusted to 4, 7 and 9 with buffers; samples incubated in dark.	- Stable at pH 4, 7 and 9 in 50 °C for 5 days, $t_{1/2}$ > 1 year at 25 °C.	(Tonski et al., 2019)
Others				
Atenolol (beta-blocker)	Atenolol acid	13 types of soil, 50 g of each put in a plastic flask and spiked with parent compound. Incubated at 20 °C in dark for max. 61 days.	- Quick formation in each soil from atenolol and quite quick degradation in almost all soils, which indicates low stability.	(Koba et al., 2016)
Metoprolol (beta-blocker)	Metoprolol acid	Experiments conducted in flume filled with water and sediments from a lake. Water was recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples of water from upper and lower levels as well as from pores in sediments were taken time intervals. TPs formation was monitored. Test duration: 30 days.	- Quick formation in most of the soils from metoprolol and significant degradation in almost all soils during test. - Quickly created in sediment pore water, exhibited high mobility to the surface water. Approximately midway the test, after reaching maximum concentration, concentration decrease was observed in surface and sediment pore water.	(Li et al., 2015)
	α-Hydroxymetoprolol	Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals	- The most abundant among all transformation products of metoprolol found in this study, - Nevertheless, after 20 days of experiment quick disappearance was observed, indicating limited persistence. - Its amount was increasing during first 10 days, then started to decrease; formation of other transformation products α-ketometoprolol and one unknown during disappearance of α-hydroxymetoprolol suggests, that this compound may be a precursor for other transformation products.	(Li et al., 2014)
	α-Ketometoprolol		- Its abundance was increasing for around 15 days of the experiment, to achieve constant level afterwards, - It is possible that it is formed from α-hydroxymetoprolol, which disappears during the experiment, while α-ketometoprolol amount increases. - Potentially stable. - Formed gradually during entire experiment.	
Propranolol (beta-blocker)	1-Naphthol	Experiments conducted in flume filled with water and sediments from a lake. Water was	- Formed in sediment and transported to the surface water, - Quick degradation, completely removed at the end of the test.	(Li et al., 2015)

		recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples of water from upper and lower levels as well as from pores in sediments were taken time intervals. TPs formation was monitored. Test duration: 30 days.		
	Bezafibrate (lipid-lowering agent)	Experiments conducted in flume filled with water and sediments from a lake. Water was recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples of water from upper and lower levels as well as from pores in sediments were taken time intervals. TPs formation was monitored. Test duration: 30 days.	- Quick formation from bezafibrate and quick elimination within several days after reaching maximum concentration. - There was observed formation in control experiments with water-only system and sterile water and sediments so abiotic formation is also possible.	(Li et al., 2015)
	4-Chlorobenzoic acid	Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals	- Quick formation with maximum concentration after around 15 days, then fast disappearance within 5 days.	(Li et al., 2014)
		1 L of deionized water with 5 mg of solid form of PC was mixed with 10 mg/L of fresh sludge from a membrane bioreactor used to treat wastewater. Mixture kept in glass bottle for 28 days. External carbon source was added to investigate co-metabolism of parent compound, since no degradation occurred without it.	- Formed very quickly, with maximum after 4 days, after that a rapid, complete degradation within two days occurred – high vulnerability to biodegradation.	(Quintana et al., 2005)
	Furosemide (diuretic)	Saluamine	Experiments conducted in flume filled with water and sediments from a lake. Water was recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples	- Formed in sediment, transported to surface water, - Concentration was increasing during entire test, potentially stable.	(Li et al., 2015)

(continued on next page)

Table 1 (*continued*)

Parent compound (PC)	Transformation product (TP)	Experimental/matrix	Results/conclusions	Ref.
		of water from upper and lower levels as well as from pores in sediments were taken time intervals. TPs formation was monitored. Test duration: 30 days.		
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals	- Observed from the beginning of the test, - Constant amount increase throughout entire experiment.	(Li et al., 2014)
Hydrochlorothiazide (diuretic)	4-Amino-6-chloro-1,3-benzenedisulfonamide	Experiments conducted in flume filled with water and sediments from a lake. Water was recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples of water from upper and lower levels as well as from pores in sediments were taken time intervals. TPs formation was monitored. Test duration: 30 days.	- Formed in water-only control experiment, sterile control experiment and in the appropriate test, which indicates abiotic formation from hydrochlorothiazide, - Concentration was increasing during entire test	(Li et al., 2015)
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals	- Probable hydrolysis and biotransformation product of hydrochlorothiazide transformation, - Accumulated throughout entire experiment.	(Li et al., 2014)
	Chlorothiazide	Experiments conducted in flume filled with water and sediments from a lake. Water was recirculated to imitate conditions in a stream. Water was spiked with pharmaceuticals, samples of water from upper and lower levels as well as from pores in sediments were taken time intervals. TPs formation was	- Formed in water-only control experiment, sterile control experiment and in the appropriate test, which indicates abiotic formation from hydrochlorothiazide, - Concentration was increasing during entire test.	(Li et al., 2015)

		monitored. Test duration: 30 days.		
		Sediments collected from rivers mixed with artificial river water spiked with 9 parent compounds – incubated in glass bottles (200 g sediment + 600 mL artificial river water) in the dark in 15 °C for 35 days. Samples collected in time intervals	- Accumulated through entire batch test, - Probably a product of hydrolysis, potential intermediate product during hydrochlorothiazide degradation; however, faster formation with microbial presence, indicating 2 routes of formation.	(Li et al., 2014)
	Albendazole (antiparasitic)	Ricobendazole (albendazole sulfoxide)	Pure water with phosphate buffers	- Degradation followed pseudo first-order kinetics in the pH range 2–12 and temp. 55 °C, - V-shaped pH rate profile, with slowest degradation at pH 4.8 ($k_{obs} = 2.4 \times 10^{-4}$ [1/day], $t_{1/2} = 96$ months), - More rapid degradation at basic conditions, - E_a at pH 11 was ~80 kJ/mol.	(Wu et al., 2009)

source or occurrence. Therefore in our paper, all of the available worldwide literature data on the stability and (eco)toxicity of selected TPs of pharmaceuticals have been presented in Tables 1 and 2, respectively. This data has been however discussed for each therapeutic group separately. Ultimately, our work aims to identify key knowledge gaps in this field, which will be useful in taking the correct scientific path to fully understand the consequences of the presence of these compounds in surface waters.

2. State of knowledge on the environmental fate and effects of pharmaceuticals' TPs

2.1. TPs of selective serotonin reuptake inhibitors (SSRIs)

SSRIs are one of the most commonly prescribed pharmaceuticals around the world. These compounds are used in the treatment of depressive disorders and work by inhibiting the reuptake of serotonin from cell membranes (Carlsson, 1999). The most commonly prescribed SSRIs include fluoxetine, sertraline, venlafaxine and citalopram, which correspond to the predominant metabolites: norfluoxetine, desmethylsertraline, desmethylvenlafaxine desmethylcitalopram, and didesmethylcitalopram. Although there are no conclusive data on this, numerous studies suggest that even more than 90% of SSRI drugs are excreted as metabolites (e.g. Vaswani et al., 2003; Calisto and Esteves, 2009). It must be also noted that in 2020 metabolite of venlafaxine, *O*-desmethylvenlafaxine was added to the 3rd Watch List (Commission Implementing Decision (EU) 2020/1161), which highlights the importance and necessity for monitoring the presence not only of the parent compounds but also their metabolites in the environmental waters, with special attention to this specific group of pharmaceuticals.

2.1.1. Transformation in the environment

The work of Mathon et al. (2021) showed that of the 36 different micropollutants analysed, norfluoxetine is one of the most resistant to photodegradation. Under laboratory conditions, the half-life of this compound was 94.7 h, classifying this compound in the slow photodegradation group. Similar results were obtained in the work of Kwon and Armbrust (2005), where the stability and environmental fate of fluoxetine and norfluoxetine were analysed. The study showed that norfluoxetine is resistant to hydrolytic processes, but its photodegradation is more rapid than that of fluoxetine (the half-life was set at 9 days). The analyses confirmed that norfluoxetine may be formed from fluoxetine in the environment and then converted into further photoproducts, which, however, were not identified.

On the other hand, the work of Rühmland et al. (2014) analysed the fate of three venlafaxine transformation products: *N*-desmethylvenlafaxine, *N*,*O*-didesmethylvenlafaxine and *O*-desme thylvenlafaxine. In this study it was shown that all these compounds, like the parent compound, are susceptible to photodegradation, which occurs in the layer up to 20 cm from the water surface, while at greater depths it decreases dramatically.

2.1.2. (Eco)toxicity and bioconcentration

In the study of Rodrigues et al. (2020) a wide range of molecular markers was used to evaluate the toxicity of norfluoxetine alone and in combination with venlafaxine in zebrafish larvae. However, no effect of norfluoxetine on the expression of the genes studied was noted at any of the concentrations tested (ranging from 0.64×10^{-6} to 400×10^{-6} mg/L). On the other hand, analyses showed that at 80 h post-fertilization, all metabolite concentrations had a statistically significant effect on the number of malformations in embryos, but this effect was less drastic than for the parent compound, as shown in other work (Rodrigues et al., 2020). Additionally, although neither norfluoxetine nor venlafaxine individually caused increased mortality in larvae, statistically significant changes in mortality were noted for their mixture at environmentally relevant concentration (3.2×10^{-6} mg/L of

Table 2
Toxic effects of transformation products of pharmaceuticals.

Parent compound	Transformation product	Tested concentration	Tested organism	Effects	OECD/ISO	References
SSRIs						
Fluoxetine	Norfluoxetine	6.4×10^{-7}–4×10^{-4} mg/L	*D. rerio*	Increased number of malformations in embryos	OECD 212; OECD 236	Rodrigues et al. (2020)
			D. rerio	Changes in locomotor performance	–	Huang et al. (2019)
		1.1×10^{-5}–1.1×10^{-3} mg/L	*D. magna*	Changes in cholinesterase activity	OECD 202	Över et al. (2020)
Sertraline	Norsertraline		*D. rerio*	Changes in locomotor performance	–	Huang et al. (2019)
Venlafaxine	Des-venlafaxine		*D. rerio*	Changes in locomotor performance	–	Huang et al. (2019)
Anticancer drugs						
Tamoxifen	4-Hydroxytamoxifen	1.8×10^{-3}–9.89×10^{-2} mg/L	*D. magna*	NOEC for reproduction = 1.8×10^{-3} mg/L	OECD 211	Borgatta et al. (2015)
		1.6×10^{-4}–7.299×10^{-2} mg/L	*D. magna*	Size reduction, Morphological abnormalities	OECD 211	Borgatta et al. (2016)
	Endoxifen	4×10^{-4}–2.024×10^{-1} mg/L	*D. magna*	NOEC for reproduction = 4.3×10^{-3} mg/L	OECD 211	Borgatta et al. (2015)
5-Fluorouracil	a-fluoro-b-alanine	Up to 500 mg/L	*P. putida, D. subspicatus, D. magna*	Non-toxic	OECD 211	Zounkova et al. (2010)
Cytarabine	Uracil-1-b-D-arabinofuranoside			Non-toxic		
Gemcitabine	20,20-Difluorodeoxyuridine			Non-toxic		
Methotrexate	7-Hydroxymethotrexate	Up to 10 mg/L	*V. fischeri*	$EC_{50} \geq 10$ mg/L	–	Bialk-Bielinska et al. (2017a, 2017b)
			R. supcapitata	$EC_{50} \geq 10$ mg/L		
			L. minor	$EC_{50} = 8.3$ mg/L		
Cyclophosphamide & Iphosphamide	Carboxycyclophosphamide	3.05–320 mg/L	*S. leopoliensis*	$EC_{50} = 17.1$ mg/L	OECD 201	Cesen et al. (2016)
			S. typhimurium	Genotoxic		
		Up to 100 mg/L	*C. dubia*	Non-toxic	ISO 20666; ISO 20665	Russo et al. (2018)
			B. calyciflorus	$EC_{50} = 23.66$ mg/L		
	4-Ketocyclophosphamide	3.05–320 mg/L	*S. leopoliensis, S. typhimurium*	Non-toxic	OECD 201	Cesen et al. (2016)
		Up to 100 mg/L	*C. dubia, B. calyciflorus*	Non-toxic	ISO 20666; ISO 20665	Russo et al. (2018)
	3-Dechloroethylifosfamide	3.05–320 mg/L	*S. leopoliensis, S. typhimurium*	Non-toxic	OECD 201	Cesen et al. (2016)
		Up to 100 mg/L	*C. dubia, B. calyciflorus*	Non-toxic	ISO 20666; ISO 20665	Russo et al. (2018)
Antibiotics						
Amoxicillin	Amoxicilloic acid	10 mg/L	*C. carpio*	Oxidative stress	–	Elizalde-Velázquez et al. (2017)
Clarithromycin	14-Hydroxy(*R*)-clarithromycin	2 mg/L	*D. rerio*	$EC_{50} \geq 2$ mg/L	DIN EN ISO 15088	Baumann et al. (2015)
			D. magna	$EC_{50} \geq 2$ mg/L	OECD 211	
			D. subspicatus	$EC_{50} = 4.63 \times 10^{-2}$ mg/L	DIN EN ISO 8692	
			Flos-aquae	$EC_{50} = 2.72 \times 10^{-2}$ mg/L	OECD 201	
	N-desmethyl-clarithromycin	2 mg/L	*D. rerio*	$EC_{50} \geq 2$ mg/L	DIN EN ISO 15088	Baumann et al. (2015)
			D. magna	$EC_{50} = 0.7$ mg/L	EN ISO 6341-L40	
			D. subspicatus	$EC_{50} = 5.752 \times 10^{-2}$ mg/L	DIN EN ISO 8692	
			Flos-aquae	$EC_{50} = 0.134$ mg/L	OECD 201	
Sulfamethoxazole	Sulfanilic acid	Up to 100 mg/L	*D. magna*	$EC_{50} = 90$ mg/L	–	Trovó et al. (2009)
		0.1–100 mg/L	*S. vacuolatus*	Non-toxic	–	Bialk-Bielinska et al. (2017a, 2017b)
			L. minor	Non- toxic	OECD 221	
	Sulfanilamide	0.1–100 mg/L	*S. vacuolatus*	$EC_{50} = 25.89$ mg/L	–	Bialk-Bielinska

			L. minor	EC_{50} = 5.09 mg/L	OECD 221	et al. (2017a, 2017b)
	3-Amino-5-methylisoxazole	Up to 100 mg/L	*D. magna*	EC_{50} = 100 mg/L	–	Trovó et al. (2009)
	N4-acetylsulfamethoxazole	5 mg/L	*R. subcapitata*	Growth inhibition (27%)	OECD 201	Grabarczyk et al. (2020)
		3 mg/L	*L. minor*	Growth inhibition (17%)	OECD 221	
		3 mg/L	*V. fischeri*	Luminescence inhibition (8%)	ISO11348-3:2007	
Sulfapyridine	N4-acetylsulfapyridine	48 mg/L	*V. fischeri*	EC_{50} = 8.2 mg/L	EU-EN ISO 11348	García-Galán et al. (2012a, 2012b)
Tetracycline	Mixture of photolysis TPs	20 mg/L	*V. fischeri*	Immobilization (49%)	–	Jiao et al. (2008)
Antiepileptic drugs						
Carbamazepine	Acridine		*V. fischeri*	EC_{50} = 5.34–6.90 mg/L	ISO 11348-3	Donner et al. (2013)
			P. subcapitata	EC_{50} = 0.61 mg/L	ISO 8692	Donner et al. (2013)
		1–30 mg/L[b]	*D. magna*	EC_{50} = 0.71 mg/L[a]	ISO 6341	[a]Donner et al. (2013)
				EC_{50} = 4.6 mg/L[b]	ISO 6341	[b]Eisentraeger et al. (2008)
		1–30 mg/L[a]	*Algae (multiple spieces studied)*	EC_{50} = 2.1 mg/L[a*]	ISO 8692	[a]Eisentraeger et al. (2008)
		0.04–10 mg/L[b]		EC_{50} = 0.08–0.79 mg/L[b*]	–	[b]Dijkman et al. (1997)
	Acridone	0.16–2.5 mg/L	*D. polymorpha*	EC_{50} = 0.96 mg/L	–	Kraak et al. (1997)
			D. magna	EC_{50} = 1.49 mg/L	ISO 6341	Donner et al. (2013)
	Carbamazepine-10-11-epoxide	100 mg/L	*V. fischeri*	Luminescence inhibition (30%)	ISO11348-3:2007	Grabarczyk et al. (2020)
		50 mg/L	*L. minor*	Growth inhibition (32%)	OECD 221	Grabarczyk et al. (2020)
		0.17–1.2 mg/kg	*Ch. riparius*	LC_{50} = 0.2 mg/kg	OECD 233	Heye et al. (2016)
	10,11-Dihydro-10-hydroxycarbamazepine	50 mg/L	*L. minor*	Growth inhibition (11%)	OECD 221	Grabarczyk et al. (2020)
	10,11-Dihydro-10,11-dihydroxycarbamazepine	0.17–1.2 mg/kg	*Ch. riparius*	Non-toxic	OECD 233	Heye et al. (2016)
NSAIDs and analgesic						
Ibuprofen	4-Acetylbenzoic acid	15.6 mM–1 mM; 10 mM*	*Chlorella* sp., *B. megaterium* and *P. atlantica*	Growth stimulation of *B. megaterium* and *P. atlantica* by MpI	–	Rubasinghege et al. (2018)
	Non identified mixture of phototransformation products (MpI)			significant affection of *Chlorella* sp. viability by 4-acetylbenzoic acid		
	2-hydroxyibuprofen; carboxyibuprofen	15 mg/L *L. minor* test; 10 mg/L *V. fischeri* test; 100 mg/L *R. subcapitata* test; 50 mg/L *D. magna* test	*V. fischeri*, *D. magna*, *L. minor* and *R. subcapitata*	Slight inhibition of *L. minor* and *R. subcapitata* growth; luminescence of *V. fischeri* and slight immobilization of *D. magna*	OECD 201; OECD 202; OECD 221	Grabarczyk et al. (2019)
Diclofenac	Non identified mixture of phototransformation products (MpI)	50 mg/L*	*S. vacuolatus*	Significant inhibition of *S. vacuolatus* growth in a concentration- and time-dependent manner; 5–6 times higher toxicity after 53 h of diclofenac irradiation	–	Schmitt-Jansen et al. (2007)
	F1–F6 fractions	50 mg/L*	*S. vacuolatus*	Fraction F5 (CPAB) inhibited algal growth by 92% and mixture of fractions by 73.1%. Calculated EC_{50} for CPAB was 4.8 mg/L	–	Schulze et al. (2010)
	F5 - 2-[(2-chlorophenyl)amino]benzaldehyde (CPAB)	0.49–250.0 mg/L for DIC 0.1–98.91 mg/L for CPAB				
	4-Hydroxydiclofenac	15 mg/L *L. minor* test; 10 mg/L *V. fischeri* test; 50 mg/L *R. subcapitata* test; 60 mg/L *D. magna* test	*V. fischeri*, *D. magna*, *L. minor* and *R. subcapitata*	Significant inhibition of *R. subcapitata* growth and *V. fischeri* luminescence; slight immobilization of *D. magna*	OECD 201; OECD 202; OECD 221	Grabarczyk et al. (2019)

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Table 2 (*continued*)

Parent compound	Transformation product	Tested concentration	Tested organism	Effects	OECD/ISO	References
Naproxen	Photoproducts 1–4 (P1, P2, P3, P4)	8×10^{-4} M*	*P. subcapitata*, *B. calyciflorus*, *T. platyurus* and *C. dubia*	Chronic and acute test showed toxic effect of 4 identified photoproducts of diclofenac on all selected model organisms. Acute LC_{50} (mg/L) for *T. platyurus* and *B. calyciflorus* P1-9.45; 8.23 P2-11.37; 5.30 P3-14.46; 14.01 P4-4.51; 11.63 Acute EC_{50} (mg/L) for *C. dubia* P1-16.70 P2-10.09 P3-16.49 P4-6.30	–	Isidori et al. (2005)
	Photoproducts 1–7 (P1, P2, P3, P4, P5, P6, P7)	7.8×10^{-4} M*	*D. magna*, *V. fischeri*	EC_{50} (mg/L) for *D. magna* and *V. fischeri*, respectively P1-59.44; 42.95 P2-12.61; 20.61 P3-13.65; 16.17 P4-10.51; NE 50 P5-6.43; 30.41 P6-NE 60; ND P7-50.00; ND	–	DellaGreca et al. (2004)
	Mixture of phototransformation products (MpI) (P1–P7)	50 mg/L*	*V. fischeri*	EC_{20} value (mg/L) decreases with the increasing time of naproxen irradiation from 11.7 at t0 to 7.2 at 80 min.	–	Cazzaniga et al. (2020)
Acetylsalicylic acid	Salicylic acid	0.004, 0.04, 0.4, 4 and 20 mg/L	*C. carpio*	Oxidative stress; lipid peroxidation; hatching disruption; early ontogeny; the condition decrease; changes in skin tissues	OECD 210	Zivna et al. (2015)
		4 mg/L	*M. galloprovincialis*	Influence the metabolic capacity; oxidative stress; loss of redox balance	–	Freitas et al. (2020),
		0.005; 0.05; 0.5 and 5 mg/L	*M. galloprovincialis*	Respiratory capacity and metabolism disruption; oxidative stress; neurotoxic effect	–	Freitas et al. (2019)
		5×10^{-2}–2.53125×10^{-1} mg/L	*H. diversicolor*	Lipid peroxidation; acetylcholinesterase activity disruption; oxidative stress	–	Nunes et al. (2018)
Opioids						
Tramadol	*O*-desmethyltramadol	100 mg/L *L. minor* test; 100 mg/L *V. fischeri* test; 100 mg/L *R. subcapitata* test; 70 mg/L *D. magna* test	*V. fischeri*, *D. magna*, *L. minor* and *R. subcapitata*	Significant inhibition of of *R. subcapitata* growth; slight immobilization of *D. magna*	OECD 201; OECD 202; OECD 221	Grabarczyk et al. (2019)
	N-oxidetramadol and *N*-desmethyltramadol	2×10^{-3} mg/L (N-oxidetramadol) 5.2×10^{-2} mg/L (*N*-desmethyltramadol)	*V. fischeri*	Slight inhibition of *V. fischeri* growth by both tested metabolites	–	Antonopoulou and Konstantinou (2016)
	N-oxidetramadol, *N*-desmethyltramadol and tramadol mixture and mixture of phototransformation products (MpI)	10 mg/L* and 0.1 mg/L*	*V. fischeri*	Significant inhibition of *V. fischeri* growth by MpI and mixture of N-oxidetramadol, *N*-desmethyltramadol and tramadol; slight inhibition of *V. fischeri* by N-oxidetramadol and *N*-desmethyltramadol separately	–	Antonopoulou et al. (2020)
	O-desmethyltramadol, *N*-desmethyltramadol	1×10^{-3}, 10×10^{-3}, and 100×10^{-3} mg/L*	*Pimephales promelas*	Metabolism of tramadol to *O*-desmethyltramadol (0.88 ± 0.60 ng/mL and 3.8 ± 0.99 ng/mL) and *N*-desmethyltramadol (0.48 ± 0.21 ng/mL, 1.2 ± 0.43 ng/mL, and 7.2 ± 1.7 ng/mL) – metabolites were detected in fish plasma	–	Tanoue et al. (2017)
Others						
Thioridazine	Thioridazine-2-sulfoxide		*V. fischeri*	EC_{50} = 18.1–211.2 μmol/L	–	Wilde et al. (2016)
	Thioridazine-5-sulfoxide		*V. fischeri*	$EC_{50} \geq 320$ μmol/L	–	Wilde et al. (2016)

Metoprolol	Metoprolol acid	100 mg/L	*D. magna*	Non-toxic	OECD 202	Grabarczyk et al. (2020)
		100 mg/L	*V. fischeri*	Non- toxic	ISO11348-3:2007	Grabarczyk et al. (2020)
		100 mg/L	*L. minor*	Growth inhibition (5%)	OECD 221	Grabarczyk et al. (2020)
		100 mg/L	*R. subcapitata*	Non-toxic	OECD 201	Grabarczyk et al. (2020)
Clofibrate	Clofibric acid	≤1000 mg/L	*D. tertiolecta*	Non-toxic	–	Embldge and DeLorenzo (2006)
		≤1000 mg/L	*P. pugio*	Non-toxic	–	Embldge and DeLorenzo (2006)
		≤1000 mg/L	*F. heterocliticus*	Non-toxic	–	Embldge and DeLorenzo (2006)
		Up to 200 mg/L	*D. magna*	$EC_{50} \geq 200$ mg/L	–	Ferrari et al. (2003)
		Up to 200 mg/L	*C. dubia*	$EC_{50} \geq 200$ mg/L	–	Ferrari et al. (2003)
Dexamethasone	Different photo-TPs	50 mg/L	*D. magna*	EC_{50} = 10.88–48.30 mg/L	ISO 6341-ASTM E1440-91	DellaGreca et al. (2004)
			T. platyurus	LC_{50} = 20.9–60.11 mg/L		
			B. calyciflorus	LC_{50} = 18.20–48.22 mg/L		
Prednisolone	Different photo-TPs	50 mg/L	*D. magna*	EC_{50} = 1.79–17.88 mg/L	ISO 6341-ASTM E1440-91	DellaGreca et al. (2004)
			T. platyurus	LC_{50} = 0.71–40.77 mg/L		
			B. calyciflorus	LC_{50} = 1.43–35.46 mg/L		

norfluoxetine and 2000×10^{-6} mg/L of venlafaxine). This means that these compounds, although harmless on their own, may be much more harmful in an environmental situation, where they occur in mixtures.

In another study, Över et al. (2020) analysed the effects of norfluoxetine and fluoxetine on *Daphnia magna*. Environmentally relevant concentrations of 0.011×10^{-3} mg/L norfluoxetine and 0.091×10^{-3} mg/L fluoxetine were analysed, as well as their tenfold and one hundredfold concentrations. Already at the environmental concentration, a statistically significant effect of norfluoxetine was observed on cholinesterase activity – an indicator of apoptosis. In contrast, for the other markers, Gpx (glutathione peroxidase), TBARS (thiobarbituric acid reactive substances) and total protein content, no effect was observed at the environmental concentration, but statistically significant changes were noted at higher concentrations, especially 100-fold. Additionally, at environmental concentrations of both norfluoxetine and fluoxetine, statistically significant reproduction increase in four consecutive generations was noted. The effect of both drug and metabolite on changes in carapace length was also observed.

A study performed by Fong and Molnar (2008) showed that norfluoxetine, at concentrations as low as 5 μM, can induce spawning in the freshwater bivalves *Dreissena polymorpha* and *Mytilopsis leucophaeata*. It is worth noting, however, that the parent compound, fluoxetine, caused the same effects at concentrations 10 times lower. These studies confirm that both fluoxetine and its main metabolite can induce reproductive processes in bivalves.

Also Neuwoehner et al. (2009) analysed the effects of fluoxetine metabolites on aquatic organisms. In tests using green algae *Scenodesmus vacuolatus*, the toxicity of norfluoxetine was shown to be similar to that of the native compound, while the toxicity of p-trifluoromethylphenol was much lower. The study also examined the effects of mixtures of the native compound with metabolites, resulting in a concentration-additive effect.

In the work by Huang et al. (2019), the effects of three metabolites (norfluoxetine, des-venlafaxine and norsertraline) of SSRIs on *Danio rerio* larvae were analysed. The obtained results in this study proved that these major metabolites of commonly consumed antidepressants can significantly affect locomotor performance in fish larvae, and this effect may be at a similar level to the parent compounds.

The potential for bioaccumulation of fluoxetine metabolites was analysed in the work of Zindler et al. (2020), where *D. rerio* larvae were exposed to fluoxetine at three concentrations: 0.01, 0.05, and 5 mg/L. Interestingly, it was observed that after 96 h of exposure and then 24 h of depuration, norfluoxetine concentrations in larval tissues were higher than for the native compound. This situation occurred in organisms exposed to 0.01 and 0.05 mg/L. In contrast, a much faster accumulation of the native compound was observed at the highest concentration, most likely due to the limited metabolic capacity. However, this work confirms that even the early life stages of aquatic vertebrates can metabolize fluoxetine and accumulate its major metabolite in tissues. Similarly, in a study by Nakamura et al. (2008) in which juvenile Japanese medaka (*Oryzias latipes*) were exposed for 30 days to fluoxetine (0.01 mg/L), greater bioaccumulation of norfluoxetine was observed. The value of bioaccumulation factor (BAF) for this compound in the liver reached the value of 3700 at pH 9, 3300 at pH 8, and 1500 at pH 7. In this study, bioaccumulation increased with increasing pH, which may be due to the higher concentration of nonionized species at higher pH.

The bioaccumulation of metabolites of antidepressants in aquatic organisms was also confirmed in the environmental analysis. For example, Arnnok et al. (2017), who analysed tissues from 11 species of fish inhabiting the waters of the Niagara River, showed significant accumulation of norsetraline in the brains and livers of fish. Bioaccumulation factor (BAF) in these organs ranged from several hundred to several thousand (maximum 3000 in rudd liver). This is particularly noteworthy considering that no significant bioaccumulation of the native drug,

sertraline, was observed in any of the fish studied. Bioaccumulation of norfluoxetine was also analysed in this study, but was only observed in a few species, and the BAF generally did not exceed a value of 100. Also, Brooks et al. (2005) analysed the presence of antidepressant metabolites in three species of fish from the environment (Pecan Creek, Texas, USA). Although they did not determine BCF and did not analyse the concentration of the test compounds in water, it showed that both norsertraline and norfluoxetine reached higher concentrations in fish tissues than parent compounds, exceeding 10 ng/g for liver and brain. Similar concentrations were reported in fish tissues from the Parana and Acarangua rivers of Argentina, in which norfluoxetine reached concentrations ranging from 1.1×10^{-6} to 9.1×10^{-6} mg/g (Ondarza et al., 2019).

Also Lajeunesse et al. (2011) observed a significant bioconcentration of norsertraline in fish tissues. The BCF determined for brook trout was as high as 12,250 for liver and 2476 for brain. In the same work, bioconcentration of norfluoxetine was also observed in fish livers and the BCF was determined to be 1750. Nortriptyline was also analysed but was not observed in fish tissues.

An interesting study in the environmental context was performed by co-exposing fish to venlafaxine, *O*-desmethylvenlafaxine and microplastic (Qu et al., 2019). Although neither the parent compound nor the metabolite showed significant potential for bioconcentration (BCF < 1), when co-exposed with microplastic, the bioconcentration factor increased to over 20 for the metabolite (after 16, 25, and 40 days). This indicate that the compound may be adsorbed on microplastics present in the environment and thus enter and accumulate in fish tissues to a greater extent.

2.2. TPs of anticancer drugs

According to global estimates, cancer is the second most common cause of death in humans, and the incidence of these conditions is rising regularly (Ferlay et al., 2013). Therefore, it is expected that the consumption of anticancer drugs, being already high, will also increase rapidly in the coming years. Although anticancer drugs are a large and diverse group of pharmaceuticals, they include many compounds, which are extensively metabolized by humans and excreted mainly as metabolites. Up to 90% of the therapeutic dose is excreted as metabolites for such commonly used drugs as cytarabine (Balis et al., 1983), gemcitabine (Abbruzzese et al., 1991) or 5-fluorouracil (Heggie, 1985). Hence the presence in environment of the TPs of anticancer drugs (including metabolites and produced degradation products) is also of special concern.

2.2.1. Transformation in the environment

The stability of anticancer drugs under environmental conditions is still not fully evaluated; even less is known about the stability of their TPs. However, derivatives of cyclophosphamide and ifosfamide seem to be most thoroughly examined in the past century. It could be due to high popularity of these drugs in the past along with the acknowledged health repercussion of using them, which encouraged researchers to investigate their metabolites and degradation products.

Phosphoramide mustard, main alkylating metabolic product of cyclophosphamide, was found to be rather hydrolytically unstable in various conditions (Watson et al., 1985). Degradation rates were much lower at acidic pH, whereas at environmentally relevant conditions of pH 7.4 and 25 °C the half-life was only 93.2 min. However, it was found that presence of chloride ions causes a stabilization effect and half-life of a compound significantly increases. It results from the degradation pathway, in which the first step is a formation of an aziridine ion after Cl^- break off, which may be reversible; therefore, the addition of chloride ions slows down the hydrolysis of phosphoramide mustard.

2-Dechloroethylifosfamide, 3-dechloroethylifosfamide and 2,3-didechloroethylifosfamide have also been investigated. Kaijser et al. (1996) described their hydrolysis rates at high temperature (70 °C) and wide pH range of 1–13 to assess their degradation kinetics. Hydrolysis was pH-dependent. 2-dechloroethylifosfamide was more susceptible to hydrolysis at low pH, while the highest stability was observed at the pH 6 up to 11. However, at pH over 11 there was an increase in degradation rate. 3-dechloroethylifosfamide was more stable and there was a V-shaped curve of degradation rates at the measured pH range. High degradation rates were found in both acidic and alkaline conditions. These findings suggest, that there is an acid and base-catalysed hydrolysis. Gilard et al. (1999) conducted experiments in much more environmentally relevant temperatures (20 or 25 °C). They also confirmed that 3-dechloroethylifosfamide ($t_{1/2}$ = 49 days at pH 5.5, 20 °C) was more stable than 2-dechloroethylifosfamide ($t_{1/2}$ = 3.8 days at pH 5.5, 20 °C); moreover, 2,3-didechloroethylifosfamide was found to be the most unstable compound ($t_{1/2}$ = 32 min at pH 5.4, 25 °C). The enhanced stability of these compounds with pH becoming more neutral (from 3 to 6.8) indicated that they might be averagely persistent in the environment. Additionally, some hydrolysis products of these metabolites have been identified, but they did not possess antitumor properties and were less toxic than ifosfamide, which indicates that they will not be harmful to biota.

Other anticancer drugs' TPs were only investigated by Negreira et al. (2014), who investigated the TPs of tamoxifen: 4-hydroxy-*N*-desmethyltamoxifen (endoxifen) and (Z)-4-hydroxytamoxifen; methotrexate: 7-hydroxymethotrexate; temozolomide: 5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide; paclitaxel: 6(α)-hydroxypaclitaxel. Samples prepared in HPLC water and wastewater were incubated for 24 h at 4 °C. Moreover, samples in wastewater were also incubated for 3 months at −20, 4 and 25 °C with aliquots taken out at several time intervals. All samples were incubated in amber glass vials. During 24 h stability test in pure water at 4 °C all of the TPs besides 5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide (78% degradation) were stable. Also under the same conditions, but with the usage of wastewater, 4-hydroxy-*N*-desmethyltamoxifen (over 80% stability), 6(α)-hydroxypaclitaxel (100% stability) and 7-hydroxymethotrexate (over 80% stability) remained highly stable. Only over 50% of (Z)-4-hydroxytamoxifen remained, which may suggest biological degradation, whereas previously highly unstable 5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide was completely degraded. Extended test at higher temperature and both acidic and neutral pH confirmed, that TPs of tamoxifen are the most stable of all investigated; they were still quite high amounts present in wastewater at 25 °C after 3 months, which makes them fairly resistant to biological and hydrolytic degradation. 6(α)-hydroxypaclitaxel degradation can be inhibited to a certain extent by acidification; however, it was clearly visible only at higher temperature. 7-hydroxymethotrexate concentration declined significantly after a month at 25 °C, but it seems quite stable at 4 °C, which makes it quite persistent. Also in the study performed by Bialk-Bielinska et al. (2017a, 2017b) it was observed that 7-hydroxymethotrexate is susceptible to degradation under the ecotoxicological test condition with duckweed (*Lemna minor*) – with continuous illumination (6000 lx) at the 25 °C for 7 days. Based on the observed high stability of this compound in the samples kept in the dark but also in the 25 °C for 7 days it was concluded that this TP is susceptible to photolysis. However, in the study conducted by Tonski et al. (2021), 7-hydroxymethotrexate was stable for 5 days at pH 4, 7 and 9 at 50 °C, which is an equivalent of $t_{1/2} > 1$ year at 25 °C. It shows that it is hydrolytically stable.

2.2.2. (Eco)toxicity and bioconcentration

In the study of Borgatta et al. (2015) the effects of tamoxifen metabolites 4-hydroxytamoxifen and endoxifen on *D. magna* were investigated. In a two-generation experiment, organisms were exposed to concentrations of 1.8×10^{-3}–98.9×10^{-3} and 0.4×10^{-3}–202.4×10^{-3} mg/L for 4-hydroxytamoxifen and endoxifen, respectively. For both compounds, the highest tested concentrations proved to be lethal to crustaceans. In contrast, concentrations as low as

6.8×10^{-3} mg/L of 4-hydroxytamoxifen and 51.8×10^{-3} mg/L of endoxifen caused reproductive impairment in both the first and second generations of *D. magna*. In a subsequent paper published by Borgatta et al. (2016), a four-generation analysis of the effects of 4-hydroxytamoxifen on *D. magna* was conducted. This work confirmed that both parent compound and metabolite can affect the reproductive ability of crustaceans. A statistically significant effect of 4-hydroxytamoxifen on body size of organisms in subsequent generations as well as morphological deformations was also observed. Although it was not the purpose of the study, some morphological deformations were also observed in organisms exposed to 4-hydroxytamoxifen during the experiments.

Also Zounkova et al. (2010) evaluated the ecotoxicity of metabolites of three antineoplastic drugs: 5-fluorouracil, cytarabine and gemcitabine. The study showed that all of the tested metabolites had significantly lower toxicity than parent compounds or no toxicity in both effects on crustaceans, algae, and bacteria, even at concentrations of several hundred mg/L. These studies suggest that the metabolites of these compounds pose little environmental risk. However, it should be noted that these drugs are mostly excreted as metabolites, so the environmental concentrations of metabolites may be much higher than parent compounds.

Also in the work by Bialk-Bielinska et al. (2017a, 2017b) it was shown that the main metabolite of methotrexate - 7-hydroxymethotrexate shows much lower toxicity than parent compound. In tests on various aquatic organisms, it was shown to cause no acute toxicity in either *D. magna*, *V. fischeri* or *R. supcapitata*, while in the case of *L. minor* the EC_{50} value was two orders of magnitude lower than for the parent compound.

In contrast, Cesen et al. (2016) evaluated the toxicity of cyclophosphamide and ifosfamide metabolites on the alga *Pseudokirchneriella subcapitata* and cyanobacterium *Synecococcus leopoliensis*. The study showed that neither parent compounds nor two metabolites, ketocyclophosphamide and N-dechloroethylcyclophosphamide, showed acute toxicity in the concentration range tested (up to 320 mg/L). However, carboxycyclophosphamide was found to be toxic, for which the determined EC_{50} was 17.1 mg/L. Additionally, in a test with *Salmonella typhimurium*, it was shown that carboxycyclophosphamide can have direct genotoxic effects. Additionally, in this study, parent compounds were subjected to UV treatment and then the toxicity of the resulting products was tested. The demonstrated lack of toxicity suggests that stable compounds with harmful effects are not formed under UV exposure. In another study (Russo et al., 2018), the chronic toxicity of the same compounds was evaluated, confirming that carboxycyclophosphamide is the only metabolite showing negative effects in aquatic organisms. The EC_{50} for *B. calyciflorus* reproduction was 23.66 mg/L, which was more than 3 times lower than that of parent compounds. Interestingly, in this work, in contrast to the study of Cesen et al. (2016), increased toxicity of UV-formed mixtures to ifosfamide and cyclophosphamide was observed. This highlights the fact that the transformation products of anticancer drugs need more attention.

To date, no work exploring the potential bioconcentration of anticancer drug metabolites has been found. However, given the reports of their toxicity and the fact that they are recorded in the environment, it should be concluded that this is an important gap in knowledge that should be investigated in the nearest future.

2.3. TPs of NSAIDs and analgesics

NSAIDs are a group of pharmaceuticals widely used throughout the world (Nikolaou et al., 2007; Kosjek et al., 2005; Bonnefille et al., 2018; Kümmerer, 2008). They are characterised by their anti-inflammatory, antipyretic and analgesic effects, making them used in the treatment of many health ailments such as fever caused by viruses or bacteria, rheumatoid arthritis, menstrual pain, post-traumatic pain, and many others (Derry et al., 2009; Crofford, 2013). In addition to the pharmaceutical industry, NSAIDs are also commonly used in veterinary medicine and animal husbandry (Nikolaou et al., 2007). Most NSAIDs are sold over-the-counter, which encourages their high consumption and release into the environment through WWTPs, rivers, surface run-off or from farm landings (Bonnefille et al., 2018; Nikolaou et al., 2007). The most commonly detected non-steroidal drugs in the aquatic environment are diclofenac and ibuprofen (Kosjek et al., 2005; Bonnefille et al., 2018; Maskaoui and Zhou, 2010; Kasprzyk-Hordern et al., 2008; Ngubane et al., 2019). NSAIDs enter the aquatic environment in primary form as well as metabolites (Fent, 2008; Kümmerer, 2008; Brausch et al., 2012).

2.3.1. Transformation in the environment

Taking into account the number of available literature data it might be concluded that the stability of selected TPs of NSAIDs has been investigated more often in comparison to other groups of pharmaceuticals, which most probably results from the wide consumption and hence spread of the native forms of NSAIDs as well as their TPs in the environment. For example, Tonski et al. (2019) investigated the hydrolytic stability of 2-hydroxyibuprofen, carboxyibuprofen, 4-hydroxydiclofenac and *O*-desmethylnaproxen according to the OECD 111 guideline. The preliminary experiments at pH 4, 7 and 9, 50 °C and 5 days of incubation in the dark showed, that most of the TPs were stable, which can be described as $t_{1/2} > 1$ year at 25 °C. Only 4-hydroxydiclofenac was slightly unstable at pH 4 and 9. However, the results of extended test indicated, that there was no degradation at 20 °C for 30 days, which suggests that at environmentally relevant conditions, hydrolysis does not have an impact on removal of these contaminants from water.

Moreover, very interesting results were presented by Li et al. (2014), who described batch tests with river sediments and artificial river water spiked with PCs, like ibuprofen and diclofenac and incubated in the dark at 15 °C for more than a month. Quick disappearance of ibuprofen was observed with a simultaneous appearance and concentration increase of 2-hydroxyibuprofen and carboxyibuprofen. However, when ibuprofen was completely removed from water, quick degradation of its TPs was noticed. It suggests that biological processes are responsible for their formation as well as efficient removal. 4-hydroxydiclofenac was formed in small amount from diclofenac, which suggests higher resistance of this pharmaceutical to microbial transformation; nevertheless, the amount of its TPs was constantly increasing for first 25 days (probably due to continuing formation), but after that time rapid disappearance occurred, probably due to increased biological activity of microorganisms. Since no TPs were found in control samples with sterile sediment and water, biological degradation may be acknowledged as a main route of elimination of these compounds in the environment.

Similar study was also performed by Quintana et al. (2005), who investigated biodegradation of ibuprofen, ketoprofen and naproxen, formation of TPs and their fate in a batch test with a water mixed with fresh sludge from an actual membrane reactor, used to treat wastewater. Interestingly, in initial experiment only ketoprofen was degraded. Firstly, formation of one TP, namely 3-(hydroxy-carboxymethyl) hydratopic acid was observed, which reached maximum concentration after 14 days. After that time quick and full degradation occurred. Meanwhile, after 10 days another TP, 3-(keto-carboxymethyl)hydratopic acid was detected. Its amount was increasing until the end of an incubation. Taking into account that there was constant degradation of ketoprofen and 3-(hydroxy-carboxymethyl)hydratopic acid observed, while the amount of 3-(keto-carboxymethyl)hydratopic acid was constantly increasing, it suggests that 3-(keto-carboxymethyl)hydratopic acid is a final product of a direct biodegradation of ketoprofen. 3-(keto-carboxymethyl)hydratopic acid may be potentially persistent; however, it is not sure if the increasing amount was only due to higher increase rate than degradation, or is it unsusceptible to hydrolysis and biodegradation. Nevertheless, these authors have also performed additional experiments with an addition of external carbon source. In this case,

ketoprofen was stable during the entire test. On the other hand, ibuprofen and naproxen turned out to be unstable, which indicates that co – metabolism is the process that is a microbiological source of TPs of these compounds. In this arrangement, ibuprofen was fully degraded in two weeks, along with a formation of hydroxylated TPs. However, with a disappearance of pharmaceutical, after reaching maximum level, its TPs underwent quick degradation, which supports data presented by Li et al. (2014). Moreover, degradation of naproxen was much slower. It was detected in the water at the end of the test. However, quick formation of its TP *O*-desmethylnaproxen was observed in first 5 days, and then the increase was much slower in the next 20 days. It seems that after 25 days, the concentration of *O*-desmethylnaproxen started to decline, but the test was only 28 days long, so more study is needed to confirm its susceptibility to biodegradation.

Interesting observation was made for p-benzoquinone imine of 5-hydroxydiclofenac, which was found as a biodegradation product of diclofenac (probably through a by-product – 5-hydroxydiclofenac) in a fixed-bed column bioreactor containing sediment from a creek (Gröning et al., 2007). p-Benzoquinone imine of 5-hydroxydiclofenac was separated and incubated with both natural and sterilized sediment from river, as well as just water (without sediment). No degradation was observed in water, so it is hydrolytically stable; however, similar and significant loss of this compound was observed during batch test with native and sterile sediment (depletion rates 0.051 [1/d] and 0.048 [1/d], respectively), which indicates that sorption to sediment/biofilm is important factor in its environmental fate. The persistency of this TP and its reactivity in biological systems may be potentially harmful to the environment.

Another interesting study included phenazone-type pharmaceuticals and their TPs, which stability was investigated in a test with biologically active filter material (clay) from open bed filter used in drinking water purification (Zuehlke et al., 2007). 1,5-dimethyl-1,2-dehydro-3-pyrazolone, TP of phenazone and 4-(2-methylethyl)-1,5-dimetyl-1,2-dehydro-3-pyrazolone, a derivative of propyphenazone were formed during degradation of its pharmaceuticals, however they were also degraded by the end of the test. There was also quick and full degradation in a batch tests with only 1,5-dimethyl-1,2-dehydro-3-pyrazolone or 4-(2-methylethyl)-1,5-dimetyl-1,2-dehydro-3-pyrazolone, indicating high instability of these products. The authors stated that degradation was also observed in control samples with inactivated material, suggesting abiotic transformation, but no supporting data was shown. Nevertheless, 1,5-dimethyl-1,2-dehydro-3-pyrazolone was found in drinking water after treatment in a big-scale water treatment facility with the same active clay that was used in the experiments at high concentrations (up to 1 μg/L). Moreover, 4-(2-methylethyl)-1,5-dimetyl-1,2-dehydro-3-pyrazolone was found at low levels, but higher after the water purification than before. The authors proved that it was probably due to too short contact time of raw water and filter material. 1-acetyl-1methyl-2-phenylhydrazide and 1-acetyl-1-methyl-2-dimethyloxamoyl-2-phenylhydrazide, TPs of dimethylaminophenazone, where formed during batch test conducted with their pharmaceuticals and remained on constant level throughout entire test. In dedicated batch tests, 1-acetyl-1methyl-2-phenylhydrazide was completely degraded, but 1-acetyl-1-methyl-2-dimethyloxamoyl-2-phenylhydrazide was stable during entire experiment. 1-acetyl-1methyl-2-phenylhydrazide was found in purified drinking water at low levels of concentration. However, 1-acetyl-1-methyl-2-dimethyloxamoyl-2-phenylhydrazide, which was found stable during batch test, was detected at high levels up to 1×10^{-3} mg/L in purified drinking water, which did not differ from raw water. It indicates high stability and persistency, even in drinking water. Additionally, acetoaminoantipyrine and formylaminoantipyrine, TPs dimethylaminophenazone or metamizole were found at low levels during dimethylaminophenazone batch test, but they were degraded by the end of the test. Moreover, batch tests with these two TPs showed that they were fully degraded.

2.3.2. Ecotoxicity and bioconcentration

Only a few studies have investigated the toxicity of NSAIDs' metabolites that are present in the aqueous environment (Scheurell et al., 2009), while more work focuses on the toxicity of phototransformation products of this group of pharmaceuticals. However, not only the metabolites but also the metabolic processes of NSAIDs and analgesics leading to the formation of acyl glucuronides can cause toxic effects such as liver damage (Van Vleet et al., 2017; Grillo et al., 2003; Park et al., 2006). Kim et al. (2018) investigated toxic effect of acetaminophen metabolic processes in *Daphnia manga*. Crustaceans were exposed to acetaminophen at 50 and 100 mg/L for 12 and 24 h. The most commonly produced acetaminophen metabolite *N*-acetyl-*p*-benzoquinoneimine was then detected and identified. Kim et al. (2018) focused on detecting only *N*-acetyl-*p*-benzoquinoneimine and it would be worthwhile to screen for other common acetaminophen metabolites such as the previously mentioned glucuronides or hydroxy derivatives. The toxicity markers tested were specifically related to the metabolic processes of xenobiotic like tested acetaminophen. Due to acetaminophen metabolism, increases in thioredoxin reductase activity, reactive oxygen species production and decreases in glutathione levels were observed. Furthermore, Kim et al. (2018) noted upregulation of CYP307A1 and CYP370A13 genes and linked this to *N*-acetyl-*p*-benzoquinoneimine production. However, the production of other metabolites by *D. manga*, which were not analysed in this study, could also cause modification in CYP307A1 and CYP370A13 genes. Moreover, observed upregulation of CYP like genes was related to acetaminophen concentration and exposure time. In fact, CYPs enzymes play a key role in metabolism of most xenobiotic including pharmaceuticals (Kim et al., 2018; Zanger and Schwab, 2013). Metabolic processes of NSAIDs as well as other pharmaceutical compounds in aquatic organisms are almost unknown, which raises the need for research in this direction.

Rubasinghege et al. (2018) investigated the toxicity of ibuprofen and its secondary products of photodegradation on the algae *Chlorella* sp. and two bacterial strains *Bacillus megaterium* and *Pseudoaltermonas atlantica*. Growth rate and viability were the parameters used to assess the toxicity of pharmaceuticals using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, optical density measurement and flow cytometry methods. The toxicity of the mixture of all the obtained ibuprofen photodegradation products was studied, as well as the toxicity of the main photodegradation product 4-acetylbenzoic acid alone. No effect of the mixture of secondary products on bacterial and algae viability was observed in comparison to ibuprofen. Interestingly the hormesis effect was observed. Mixture of secondary photodegradation products of ibuprofen at 1 mM stimulated the growth of two tested species of bacteria *B. megaterium* and *P. atlantica*. Furthermore, no toxic effects of 4-acetylbenzoic acid on bacterial strains were observed. On the other hand, 4-acetylbenzoic acid significant affects viability of *Chlorella* sp., starting from a concentration of 0.25 mM caused death of algae. Concentrations above 0.5 mM caused death of all algal cells.

Schmitt-Jansen et al. (2007) also tested toxicity of NSAIDs' phototransformation products using algae *Scenedesmus vacuolatus*. Diclofenac at 50 mg/L was exposed to natural sunlight for up to 145 h and water samples were collected each day to assess toxicity, namely the effect on algal growth. The resulting mixtures of secondary compounds were found to significantly affect the growth of *S. vacuolatus* in a concentration- and time-dependent manner. Maximum toxicity was observed after 53 h of diclofenac exposure. At the 53rd hour of diclofenac exposure to sunlight, the toxicity of the mixture was 5–6 times higher compared to the unexposed control. In another work by the same team (Schulze et al., 2010) phototransformation products of diclofenac were identified. A solution of diclofenac in distilled water was exposed to simulated sunlight (290–800 nm) for 34 h. Diclofenac metabolites were then identified by mass spectra analysis. In this study, six fractions (F1–F6) produced by phototransformation of diclofenac were detected. The toxicity of all fractions as well as the

initial non-fractionated extract and the reconstituted mixture of fractions was assessed. The fraction that inhibited algae growth the greatest (by 92%) was F5. The inhibition of algal growth by other fractions was similar to control and did not exceed 20%. In turn, non-fractionated extract inhibited algal growth by 80.5% and the reconstituted mixture of fractions by 73.1% and there were no statistically significant differences in algal growth inhibition between F5, non-fractionated extract and the reconstituted mixture of fractions. The major toxic fraction F5 was tentatively identified as 2-[(2-chlorophenyl)amino]benzaldehyde. Furthermore, the EC_{50} was calculated for diclofenac and 2-[(2-chlorophenyl)amino]benzaldehyde, which was 48 mg/L for diclofenac and only 4.8 mg/L for 2-[(2-chlorophenyl)amino]benzaldehyde, respectively. The EC_{50} value for both substances can be related to lipophilicity. 2-[(2-chlorophenyl)amino]benzaldehyde is more lipophilic than diclofenac and may also be more toxic, as evidenced by 10 times lower EC_{50} value.

On the other hand, Isidori et al. (2005) investigated the acute and chronic toxicity of naproxen phototransformation products. For this purpose, algae (*Pseudokirchneriella subcapitata*), rotifers (*Brachionus calyciflorus*) and microcrustaceans (*Thamnocephalids platyurus*, *Ceriodaphnia dubia*) were used. First, sodium salt of naproxen was dissolved in drinking water and was irradiated with a solar stimulator for 72 h. Then, the photodegradation products of naproxen were identified by GC–MS. In the second step, toxicity of four detected phototransformation products, naproxen and naproxen sodium salt was evaluated. For acute toxicity assessment rotifer and crustaceans were exposed to the four obtained naproxen phototransformation products for 24 h (*B. calyciflorus*, *T. platyurus*) and 48 h (*C. dubia*). Next, mortality and LC_{50} (*B. calyciflorus*, *T. platyurus*), mobility and EC_{50} (*C. dubia*) were used to assess acute toxicity. Measured acute toxicity (EC_{50} or LC_{50}) was significantly higher for naproxen photoproducts than parent compounds for all three organisms tested (*B. calyciflorus*, *T. platyurus*, and *C. dubia*). The toxicity of all four photoproducts was similar. Furthermore, the toxic effect of naproxen sodium salt was higher than naproxen. Interestingly, the sensitivity to pharmaceuticals and its photoproducts for different model species was similar, as evidenced by comparable data of acute test. For chronic toxicity assessment algae (*P. subcapitata*), rotifer (*B. calyciflorus*) and crustacean (*C. dubia*) were used. *P. subcapitata* was exposed for 96 h and then IC_{50} was measured and effect on growth was assess. In contrast, *B. calyciflorus* and *C. dubia* were exposed for 48 h and 7 days, respectively, after which the EC_{50} for both species was calculated and effect on reproduction was evaluated. Similar to the acute test, toxicity was significantly higher for some naproxen photoproducts in chronic test. P4 and P2 had the greatest measured effect on *C. dubia*, while P3 and P1 had the greatest effect on *P. subcapitata*. *B. calyciflorus*, compared to the above species, was the least sensitive to naproxen and its photoproducts, and was only slightly affected by P3. In contrast to the acute test, in which the same sensitivity was observed in selected model species, in the chronic test the species showed significantly different sensitivities to naproxen and its photodegradation products. Furthermore, chronic test showed higher toxicity of naproxen photoproducts than the acute test, as indicated by the positive values of ratio (acute EC_{50}/chronic EC_{50}) calculated for *B. calyciflorus* and *C. dubia*. DellaGreca et al. (2003) and Cazzaniga et al. (2020) also confirmed higher acute toxicity (EC_{50}, EC_{20}) of naproxen photoproducts on *D. magna* and *Vibrio fischeri* compared to parent compound

On the other hand, Grabarczyk et al. (2020) investigated the effects of human metabolites of diclofenac and ibuprofen, namely 4-hydroxydiclofenac, 2-hydroxyibuprofen and carboxyibuprofen. To investigate the ecotoxicity of drugs, aquatic organisms that are most at risk from exposure to xenobiotics were used (*V. fischeri*, *D. manga*, *L. minor* and *Raphidocelis subcapitata*). Grabarczyk et al. (2019) reported a 38% inhibition of *V. fischeri* luminescence by 4-hydroxydiclofenac, whereas 2-hydroxyibuprofen and carboxyibuprofen inhibited it by only 4 and 2%, respectively. The parent compounds (diclofenac and ibuprofen) inhibited luminescence by 45 and 42%, respectively. In contrast, 4-hydroxydiclofenac did not affect *L. minor* growth, whereas 2-hydroxyibuprofen and carboxyibuprofen slightly inhibited growth by 11% and 6%. In the case of immobilization, only minor effects of metabolites were observed. 4-hydroxydiclofenac and carboxyibuprofen immobilized *D. manga* by 8%, while the parent compounds immobilized by 50 and 52%, respectively. The greatest toxic effect of metabolites was observed in inhibiting algal (*R. subcapitata*) growth. 4-hydroxydiclofenac, 2-hydroxyibuprofen, and carboxyibuprofen inhibited growth of *R. subcapitata* by 45, 16, and 14, respectively, while ibuprofen inhibited growth by 52% (for diclofenac, data are not available). The observed toxic effects of 4-hydroxydiclofenac in some cases were almost as high as those of the parent compound. In contrast, the observed effects of ibuprofen metabolites were always much lower than those of ibuprofen. Interestingly, 4-hydroxydiclofenac inhibited growth of soil bacteria's (*Arthrobacter globiformis*) by 14%. However, this inhibition was greater for parent compound (30%).

Salicylic acid is one of the best known and widely studied metabolite of NSAIDs in terms of toxicity. This compound is commonly used as a treatment for acne and food preservative. Zivna et al. (2015) investigated toxic effect of salicylic acid on early life stages of *Cyprinus carpio*. Individuals were exposed to 0.004, 0.04, 0.4, 4 and 20 mg/L for 34 days. Salicylic acid caused, among other things, oxidative stress induction (decrease in glutathione reductase and catalase activity), lipid peroxidation, hatching disruption, early ontogeny, the weight decrease, lesions in skin tissues and higher numbers of mucous cells. However, this pharmaceutical did not influence the mortality. Freitas et al. (2020), in turn, tested toxic effect of salicylic acid on *Mytilus galloprovincialis* under different temperature conditions (17 ± 1 °C control; and 21 ± 1 °C). Freitas et al. (2020) observed that higher temperature significantly influence the metabolic capacity of mussels and toxicity of salicylic acid. Interestingly, higher metabolic capacity was observed only in control mussels (17 ± 1 °C) exposed to pharmaceutical. In addition, mussels from higher temperature (21 ± 1 °C) were not able to increase metabolic capacity as in the case of control group. In experimental group (21 °C) treated with the pharmaceutical oxidative stress was revealed as a significant increase in superoxide dismutase. On the other hand, in control mussels (17 °C) treated with salicylic acid oxidative stress was observed as a decrease in catalase activity. In addition, loss of redox balance due to exposure to salicylic acid was observed. In previous work of the same author (Freitas et al., 2019) salicylic acid affected respiratory capacity of *M. galloprovincialis*, induced oxidative stress, disrupt metabolism, and revealed neurotoxic effect. In contrast, Nunes et al. (2018) used polychaeta species *Hediste diversicolor* to evaluate the ecotoxicity of salicylic acid. Individuals were exposed for 96 h to concentrations in the range of 50×10^{-3}–253.125×10^{-3} mg/L. Metabolite at 50×10^{-3} mg/L significantly affect the catalase activity, as well as acetylcholinesterase activity at all concentrations starting from 0.112 mg/L. Other effects observed were an increase in lipid peroxidation and modulation of glutathione peroxidase activity. The observed endpoints were concentrations-dependent.

Although knowledge of the potential bioconcentration of metabolites of NSAIDs is very limited, the recent work of Fu et al. (2020) provides extremely important information. In this study, diclofenac methyl ester (DCF-M310.03) was shown to have a significantly higher bioaccumulation capacity than the parent compound, as demonstrated in two crustacean species, *H. azteca* and *G. pulex*. The BCF for diclofenac in this study was 0.5 and 3.2 for *H. azteca* and *G. pulex*, respectively, while for diclofenac methyl ester it was as high as 164.5 and 104.7, respectively. Although this is only a preliminary study on the subject, it confirms that transformation products of NSAIDs deserve special attention also in the context of potential bioaccumulation, with particular emphasis on methylated derivatives. Methylation leads to the formation of compounds with higher hydrophobicity and thus may cause higher bioaccumulation potential and toxicity (Fu et al., 2020).

2.4. TPs of opioids

In brief, opioids are a group of compounds (natural or synthetic) that bind to the opiate receptors in the body and produce an agonist effect (Duarte, 2005; Fontes et al., 2020; Melis et al., 2011). Opioids are mainly used for the relief of mild to severe, chronic or acute pain e.g. during cancer treatment and postoperative pain (Smith, 2009; Melis et al., 2011). However, other common symptoms that are treated with opioids are breathlessness and strong cough (Melis et al., 2011; Nicholson, 2003). The most common used opioids in medicine are tramadol, codeine, and morphine (Melis et al., 2011; Fontes et al., 2020). Tramadol and other synthetic opioids are often used as prescribed medicine, while codeine, morphine and other natural or semi-synthetic opioids are usually used in severe medical conditions in hospitals. According to UNODC (2019) the number of people, which are treated with opioids is estimated in 35 million around the world (Fontes et al., 2020). According to Fontes et al. (2020) morphine (heroine metabolite), 6-monoacetyl morphine (heroine metabolite), codeine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (metadone metabolite) and tramadol are the most frequently detected in aquatic environment opioids. Hence, not only primary opioids but also their metabolites pose a risk to the aquatic environment.

2.4.1. TPs of opioids – transformation in the environment

In general, only the information of hydrolytic stability of *O*-desmethyltramadol is available in the literature for this group of pharmaceuticals. It was proved that this metabolite is hydrolytically stable ($t_{1/2} > 1$ year at 25 °C) at the investigated pH 4, 7 and 9 at 50 °C for 5 (Tonski et al., 2019).

2.4.2. (Eco)toxicity and bioconcentration

Grabarczyk et al. (2019) studied the toxic effects of selected illicit drugs, including opioids and their selected metabolite. The toxic effects of tramadol and, its metabolite, *O*-desmethyltramadol were investigated. *O*-desmethyltramadol is an example of opioid metabolite frequently detected in the aquatic environment that has a high persistence in the aqueous matrix and surface waters. This metabolite may therefore pose a risk to aquatic organisms (Archer et al., 2017; Chen et al., 2016; Han and Lee, 2017; Grabarczyk et al., 2019; Campos-Mañas et al., 2018). For ecotoxicity assessment, *V. fischeri*, *D. manga*, *L. minor* and *R. subcapitata* were used. *O*-desmethyltramadol inhibited the growth of *R. subcapitata* by 35%, while the parent compound tramadol, inhibited bacterial growth by 93%. In contrast, *O*-desmethyltramadol similarly to tramadol did not significantly affect the growth of *L. minor* and did not inhibit the luminescence of *V. fischeri*. Furthermore, the tramadol metabolite only immobilized *D. manga* in 8%, whereas tramadol itself immobilized crustaceans in 44%. Thus, the observed toxicity of the metabolite was much lower than that of the parent compound.

Similar effects of other tramadol metabolites N-oxidetramadol and *N*-desmethyltramadol were observed in study of Antonopoulou and Konstantinou (2016). N-oxidetramadol, *N*-desmethyltramadol as well as *O*-desmethyltramadol are common metabolites resulting from the photodegradation of tramadol as well as its metabolism by some organisms and humans. For the purpose of toxicity assessment standard *V. fischeri* bioassay was used. N-oxidetramadol and *N*-desmethyltramadol inhibited *V. fischeri* growth by respectively 6 and 4%. Further work by the same author (Antonopoulou et al., 2020) examined toxicity of N-oxidetramadol and *N*-desmethyltramadol using the same method as in previous research (*V. fischeri* bioassay). N-oxidetramadol and *N*-desmethyltramadol did not significantly affect bacterial growth, but their mixture with tramadol inhibited *V. fischeri* growth by 40% what can be associated with their synergistic effect. In addition, Antonopoulou et al. (2020) examined whether there is a risk associated with the photolytic transformation of tramadol. Aqueous solution of tramadol was irradiated using a Suntest XLS+ apparatus (wavelengths < 310 nm). Original solution inhibited bacterial growth only by 11%, while during photodegradation inhibition increase reaching the almost 70% in 1 h. The photodegradation products of tramadol may therefore be more hazardous to aquatic organisms than the parent compound. Bergheim et al. (2012) also demonstrated higher toxicity (22% compared to 7% inhibition growth of *Pseudomonas putida* for parent compound) of irradiated samples of tramadol. However inhibition value was negligible compared to the work by Antonopoulou et al. (2020), which may be related to the applied concentration of analyte, test duration or different model species.

On the other hand, Tanoue et al. (2017) revealed that *Pimephales promelas* fish metabolize tramadol to *N*-desmethyltramadol and *O*-desmethyltramadol. Fish were exposed to tramadol in the concentration range of 1×10^{-3}–100×10^{-3} mg/L for 24 days. In the treatment groups 10 and 100 μg/L, the metabolite *O*-desmethyltramadol was detected in fish plasma at the following concentrations $0.88 \pm 0.60 \times 10^{-6}$ mg/mL and $3.8 \times 10^{-6} \pm 0.99$ mg/mL, whereas in treatment groups 1×10^{-3}, 10×10^{-3}, and 100×10^{-3} mg/L, *N*-desmethyltramadol was detected with the following concentrations of $0.48 \times 10^{-6} \pm 0.21$ mg/mL, $1.2 \times 10^{-6} \pm 0.43$ mg/mL, and $7.2 \times 10^{-6} \pm 1.7$ mg/mL.

2.5. TPs of antibiotics

Antibiotics are a group of drugs that exhibit activity against bacteria by killing them or inhibiting their growth and division (Pauter et al., 2020). From a chemical point of view, antibiotics can be classified into different groups of compounds due to their different structure, composition and physicochemical properties. The well-known and the most widely used groups are beta-lactams, macrolides, aminoglycosides, sulfonamides and tetracyclines (Pauter et al., 2020).

2.5.1. Transformation in the environment

Even though antibiotics are widely used around the world, the data on their transformation products stability and fate is limited. There is definitely less information than for NSAIDs. Nevertheless, some derivatives of metronidazole, sulfamethoxazole and tetracyclines have been investigated.

For example, it was found that hydroxymetronidazole was hydrolytically stable at pH 4 and 7 at 50 °C for 5 days, which means that its $t_{1/2}$ can be estimated as higher than 1 year at 25 °C (Tonski et al., 2019). However, there was slight degradation at pH 9. Extended tests at this pH showed, that no degradation was observed at 20 °C for 30 days, which indicates high hydrolytic stability at environmentally relevant temperatures. High hydrolytic stability under the same conditions was also proved for N^4-acetylsulfamethoxazole ($t_{1/2} > 1$ year at 25 °C).

Very interesting observation was made by Göbel et al. (2005), who spiked sewage sludge with N^4-acetylsulfamethoxazole and then investigated its presence in this sludge. It was found that no N^4-acetylsulfamethoxazole was present after some time; however, the amount of sulfamethoxazole (parent compound of this metabolite) increased, which suggests biological transformation of TP to its parent compound, which may be environmentally relevant. It also shows that fate of the pharmaceuticals and their TPs may be very complicated and multivariable. Other study showed, that photolysis of transformation products of sulfamethoxazole may be pH – dependent (Bonvin et al., 2013). These compounds were less susceptible to photolysis at pH 8.4 rather than 3.2, due to the fact that they are in ionic form at higher pH. Moreover, the sulfamethoxazole β-D-glucuronide degradation was very fast, whereas 4-nitrosulfamethoxazole was the most stable during test.

Other photolysis study showed, that N4-acetylsulfapyridine was easily degraded by direct photolysis (García-Galán et al., 2012a, 2012b). However, the half-life increased 3 times when the analyte was dissolved in WWTP effluent.

In another study the stability of TPs of chlortetracycline (4-epi-chlortetracycline, anhydro-chlortetracycline and 4-epi-anhydro-

chlortetracycline) was investigated in different conditions (Søeborg et al., 2004). Influence of light, oxygen, temperature, pH and matrix (milliQ water and soil interstitial water – SIW) on chemical stability of these compounds was investigated. In each case, there was no difference in degradation rates between samples with or without contact with the oxygen as well as there were higher degradation rates observed at 20 °C than 6 °C. 4-epi-chlortetracycline was not susceptible to photolysis. It was much more stable at acidic conditions, where maximum half-lives were over 100 days (depending on conditions), while at pH 8.5–9.0 it was only several days. Moreover, significantly faster disappearance was noticed in SIW than in milliQ water, which could be due to the formation of the complex with Ca^{2+} and Mg^{2+}. Anhydrochlortetracycline was susceptible to light. Degradation was pH-dependent; lowest stability was observed at pH 5.6, higher was at both acidic and alkaline conditions. Effect of a matrix is unclear; it seems that in many cases it was more stable at SIW than milliQ water. 4-epi-anhydro-chlortetracycline was also susceptible to light, but it was also pH-dependable – the influence was noticeable from pH 5.6 and higher. Nevertheless, general lower stability was observed at high pH, where maximum $t_{1/2}$ of 5 days was calculated, whereas around 25 days at pH 3. Additionally, there was no significant matrix influence; however, at pH 8.5 and 9 it was more stable in SIW.

2.5.2. (Eco)toxicity and bioconcentration

For antibiotics, there are quite a few literature reports on the effects of their TPs, but most of them are limited to one group - the sulfonamides. For the others, there are only single articles on their (eco)toxicity and bioconcentration.

For example, amoxicillin is β-lactam antibiotic widely prescribed in veterinary and human medicine for the prevention and treatment of bacterial infections. Degradation of these antibiotics in water, depending on the pH, yields different products. Amoxicilloic acid is the main degradation product of amoxicillin at neutral pH (6.8–7.9). In the study of Elizade-Velazquez et al. it was observed that amoxicillin was completely degraded to amoxicilloic acid within 12 h. Moreover, amoxicilloic acid caused oxidative stress in carp. The kidney was most sensitive to its toxic effects, while the liver showed the least sensitivity to this metabolite (Elizalde-Velázquez et al., 2017).

Baumann et al. (2015) examined the aquatic toxicity of the human macrolide antibiotic clarithromycin and its two metabolites: 14-hydroxy(*R*)-clarithromycin and *N*-desmethyl-clarithromycin. 14-hydroxy(*R*)-clarithromycin is pharmacologically active and showed simillar toxic effect like clarithromycin. *D. magna* and *D. rerio* did not show sensitivity to both compounds. However the strong toxicity was observed for algae (*D. subspicatus*) and cyanobacteria (*A. flos-aquae*). *N*-desmethyl-clarithromycin generally was less toxic than parent compound. Nevertheless, only in this case a slight inhibition of *D. magna* reproduction and algal growth was observed. Taking into account these results and the fact that about 60% of clarithromycin is excreted in the metabolized form, the fate of its metabolites should be investigated more deeply (Baumann et al., 2015; Ferrero et al., 1990).

Many of the antibiotics may be photodegradable in the environment. However their photobyproducts cannot be ignored. In the study of Wang and Lin (2012), the transformation of different cephalosporins through direct photolysis was observed. In the case of all studied compounds the byproducts were less photolabile and more toxic towards *V. fischeri* than parent drugs. The strongest acute toxicity was observed for cefazolin, what was probably caused by its 5-methyl-1,3,4-thiadiazole-2-thiol moiety (Wang and Lin, 2012).

Also in the study of Jiao et al., the increase of luminescence inhibition of *V. fischeri* during the photolysis experiment of tetracycline was observed. The inhibition rate of native compound (at initial concentrations 20 mg/L) was 33% and after irradiation for 300 min was 64%. This increase indicates that the products of photolysis may have more pronounced negative effects on the bacteria than parents compound (Jiao et al., 2008).

Also in the study of Gmurek et al. (2015), the toxicity of mixture of phototransformation products of sulfamethoxazole was reported. During long-term photolysis experiments it was observed that sulfamethoxazole (at initial concentration 100 mg/L) is transformed into a varitey of TPs present in the solution at the same time. During the toxicity analysis the significant difference in the inhibition of luminescencne of *V. fischeri* was not observed. In contrast, it was showed that growth inhibition activity was slightly decreased over the irradiation time (Gmurek et al., 2015). However in another study, the products of sulfamethoxazole phototransformation showed the significantly higher toxicity than parent compounds. Authors monitored toxicity of irradiated solution of sulfamethoxazole at initial concentration 10 mg/L towards *V. fischeri* and *D. magna*. The results obtained with *V. fischeri* showed the increase of immobilization from 38% (at initial concentration of sulfamethoxazole) to 49% after 30 h of irradiation. However, the EC_{50} concentration (5 mg/L) was not exceeded in any case. *D. magna* was more sensitive organism. For the solution of sulfamethoxazole inhibition was at the level 60% and after irradiation (30 h), when sulfamethoxazole depletion was achieved, reached to 100%. In order to find out which TPs are responsible for the increase in toxicity, the authors determined the EC_{50} for the two most abundant - sulfanilic acid (C2) and 3-amino-5-methyloisoxazole (C3). However, in both cases the EC_{50} values (90 and 100 mg/L for C2 and C3 respectively) for *D. magna* after 48 h exposure was higher than this determined during the photolysis experiment. Also the mixture of standard solution of these compounds was no toxic to *D. magna*. Therefore the authors' statement, that the other TPs are responsible for the increased of toxicity, probably the compound what is the product of photoizomerization reaction of the isoxazole ring (Trovó et al., 2009).

On the other hand, in the study of Grabarczyk et al. (2020), the toxicity of sulfamethoxazole metabolite – *N*-acetylsulfamethoxazole towards five different organisms were assessed. Despite low concentrations (3 mg/L for *L. minor* and 5 mg/L for *R. subcapitata*), N^4-acetylsulfamethoxazole caused inhibition of the growth of duckweed (17%) and algae (27%). Moreover it was observed a slight toxic effect towards *V. fischeri* (8% inhibition of luminescence). *D. magna* and soil bacteria *A. globiformis* were not sensitive to this metabolite. Although in all cases the organisms showed greater sensitivity to the native form of the drug, results of this study indicate that N^4-acetylsulfamethoxazole could be also dangerous for aquatic environment.

In the study of Bialk-Bielinska et al. (2017a, 2017b), (eco)toxicity of two TPs of sulfonamides – sulfanilic acid and sulfanilamide were evaluated. The sulfanilamide presented EC_{50} concentrations at the level 25.83 and 5.09 mg/L to *S. vacuolatus* and *L. minor* respectively, whereas for the sulfanilic acid any toxic efect was observed towards both selected spieces (Bialk-Bielinska et al., 2017a, 2017b).

In the work of García-Galán et al. (2012a, 2012b), toxicity of another sulfonamides - sulfapirydine (at concentration 160 mg/L) and its acetyleted metabolite (at concentration 48 mg/L) towards *V. fischeri* were studied. Authors observed EC_{50} concentrations of 27.4 mg/L and of 8.2 mg/L for sulfapirydine and its acetyleted metabolite respectively (after 15 min exposure). According to EU legislation (Directive 93/67/EEC) the sulfapirydine was clasiffied as harmfull, and its acetyleted metabolite as toxic compound. It is yet another example, where the tested organism was more sensitive for metabolites than parent drugs (García-Galán et al., 2012a, 2012b).

The toxicity of sulfonamides' metabolites was also studied by Hiba et al. (2016). The toxicity potential was assessed by using T.E.S.T. in silico computer-based predictive model developed by the USA Environmental Protection Agency (EPA). The parameters rat lethal dose (LD_{50}) were calculated for sulfadiazine, sulfquinoxaline and their metabolites. The predicted values of LD_{50} for parent compounds were higher (3909.82 and 8370.90 mg/kg for sulfquinoxaline and sulfadiazine rsepectively) than for their acetylated (2357.62 and 6216.15 mg/kg for acetylsulfquinoxaline and acetylsulfadiazine

respectively) and hydroxylated (2456.21 and 6786.16 mg/kg for hydroxylated - sulfquinoxaline and hydroxylated - sulfadiazine respectively) metabolites. Moreover were also evaluated that all compounds exhibit developmental toxicity in the range of 0.82–1.10 Arbitrary Unit. The highest value was obtained for hydroxylated - sulfated metabolites of sulfquinoxaline, whereas most of the other derivatives were shown to be as toxic as the native form. This study showed that the in silico methods could be also helpful in the assessment of toxicity of drugs metabolites (Hiba et al., 2016).

However, knowledge on the bioaccumulation potential of antibiotics' TPs is even less studied. There are a few papers confirming the presence of metabolites or TPs in the tissues of organisms after exposure to a given drug. However, this is very perfunctory information and concerns only a few selected compounds. Liu et al. (2014a), showed that in crucian carp (*Carassius auratus*), after exposure at erythromycin (70.5×10^{-3} mg/L) the similar metabolite as those observed in mammals were produced. The presence of *N*-demethylerythromycin was found in all tissues, while its highest concentration was found in the liver. Moreover two metabolites: dehydration-di-demethylated-erythromycin and dehydration-descladinose-erythromycin were detected (Liu et al., 2014a). The same authors detected also seventeen roxithromycin metabolites in *C. auratus* after 20 days exposure (Liu et al., 2014b). In this study it was observed that interaction between roxithromycin and its metabolite may induce biochemical disturbances in fish. Moreover it is worth noting that the presence of bioactive metabolites in organisms may cause adverse effects on them as a result of continual lifecycle and multigenerational exposure. In addition, there is a risk that they will accumulate in higher levels of the trophic chain (Liu et al., 2014a, 2014b).

Zhao et al. studied the bioaccumulation of acetylsulfamethazine in medaka (*Oryzias melastigma*) after exposure to sulfamethazine (24 h, at concentrations 40×10^{-3} and 200×10^{-3} mg/L). Acetylsulfamethazine, the main of sulfamethazine metabolite, was more easily accumulated in the organism because it is less polar than parent drug. Therefore, higher amount of acetylsulfamethazine was found in the organs with large proportion of fat. Moreover, acetylsulfamethazine were accumulated mainly in the gonad and its concentration was higher in the male group (Zhao et al., 2016). This is further evidence that bioaccumulation of drug metabolites cannot be ignored because their chemical properties often result in them being more likely to accumulate in organisms than native forms.

In another study of Zhu et al. (2020) seven biotransformation products of antibiotics in sea cucumber (*Apostichopus Japonicus*) were identified (Zhu et al., 2020). Authors detected in tissues acetyleted metabolites of sulfadiazine and sulfamethoxazole and ciprofloxacin, which was formed from enrofloxacin. Moreover, another four TPs of enrofloxacin were identified, which were not reported previously in aquatic organisms. Although all biotransformation products were detected at low concentration levels, further studies with this organism are needed. Due to the fact, that sea cucumber have a high protein contents, bioaccumulation of chemicals that can bind to proteins requires special attention. This group has been shown to include, among others, organic ions (Armitage et al., 2017; Henneberger et al., 2016). Although metabolites are not permanently charged, they have functional groups that can dissociate or attach a proton. Therefore, it is likely that their binding to proteins may also have a significant effect on their bioaccumulation potential. Therefore, their presence in sea cucumber tissues is an important clue to take a closer look at the accumulation of drug metabolites in these organisms, because a mariculture organism are consumed by human and dietary intake is a major way of exposure to pollutants (Zhu et al., 2020).

2.6. TPs of antiepileptic drugs

Antiepileptic drugs are a diverse group of pharmaceuticals that are mainly used to treat epilepsy, bipolar disorder, and neuropathic pain (Al-Otaibi, 2019). Although the list of these drugs is broad, carbamazepine is mainly used in the context of environmental studies. This is probably due to its high consumption and low degradation rate which in turn results in its presence in the environment. Therefore, there are many studies confirming its adverse effects on aquatic organisms including algae, bacteria, invertebrates and fish (Ferrari et al., 2003). However, it is important to remember that it is heavily metabolized in the body and only 2% of ingested carbamazepine is excreted in the urine as the parent drug (Bahlmann et al., 2014; Brezina et al., 2017). Its metabolism in the liver leads to the formation of nearly 30 metabolites (Lertratanangkoon and Horning, 1982). Although some of these (such as 10,11-dihydro-10,11-dihydroxycarbamazepine, 10-hydroxy-10,11-dihydrocarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine and and carbamazepine-10,11-epoxide) are detected in the environment (Brezina et al., 2017), little is known about their effects on its components.

2.6.1. Transformation in the environment

To the best of our knowledge, only some data regarding TPs of carbamazepine are available. It was proved that 10-hydroxycarbamazepine and 2-hydroxycarbamazepine were hydrolytically stable at pH 4, 7 and 9, 50 °C for 5 days, which means that their $t_{1/2}$ are higher than 1 year at 25 °C (Tonski et al., 2019). Moreover, carbamazepine-10,11-epoxide was also stable in the same experiments at pH 7 and 9; however, it was highly unstable at pH 4, where it disappeared completely after 5 days. The extended tests at pH 4 showed, that at 20 °C constant rate k_{obs} was 3.5×10^{-3} 1/h, which gives $t_{1/2} = 197.0$ h (around 8 days). The results extrapolated to lower – more environmentally relevant - temperatures, were $t_{1/2} = 13$ days at 15 °C, $t_{1/2} = 23$ days at 10 °C and $t_{1/2} = 30$ days at 4 °C.

Carbamazepine-10,11-epoxide was investigated also in several other studies. Its stability was assessed in plasma separated from human blood from patients treated with carbamazepine (Mendez-alvarez et al., 1986). The analyte in samples stored at −20 °C and 4 °C was stable for 3 months (maximum test duration). Incubation at room temperature caused around 50% degradation after 2 months and over 70% after 3 months, probably due to microbial processes, which occurred from fermentation of samples. The study of Li et al. (2014) involved batch tests with sediments collected from rivers and artificial river water spiked with carbamazepine. Samples were kept in the dark at 15 °C for 35 days. Carbamazepine-10,11-epoxide was detected after a week from the beginning of the test and its concentration was increasing until the end of the experiment. However, it is not known if this compound was stable or the rate of carbamazepine degradation was faster than the elimination of its TP. There is another study of Li et al. (2015), who have conducted an experiment in a flume filled with sediments and recirculated water (spiked with parent compounds including carbamazepine) from a lake. It was found, that carbamazepine-10,11-epoxide was formed in the sediment compartment, where parent compound first accumulated. Moreover, the TP was also found at similar level in the surface water, which indicates that not only it is formed in the sediments, but also it is mobile and can transfer from pore water to the surface water. Despite the fact that concentration of the epoxide was increasing throughout entire test it is not clear if it is persistent or slow degradation of carbamazepine during entire experiment is the main reason of the increase.

Additionally, Koba et al. (2016) conducted experiments with 13 different soils, which were spiked with parent compounds and incubated in the dark at 20 °C for 61 days. A very slow degradation of carbamazepine, which resulted in very low levels of carbamazepine-10,11-epoxide was observed in every soil. The concentration of TP was increasing during entire test, but it is not clear if it is due to the high stability of this compound or constant, slow degradation of parent compound providing additional amount of TP. Nevertheless, there is a possibility of mobility of carbamazepine epoxide in the soil. Moreover, another TP of carbamazepine, 10,11-dihydroxycarbamazepine, was found in several soils (not all) at low concentration levels. It may

indicate that the composition of a soil or differentiation of microorganisms have an influence on the creation of different TPs from the same PC. Similarly to carbamazepine-10,11-epoxide, the concentration of 10,11-dihydroxycarbamazepine increased during entire test, which indicates that formation rate was higher than potential degradation rate.

2.6.2. (Eco)toxicity and bioconcentration

Carbamazepine is one of the most studied antiepileptic drug. There are many reports that confirm their presence in the environment and explain their fate. However, the knowledge about their TPs and metabolites remains scarce, Only few studies investigating the ecotoxicity of carbamazepine TPs including their photodegradation products have been performed. For example, Almeida et al. (2017) evaluated the toxicity of irradiated carbamazepine (at environmental concentration 0.00–9.00 × 10^{-3} mg/L) towards clam *Scrobicularia plana*. After 96 h of exposure to carbamazepine photoproducts the increase of toxicity was not observed (by comparison to native form). However, among the identified photoproducts was acridine, which is known as carcinogenic compound (Almeida et al., 2017).

The oposite effect was observed by Donner et al. (2013). In this study, the photo-induced degradation of carbamazepine leaded to formation more toxic TPs than parent compound. The carbamazepine solution (at initial concentration of 6 mg/L) was exposed to UV light for 120 min and samples were collected periodically throughout the experiment. The growth inhibition of *P. subcapitata* reached 100% after 20 min, for *V. fischeri* the maximum toxic effect was observed after 25 min (70% inhibition of bioluminescence). *D. magna* was less sensitive and mixture of TPs caused immobilization after 45 min. However in all cases the toxic effect was maintained at the end of the experiment. Moreover, authors evaluated toxicity of single compounds – acridine and acridone, which were identified as carbamazepine phototransformation products (Kosjek et al., 2009; Almeida et al., 2017). During the experiment EC_{50} and in some cases NOEC or LOEC were determined. However the obtained results showed that both TPs are significantly more toxic than parent compounds. For *D. magna* EC_{50} was equal 0.71 mg/L and 1.49 mg/L for acridine and acridone respectively, whereas the NOEC concentration for carbamazepine was 30 mg/L. In the tests with algae and *V. fischeri* the highest toxic effect also was observed for acridine (EC_{50} = 0.61 mg/L and 5.34–6.90 mg/L) (Donner et al., 2013). Moreover, its toxicity towards different organisms was confirmed in many studies and their results clearly indicate that these TPs of carbamazepine could be considered as more hazardous for the environment than parent compounds (Eisentraeger et al., 2008; Dijkman et al., 1997; Kraak et al., 1997).

Several papers have also described the fate of human metabolites of carbamazepine. The chronic toxicity of carbamazepine-10,11-epoxide and 10,11-dihydro-10,11-dihydroxycarbamazepine on the non-biting midge *Chironomus riparius* was assesed by Heye et al. (2016). During the 40-days sediment full life cycle test (according to OECD 233) it was observed that carbamazepine-10,11-epoxide (LC_{50} = 0.2 mg/kg) was more toxic than carbamazepine (LC_{50} = 1.1 mg/kg), whereas 10,11-dihydro-10,11-dihydroxycarbamazepine did not cause any toxic effect (Heye et al., 2016).

The relatively high toxicity of carbamazepine-10,11-epoxide towards different organisms in comparison to parent compounds was also observed in the study of Grabarczyk et al. (2020). Carbamazepine-10,11-epoxide (at concentration 100 mg/L) caused a 30% illuminescence inhibition of *V. fischeri*, whereas the effect observed for carbamazepine was at the level 42%. The toxic effect similar to the native form (at concentration 50 mg/L) was also demonstrated in the case of *L. minor* (32% and 49% of growth inhibition for carbamazepine-10,11-epoxide and carbamazepine respectively). Green algae showed also the high sensitivity towards both metabolite (37%) and parent drug (48%). However, in the case of 10,11-dihydro-10-hydroxycarbamazepine, the slight toxic effect towards *L. minor* (11%) and *R. subcapitata* (15%) was observed. *D. magna* was not sensitive towards both metabolites, whereas for carbamazepine the 24% of immobilization was demonstrated (Grabarczyk et al., 2020).

The in vivo bioconcentration of metabolite of oxcarbazepine - 10-hydroxy-10,11-dihydrocarbamazepine and carbamazepine was evaluated by Boillot et al. (2015). The mussels (*Mytilus galloprovincialis*) were exposed for compounds (at concentration 10 × 10^{-3} mg/L) for one week. Although, 10-hydroxy-10,11-dihydrocarbamazepine showed a little higher value of BCF than carbamazepine (4.5 and 3.9 L/kg dry weight). Although both compounds were weakly accumulated, the presence of two carbamazepine metabolites (carbamazepine-10,11-epoxide and acridine) in the mussels tissue was observed after the exposure. Both compounds were detected in the gills and digestive glands. Moreover, acridine was quantified in digestive glands at 3.6 × 10^{-6} ± 1.4 mg/L dry weight and detected in mantels. This may indicate that accumulation of metabolites in organisms may occur not only as a result of direct exposure to the substance, but also as a consequence of metabolizing the original form of the drug (Boillot et al., 2015). A similar phenomenon was reported by Daniele et al. (2017). In this study a moderate bioaccumulation of carbamazepine was observed. 2-hydroxycarbamazepine (3 month exposure at 0.5 × 10^{-3} mg/L carbamazepine) and acridone (6 month exposure at 5 × 10^{-3} mg/L carbamazepine) were quantified at the level 1.3 × 10^{-6} mg/g and 3.8 × 10^{-6} mg/g respectively in zebra mussel. However in the case of acridone it is also possible that it could be bioaccumulated in zebra mussel by direct exposure, because it was present in water (11.4 × 10^{-6} mg/L). Moreover the presence of acridine (1.7 × 10^{-6} mg/g) and 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (7.3 × 10^{-6} mg/g) were confirmed in fish (Stickleback) after 6 months of exposure of carbamazepine at the concentration of 5 × 10^{-3} mg/L. Carbamazepine-10,11-epoxide was found in all fish samples, regardless of exposure concentration. It should be noted that in all cases the quantified metabolites concentration in fish tissues were higher than for carbamazepine. This may indicate their higher bioaccumulation potential than parent drug (Daniele et al., 2017). In the study of Valdés et al. (2016), the presence of metabolites in fish after 48 h exposure to carbamazepine (100 × 10^{-3} mg/L) was also confirmed. 2-hydroxycarbamazepine was found in gills, intestine, liver, brine and muscle of *J. multidentata* (ranging from 48 to 107 × 10^{-6} mg/g wet weight), whereas the carbamazepine-10,11-epoxide was detected in gills and muscle (41 × 10^{-6} and 60 × 10^{-6} mg/g wet weight respectively). Summing up, it is likely to think, that carbamazepine is being biotransformed by exposed organism, and the formed metabolites are accumulated in various tissues (Valdés et al., 2016).

In last years, the new "non-classical" bioaccumulation behavior (especially for ionic and ionizable chemical) was observed. It has been proven that accumulation potential of some compounds is affected by interaction with a number of protein. In the study of Fortuna et al., the protein binding of carbamazepine metabolites were determined by using ultrafitration method. The obtained results indicated that compared to the native form (71.61%), relatively high level of protein binding (PB) for carbamazepine-10,11-epoxide (47.86%) was observed. Moreover two of oxcarbazepine metabolites showed also the affinity to protein. PB for S-licarbazepine and R-licarbazepine were 28.7 and 31.5% respectively (Fortuna et al., 2013). The affinity of drug metabolites for blood proteins was also demonstrated by Kowalska et al. In the present study, all of them showed higher affinity for proteins than for lipids, so it can be speculated that these biomolecules may have a significant effect on their bioaccumulation process. For this reason PB provides a potential opportunity to use in vitro studies in bioaccumulation assessment of drug metabolites (Kowalska et al., 2021).

2.7. TPs of other groups of pharmaceuticals

Due to the fact that for some groups of compounds there are only single literature reports on their transformation, (eco)toxicity or bioconcentration, it was decided not to separate them as individual groups but to create a section discussing them together.

2.7.1. Transformation in the environment

Among TPs belonging to other therapeutic groups there is some data on TPs of beta-blockers. It is a popular group of pharmaceuticals that are used to treat heart problems and high blood pressure (Sabbah et al., 2004). Due to a high number of heart diseases around the world, it is a highly consumed group of medicaments, which TPs should be not omitted in the environmental issues, like their stability under different environmental conditions or fate in water compartments.

Among the data found in the literature, TPs of atenolol and metoprolol – atenolol acid and metoprolol acid, chemically are exactly the same compounds, but may be formed from both pharmaceuticals. They have been found during incubation of 13 different soils spiked with their native forms (Koba et al., 2016). TPs were formed quickly in every soil and quite quickly and significantly degraded, which indicates that they probably could not be found in the environmental soil samples.

Metoprolol acid was found in two other experiments (Li et al., 2014, 2015). First one included a flume filled with water and sediments from a lake (Li et al., 2015). Water, spiked with pharmaceuticals (including metoprolol), was circulating, while concentration and presence of native forms and TPs were monitored in surface and pore water. Metoprolol acid was quickly created in sediment pore water and exhibited high mobility to surface water. However, halfway the experiments it reached maximum concentration and then the amount of metoprolol acid started to decrease in both surface and pore water. Other study included sediments collected from rivers, which were mixed with artificial river water spiked with pharmaceuticals (including metoprolol) (Li et al., 2014). The samples were kept in the dark at 15 °C for 35 days. Metoprolol acid was found at the highest amount of all TPs of metoprolol; however, after 20 days quick degradation occurred, which confirms data mentioned above. Two other metoprolol TPs were also found during this experiment. α-Hydroxymetoprolols concentration was increasing for first 10 days and then it started to decrease. In the meantime, α-ketometoprolol was being formed with an increasing amount until day 15. Correlation between disappearance of α-hydroxymetoprolol and continuous increase of α-ketometoprolol suggests that the first one may be the precursor on a transformation pathway of metoprolol to final product, α-ketometoprolol, which concentration after full degradation of α-hydroxymetoprolol was constant until the termination of the experiment. Therefore, it may be stable and persistent in the environment.

The TPs of propranolol (1-naphthol) was also found during experiments described above in both papers (Li et al., 2014, 2015). During batch test with river sediments this TP was being formed during entire experiment; however, it is not clarified if it is persistent to biological and abiotic transformation. The second paper concludes that it was formed in the sediment compartment and then transported to the surface water; however, there was a quick degradation and 1-naphthol was completely removed by the end of the experiment, which indicates that it would not be persistent in the environment.

Also the TPs of two diuretics were investigated osemide (TP – saluamine) and hydrochlorothiazide (TPs – 4-amino-6-chloro-1,3-benzenedisulfonamide and chlorothiazide). Saluamine was observed from the beginning of the batch test with river sediment and artificial river water spiked with furosemide (Li et al., 2014). Concentration of this biotransformation product was increasing throughout entire test. Similar observations were made in the second paper, where sediments and water from a lake were put in a flume and imitation of a stream was constructed (Li et al., 2015). It was observed that saluamine is formed in the sediment compartment and then it is transported to the surface water. Its concentration was increasing throughout entire experiment as well. However, furosemide was not completely removed in both tests, so it is not possible to state if furosemide is persistent or if only degradation rate of parent compound was faster than its TP.

In the case of another diuretic, both TPs of hydrochlorothiazide were formed in sterile and water-only control experiments, which shows, that abiotic formation occurs. Nevertheless, their formation was faster with non – sterilized sediments, thus, there are two routes of their formation. Their concentrations were increasing throughout entire experiments, which may indicate that they are stable in these conditions. Even though hydrochlorothiazide was not completely removed during tests, which suggests constant formation of its TPs, it seems that in flume experiments (Li et al., 2015) there is an equilibrium reached between concentration of parent compound in surface and pore water and the degradation is inhibited or at least slowed, which may indicate that both TPs are persistent.

One more TPs was found in both Li et al. experiments (Li et al., 2014, 2015), namely 4-chlorobenzoic acid, which is a derivative of lipid-lowering agent, bezafibrate. In both batch and flume experiments this TP was quickly formed and quickly degraded, which makes it vulnerable to biotransformation. Moreover, another study confirmed this data, in which water spiked with parent compound was mixed with fresh sludge from a membrane bioreactor used to treat wastewater (Quintana et al., 2005). It was found that 4-chlorobenzoic acid was a product of co-metabolism, which was formed very quickly and after reaching maximum concentration rapid disappearance occurred within 2 days.

Finally, stability in water of ricobendazole (albendazole sulfoxide), a TP of albendazole (antiparasitic) was also investigated (Wu et al., 2009). It was found that degradation followed pseudo-first order kinetics at pH 2–12 and 55 °C. The hydrolysis was pH-dependent, V-shaped, with a lowest rate constant at pH 4.8 ($k_{obs} = 2.4 \times 10^{-4}$ 1/d, $t_{1/2} =$ 96 months). In the pH range 5.5–8 there was a most rapid increase of degradation rate; at this pH range neutral form of this compound is dominant, which suggests that it is most vulnerable to OH-influence. Above pKa of 9.8, the degradation rate increase starts to slow down. Nevertheless, ricobendazole is much more vulnerable to hydrolysis at alkaline conditions than at acidic. Additionally, activation energy of this process pH 11 was around 80 kJ/mol.

2.7.2. (Eco)toxicity and bioconcentration

The available data on the ecotoxicity and bioaccumulation of TPs of other groups of pharmaceuticals is very disperse and usually concerns two or three representative compounds of a given group.

Toxicity of photochemical derivatives of two corticosteroides – prednisolone and dexamethasone towards aquatic organisms was evaluated by DellaGreca et al. (2004). The solution of prednisolone and dexamethasone (both at concentration 50 mg/L) were irradiated for 4 and 8 h respectively. Although, the values of acute toxicity found for *B. calyciflorus*, *D. magna* and *T. platyurus* were generally low, in all cases the TPs were more toxic than parent compounds. The results of the chronic exposure test showed that all metabolites caused 50% inhibition of growth in the crustacean *C. dubia*, in most cases at lower concentration levels than the native form (DellaGreca et al., 2004).

Toxicity of phototransformation products of anti-virus drug acyclovir was assessed by the acute toxicity test of *Photobacterium phosphoreum* T3 spp. (Jia et al., 2019). A gradual increase in toxicity was observed up to 6 h of the experiment, followed by a decrease after reaching the maximum (87.29% of luminescence inhibition). Moreover the inhibition rate of luminescence after 12 h (37.35%) of irradiations was higher that this at the beginning of the experiment (10.38%). As the authors suggested, the most toxic product is likely carboxyacyclovir. These results suggested that once again TPs caused higher toxic effect than parent compounds (Jia et al., 2019).

The opposite effect was observed in the case of antipsychotic drug. Toxicity of thioridazine against *V. fischeri*, decreased during the phototransformation. Furthermore, it was proven that the two most common TPs, thioridazine-2-sulfoxide and thioridazine-5-sulfoxide, were less toxic than parent compound. Thioridazine caused toxic effect towards *V. fischeri* with EC_{50} values between 5.1 and 11.6 μmol/L, whereas for thioridazine-2-sulfoxide EC_{50} was in the range 18.1–211.2 μmol/L. In the case of second metabolite – thioridazine-5-sulfoxide, EC_{50} could not be determined within the tested concentration range (Wilde et al., 2016).

The lack of greater toxicity of phototransformation products compared to the native form was also observed with mesoridazine (Wilde et al., 2018). However it must be highlighted that in both cases the applied QSAR models predicted the hazardous impact of some formed TPs. For thioridazine mutagenic activities for TPs with carbazole heterocyclic aromatic ring was observed (Wilde et al., 2016). Moreover, some of mesoridazine TPs with carbazole derivatives were revealed as potentialy persistence, bioaccumulation, and toxicity (PBT) compounds (Wilde et al., 2018). Although this required the additionally in vitro test, this is further evidence that TPs of drugs may pose risks to human health and the environment.

Some information about toxicity of metabolites and TPs of β-blockers is also avalaible. Liu et al. (2009) evaluated the toxic effect of photodegradation products of propranolol (at two starting concentrations of native form: 1 and 10 mg/L) towards algae (*P. subcapitata*) and rotifer (*B. calyciflorus*). The results for both species showed that at each point of the experiment (6, 24, 48 h), a higher EC_{50} value (lower toxicity) was determined for the irradiated sample compared to the parent compound. The toxicity was reduced of 1.8 to 3 - times during the photolysis. The opposite results were obtained in the study of photodegradation products of atenolol and metoprolol (Toolaram et al., 2017). Their photo-TPs showed higher toxic effect than natives form. The significant growth of luminescence inhibition of *V. fischeri* was observed after 16 and 32 min of irradiation for metoprolol and atenolol respectively (Toolaram et al., 2017). In the study of Grabarczyk et al. (2020), the toxicity of human metabolite of metoprolic acid towards different organisms was evaluated. However, both the metabolite and the native form did not cause toxic effects against most species. Only for algae the 90% growth inhibition after exposure of metoprolic acid was observed (Grabarczyk et al., 2020). Given the limited number of papers and their inconclusive results, it is clearly important to continue research on the fate of TPs and beta-blocker metabolites in the environment.

Clofibric acid is the active metabolite of the lipid regulators clofibrate, etofibrate and etofyllinclofibrate (Ferrari et al., 2003). Although its presence in water has been confirmed, there is very little information about its toxicity. Ferrari et al. (2003) evaluated its toxic effect towards alga (*Dunaliella tertiolecta*), crustacean (*Palaemonetes pugio*) and fish (*Fundulus heteroclitus*). No morphological changes after exposure of clofibric acid (at concentrations ≤ 1000 mg/L) in the algal cells were observed. In the case of *P. pugio* and *F. heteroclitus*, this metabolite did not significantly affect their survival and whole body protein and cholesterol content (Emblidge and DeLorenzo, 2006). In the another study, EC_{50} concentrations determined in the 48 h acute ecotoxicity test for clofibric acid was higher than 200 mg/L (for *D. magna* and *C. dubia*). Chronic toxicity tests for four species showed that *P. subcapitata* (NOEC = 75×10^{-3} mg/L) was more sensitive than *B. calyciflorus* (NOEC = 246×10^{-3} mg/L), *C. dubia* (NOEC = 640 μg/L) and *D. rerio* (NOEC = 700 μg/L). The results from this study showed that clofibric acid is relatively low toxicity towards aquatic organism (Ferrari et al., 2003). Ecotoxicological impact of clofibric acid in carp (*C. carpio*) was evaluated in the study of Saravanan et al. (2011). Fish was exposed at different concentrations of clofibric acid (0.001, 0.01, 0.1 mg/L) for a period of 96 h. This study showed that it has significant effects on hematological, ionoregulatory and enzymological profiles in fish. Changes were observed, among others, in the levels of white and red blood cells, electrolytes and various enzymes. This clearly indicates the effects on fish body functions during exposure to different concentrations of clofibric acid. Furthermore, the results of this study indicate that changes in biochemical parameters can be used as potential indicators of metabolite toxicity in fish (Saravanan et al., 2011). Although according to the results of the presented study, clofibric acid did not show a strong toxic effect on aquatic organisms, long-term effects cannot be excluded (e.g. reproductive toxicity). Therefore, further studies are needed in which other metabolites and TPs of lipid regulator drugs should also be included.

Liu et al. (2016) determined the bioconcentration of antifungal pharmaceuticals – ketonazole and its metabolites in fish (*C. auratus*). Seven metabolites were detected in tissues after exposure to a series concentrations of native form (0.2×10^{-3}, 2×10^{-3}, 20×10^{-3} mg/L) for 14 days. *N*-deacetylketoconazole was the most dominant metabolite in the liver and muscle, whereas the amount of the rest of compounds was small. However, it must be highlighted that the accumulation of all metabolites increased according to an increased exposure time and they may affect the biological effect induced by ketonazole (Liu et al., 2016).

Another important consideration is the potential to accumulation of drug metabolites in plants, especially those that provide food for animals. The presence of albendazole (a widely used anthelmintic drug) metabolites in food plants were confirmed in many studies (Stuchlíková et al., 2020; Podlipná et al., 2013; Raisová et al., 2017; Stuchlíková et al., 2016). Consumption of plants containing albendazole residues or its metabolites poses a risk to helminth-infected animals, as it may result in the evolution of drug-resistant strains (Stuchlíková et al., 2020). This is another possible negative effect of the presence of drug metabolites in the environment that should be considered when assessing their environmental impact.

3. Conclusions and future perspectives

To the best of our knowledge, in this paper we have reviewed all the existing data on the environmental fate, (eco)toxicity and bioconcentration of TPs of pharmaceuticals. Although the state of knowledge on this subject is still insufficient, it is possible to draw first conclusions and point out future perspectives.

First of all, it must be highlighted that the knowledge on the stability of pharmaceuticals' TPs is very limited. Two processes are investigated the most – hydrolysis and biodegradation, sometimes they are included in one experiment. The influence of light on the stability of TPs is much less studied, whereas sorption potential to e.g. soil or sediment is hardly described. Moreover, the experiments are diversified; some are strictly focused on the evaluation of degradation parameters like rate constant, half-life or the influence of various parameters on degradation rates, like pH, temperature or time of incubation. On the other hand, the experiments regarding biotransformation are mostly batch tests with natural sediments, wastewater active sludge or similar material. However, in most cases the incubation is performed with parent compounds and only those TPs, which were formed during experiments, are further investigated. Even though, this is valuable information on their possible appearance in the environment, studies on single TPs are desperately needed to be performed to get full information.

From all TPs described in our paper, there are five that seem to be the most stable and persistent: α-ketometoprolol, carbamazepine-10,11-epoxide, 1-acetyl-1-methyl-2dimethyloxamoyl-2-phenylhydrazide, 3-(keto-karboxymethyl)hydratopic acid, endoxifen, (*Z*)-4-hydroxytamoxifen, however more data is required to confirm this. Moreover, it must be also pointed out that most of the data reviewed in our paper refers to the model experiments, hence the extrapolation to the environmentally relevant conditions may be difficult. The experiments conducted in wastewater or with material from some kind of water treatment facility may point out most vulnerable and stable compounds, but the microbial diversity and amount in natural waters makes it difficult to predict if the same mechanisms will occur under natural conditions.

Nevertheless, the original experiments involving especially natural sediments present more light on the origins and fate of TPs. They prove, that TPs can be formed in natural waters, so poorly treated wastewater containing metabolites are not the only source of these compounds in the environment. Scarce, unique investigations, like this of Li et al. (2015) or its continuation made by Posselt et al. (2020) conducted in a flume filled with natural sediment and artificial river water could be very important signals of how complicated it can be to predict

the fate of the compound. In these studies, it was shown that bacterial diversity has an influence on dissipation time of compounds and that some parent compounds are transferred to the waters near the bottom of a watercourse, there they are transformed into their TPs, which then may be transferred back to the surface water. Similar observations were made during incubation with various soils (Koba et al., 2016), where also formation of TPs was observed. Other interesting study showed, that TPs can be transformed back to the parent compound under the influence of microorganisms (Göbel et al., 2005). The experiments of Zuehlke et al. (2007) showed that there are stable and unstable TPs formed during drinking water treatment; however, even those unstable were found after the treatment, due to too short treatment time. These reports show, that investigation of fate of pharmaceuticals and their transformation products is multidimensional and there is much more to investigate.

Furthermore, for many groups of pharmaceuticals the available literature data for the (eco)toxicity is focused on one most important TP such as norfluoxetine (for SSRI drugs), *O*-desmethyltramadol (for opoids) or sulfonamides (for antibiotics). According to the information presented in the Fig. 1, in most cases, TPs of SSRI and anticancer drug show lower or similar toxicity compared to the parent forms. However, in the case of TPs of NSAID, antibiotics and antiepileptic drugs, the toxicity of their products was often higher than that of the native forms. It is also important to note the emerging discrepancies in the data. Using carbamazepine-10,11-epoxide as an example, it can be seen that it is assigned to a group of TPs with both lower, similar and higher toxicity with respect to the native form. Therefore, in many cases, it is difficult to clearly determine how much threat a given TPs poses. It should also be borne in mind that phototransformation studies usually determine the toxicity of a mixture of TPs, without determining which of them has the greatest effect on its properties. Moreover for many groups of compounds (e.g., antiviral drugs, beta-blockers, corticosteroids), there are only single literature reports about their (eco)toxicity. Furthermore, based on Fig. 1, it can be observed that the most frequent toxicity tests were performed towards algae, plants, bacteria, crustaceans or rotifers. However, there is little information on the toxic effects of TPs on mussels or fish. Therefore, when conducting further research, it would be advisable to follow a uniform approach and include those groups of compounds and organisms for which the state of knowledge is the least.

Finally, it must be also stated that data regarding bioconcentration of drug TPs is even more scarce. Although there are literature reports confirming the presence of some drug metabolites in aquatic tissues (e.g. TPs of SSRI, antibiotics, antiepileptic drugs), it is difficult to assess their consistency due to small amount of data. Furthermore, for many groups of pharmaceuticals, (e.g. NSAID, anticancer drugs, corticosteroids or antiviral drugs) there are no literature reports on this topic. In addition, a variety of approaches are observed in conducting research on TPs bioconcentration – sometimes organisms are exposed directly to the metabolite and sometimes to the native form and then the

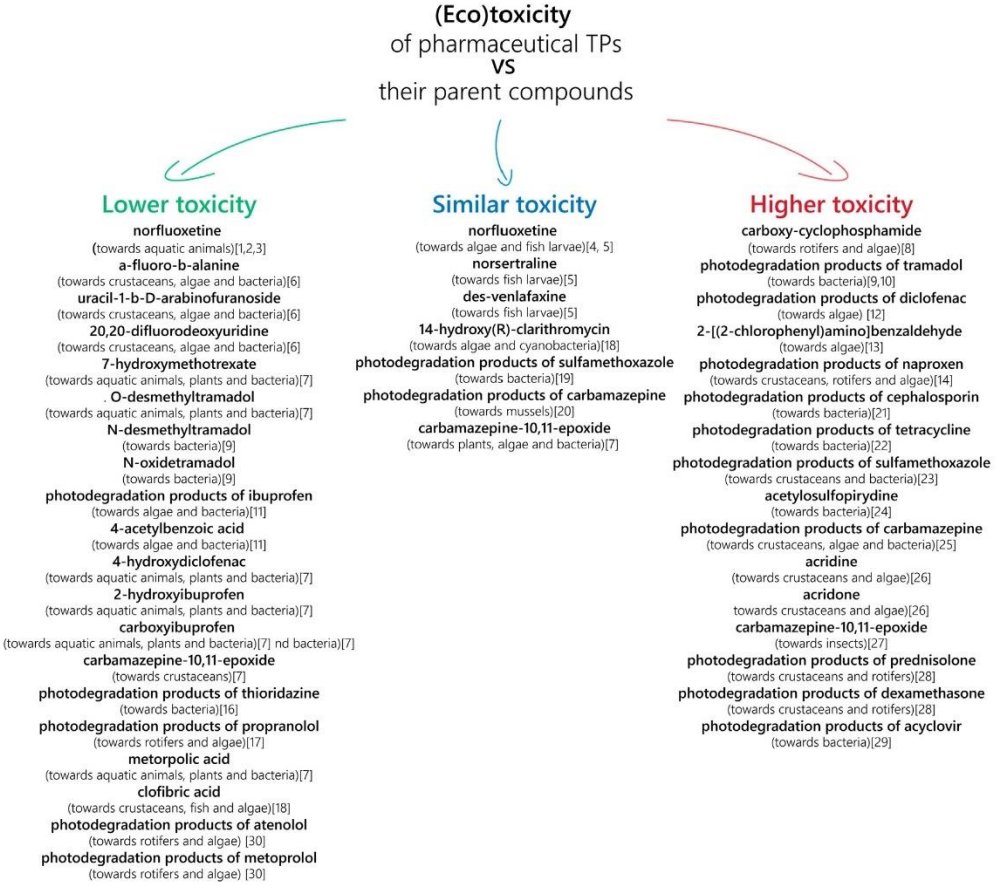

Fig. 1. (Eco)toxicity of selected TPs of pharmaceuticals in comparison to their parent compounds.

amount of TPs formed by metabolism is determined. The amount of a compound is usually only quantified in terms of its concentration in individual tissues (mainly brain and liver), while determining BAF or BCF is less common. The most commonly used organisms for this purpose are fish, less frequently mussels. There are also single studies on the presence of TPs in plants. Due to the wide disparity in how research is conducted, it would be appropriate to standardize it and propose one reference system. This would allow one to obtain reliable results that could be easily compared with each other.

In conclusion, knowledge of the environmental fate, toxicity and bioconcentration of drug TPs is very limited. Existing data is highly selective, leaving too many gaps for unambiguous risk assessment. It has been proved that there are numerous compounds whose toxicity is enhanced in relation to parent compounds. However, in order to assess their environmental risk knowledge on their stability is crucial. It should therefore be stressed that there is an urgent need to complete the data necessary for a reliable assessment of the risk posed by pharmaceutical TPs.

Declaration of competing interest

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

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Publikacja 4

Journal Pre-proof

Bioconcentration potential of ionic liquids: New data on membrane partitioning and its comparison with predictions obtained by COSMOmic

Jakub Maculewicz, Anna Białk-Bielińska, Dorota Kowalska, Piotr Stepnowski, Stefan Stolte, Stephan Beil, Agnieszka Gajewicz-Skretna, Joanna Dołżonek

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Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMO*mic*

Jakub Maculewicz[a], Anna Białk-Bielińska[a], Dorota Kowalska[a] Piotr Stepnowski[a], Stefan Stolte[b], Stephan Beil[b], Agnieszka Gajewicz-Skretna[c], Joanna Dołżonek[a*]

[a]*Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland*

[b]*Institute of Water Chemistry, TU Dresden, 01062 Dresden, Germany*

[c] *Laboratory of Environmental Chemoinformatics, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland*

*corresponding author: joanna.dolzonek@ug.edu.pl

Abstract

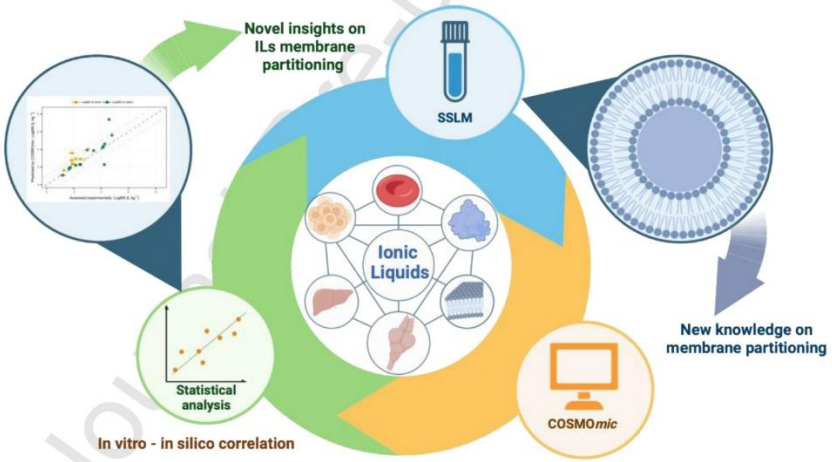
Ionic liquids (ILs) have recently gained significant attention in both the scientific community and industry, but there is a limited understanding of the potential risks they might pose to the environment and human health, including their potential to accumulate in organisms. While membrane and storage lipids have been considered as primary sorption phases driving bioaccumulation, in this study we used an *in vitro* tool known as solid-supported lipid membranes (SSLMs) to investigate the affinity of ILs to membrane lipid - phosphatidylcholine and compare the results with an existing *in silico* model. Our findings indicate that ILs may have a strong affinity for the lipids that form cell membranes, with the key factor being the length of the cation's side chain. For quaternary ammonium cations, increase in membrane affinity (logMA) was observed from 3.45 ± 0.06 at 10 carbon atoms in chain to 4.79 ± 0.06 at 14 carbon atoms. We also found that the anion can significantly affect the membrane partitioning of the cation, even though the anions themselves tend to have weaker interactions with phospholipids than the cations of ILs. For 1-methyl-3-octylimidazolium cation the presence of tricyanomethanide anion caused increase in logMA to 4.23 ± 0.06. Although some

of our data proved to be consistent with predictions made by the COSMO*mic* model, there are also significant discrepancies. These results suggest that further research is needed to improve our understanding of the mechanisms and structure-activity relationships involved in ILs bioconcentration and to develop more accurate predictive models.

Keywords:

bioaccumulation; lipid partitioning; membrane partitioning; solid-supported lipid membranes; water pollution

GRAPHICAL ABSTRACT



1. Introduction

Ionic liquids (ILs) have gained significant attention in the scientific community in recent years due to their unique properties and potential applications in various fields of chemistry (Beil et al., 2021; Guo et al., 2015). This diverse group of chemicals include various compounds that are typically composed of a complex organic cation, such as imidazolium, pyridinium or quaternary ammonium, combined with an inorganic or organic anion (Guo et al., 2015). They have gained widespread interest mainly due to their extremely low vapor pressure, high thermal stability range, and non-flammability (Singh and Savoy, 2020). These characteristics make ILs an attractive alternative to classical volatile solvents. In addition, they can be easily modified and customized for a variety of applications in modern chemistry. These applications include use as CO_2 absorbents (Aghaie et al., 2018), biomass extractants (Tan et al., 2020), electrolytes for batteries (Yang et al., 2020), corrosion inhibitors (Verma et al., 2021), additives for lubricants (Oulego et al., 2018), as well as sorbents in Solid-Phase Extraction (SPE) (Liu et al., 2021).

Despite the many potential benefits of ILs, there are also concerns about their potential environmental impact. Some ILs have been found to be toxic to microorganisms, aquatic invertebrates, and fish, but the exact mechanisms responsible for these effects are not yet fully understood. Structures of some ILs also have a high resistance to biodegradation and hydrolysis, which can be problematic in the event of environmental pollution (Gathergood et al., 2004; Stolte et al., 2012; Romero et al., 2008, Thuy Pham et al., 2010; Gomez-Herrero et al., 2018; 2019; Fabiańska et al., 2012a; 2012b). A study by Bystrzanowska et al. (2019) analyzed 319 ILs for their "greenness" based on various criteria, including biodegradability and toxicity, and found that ILs can be positioned between highly volatile non-polar solvents (e.g. cyclohexane, benzene, chloroform) and greener polar (e.g. methanol, isopropanol and acetone) classical solvents. However, more data is needed to fully assess the safety and environmental impact of

ILs. Overall, while ILs have the potential to be useful alternatives to hazardous organic solvents in a variety of applications, it is important to carefully consider their potential environmental effects before widespread adoption. Further research is needed to fully understand the risks and benefits of using ILs in various contexts, in particular with regards to their high persistency.

The importance of providing thorough risk assessment for ILs becomes even clearer in the view of recent studies that confirm the detection of these compounds in environmental samples (Leitch et al., 2021; Probert et al., 2018). For example, 1-methyl-3-octyl imidazolium cation has been determined in soil samples surrounding a landfill waste (Probert et al., 2018). Also perfluorinated IL anions have been detected in river water samples from Germany (Neuwald et al., 2021; 2022). In the screening of 1310 potentially persistent and mobile contaminants, 20 chemicals belonging to ILs have been determined, including 7 perfluorinated compounds (Neuwald et al., 2021; 2022). Furthermore, already mentioned 1-methyl-3-octyl imidazolium cation, being widely utilized in many fields of chemistry, has been recently detected in the blood of patients with autoimmune liver disease primary biliary cholangitis (PBC) (Leitch et al., 2021). Hepatotoxicity and the development of PBC was also linked to the presence of 1-methyl-3-octyl imidazolium cation by other authors (Probert et al., 2018). The described example of the presence of ionic liquids in human tissues is not an isolated case. Quaternary ammonium compounds (QACs) have been detected in human blood samples collected in China, with concentrations reaching a maximum of 68.6 ng/mL (Zhang et al., 2021). The concentration of QACs in samples collected in 2020 was statistically significantly higher than in those collected in 2018, suggesting an increase in human exposure to these compounds. Moreover, in another study involving 43 volunteers (Hrubec et al., 2021), QACs were detected in up to 80% of them, and were found to alter inflammatory markers, mitochondrial function, and cholesterol synthesis intermediaries in a dose-dependent manner.

Persistent chemicals released into the environment pose threats, with bioconcentration being a major concern. Bioconcentration assessment typically involves fish testing, as per legal regulations (e.g. OECD Test Guideline 305), but it is time-consuming, costly, and ethically questionable. *In vitro* methods may provide efficient and reliable alternatives. However, the commonly used octanol-water partition coefficient (log K_{ow} or log P) only considers hydrophobicity and may not accurately represent complex biological structures (Maculewicz et al., 2020). This method may underestimate bioconcentration potential, especially for ionic compounds. Such a situation has been recently confirmed in the case of ILs, the bioconcentration of which was previously excluded (Ropel et al., 2015). However, the preliminary application of more advanced methods (including our previous studies (Dołżonek et al., 2017; Kowalska et al., 2022) showed that ILs strongly interact with membrane lipids (Dolżonek et al., 2017; Droge et al., 2021) as well as proteins (Kowalska et al., 2022), what may result in bioconcentration. The structural and functional differences between proteins, storage and membrane lipids necessitate separate treatment to avoid inaccurate estimates (Treu et al., 2015; Endo and Goss, 2013). Absorption of charged species into cell membranes is influenced by electrostatic interactions with phospholipid head groups (Escher et al., 2000; Armitage et al., 2013). Therefore, artificial membranes, including solid-supported lipid membranes (SSLMs), are considered the most reliable tool to assess membrane partitioning, especially for polar and charged compounds (Golius et al., 2016; Timmer and Droge, 2017).

However, the number of reliable data regarding the bioconcentration assessment of charged compounds, as well as understanding of mechanisms and structure-activity relationship responsible for this phenomenon is not sufficient. Therefore, the aim of the present study was to provide data on the affinity of ILs to membrane lipids in the view of potential bioconcentration. Thereby diverse structures of cations, anions and their combinations have been systematically analysed. By providing experimental data on the interaction of diverse ILs

with phospholipid bilayers, we aimed to add a further contribution to a thorough understanding of how structure affects interactions of ILs with cell membranes.

Moreover, in environmental research, computer modelling is becoming increasingly important tool enabling to reduce the cost and amount of work needed to make initial estimates of the properties of chemicals of interest (Jagiello et al., 2015; Puzyn et al., 2022). One important challenge is to obtain a reliable tool to model the partitioning to phospholipid bilayers and, in a broader context, the bioconcentration potential of organic cations and anions. Among the existing tools for assessing partitioning to lipids, COSMO*mic* may be able to provide reliable results for permanently charged compounds (Bittermann et al., 2016; 2014). Therefore, in this study the experimentally obtained data was compared with data obtained by COSMO*mic*, in order to verify to what extent the existing tools allow predicting the interaction of ILs with membrane lipids.

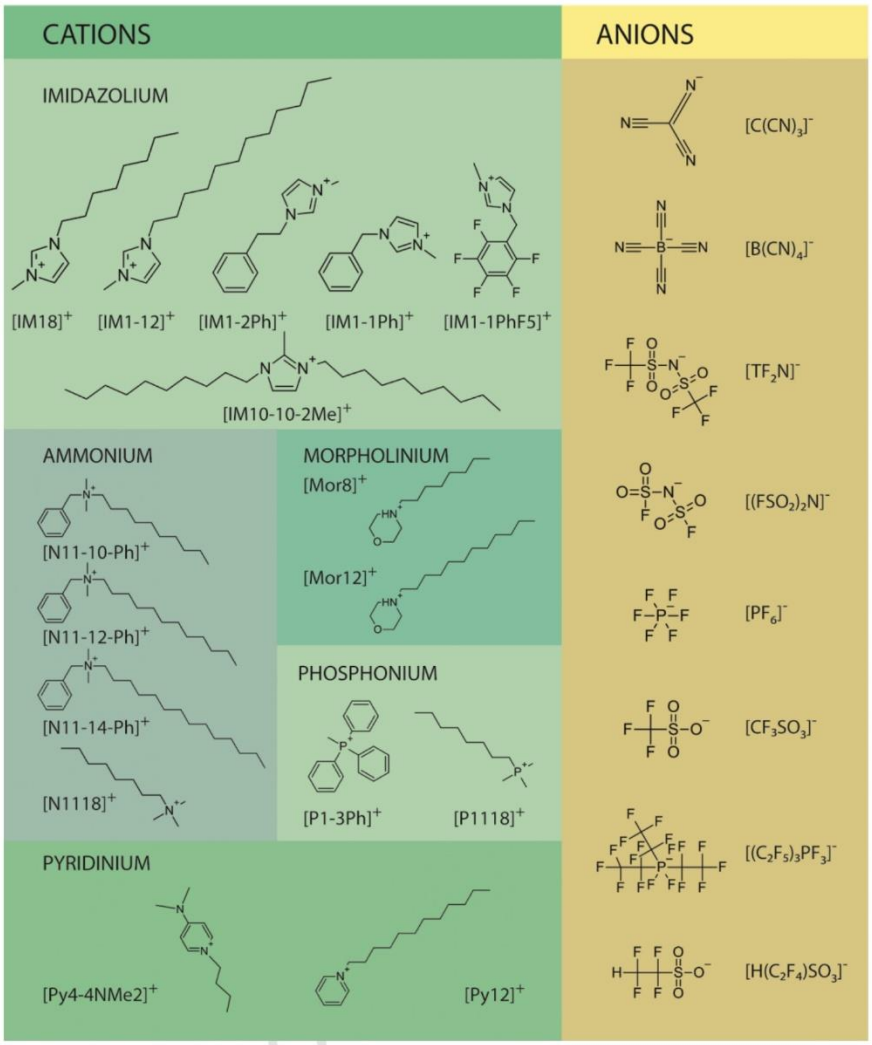


Figure 1. Structures of ions analyzed in this study.

2. Material and methods

2.1 Chemicals

The structures of ILs used are presented in Figure 1. Molecular formulas/acronyms and purity of applied reagents are presented in brackets. Acetonitrile (ACN, CAS: 75-05-8, HPLC grade), potassium dihydrogen phosphate (KH_2PO_4, CAS: 7778-77-0, ≥ 99.5%), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$, CAS: 10028-24-7, > 99%) and potassium chloride (KCl, CAS: 7447-40-7, > 99%) were obtained from POCH (Poland). Sodium chloride (NaCl, CAS: 7647-14-5, > 99%) was acquired from STANLAB (Poland). Methyltriphenylphosphonium bromide ([P1-3Ph][Br], CAS: 1779-49-3, 98%) was purchased from J&K (China). Potassium bis(fluorosulfonyl)amide ([K][$(FSO_2)_2N$], CAS: 14984-76-0 98%), sodium hexafluorophosphate ([Na][PF_6], CAS: 21324-39-0, 98%), and potassium tetracyanoborate ([K][$B(CN)_4$], CAS: 261356-49-4, 98%) were purchased from FluoroChem (Canada); 1-dodecyl-3-methylimidazolium tetrafluoroethansulfonate ([IM1-12][$H(C_2F_4)SO_3$], 97%), 1-methyl-3-octylimidazolium tetrafluoroethansulfonate ([IM18][$H(C_2F_4)SO_3$], 97%), 1-methyl-3-(2,3,4,5,6-pentafluorobenzyl)-3H-imidazolium bromide ([IM1-1PhF_5][Br], 97%), N-methyl-N-octylmorpholinium chloride ([Mor8][Cl], 97%), N-methyl-N-dodecylmorpholinium chloride ([Mor12][Cl], 97%), trimethyloctylphosphonium bromide ([P1118][Br], 95%), 1-methyl-3-octylimidazolium tricyanomethanide ([IM18][$C(CN)_3$], CAS: 1203710-60-4 >98%) were purchased from IoLiTec (Germany). 1,3-didecyl-2-methylimidazolium chloride ([IM-10-10-2Me][Cl], CAS: 70862-65-6, 98%), 1-dodecylpyridinium bromide ([Py12][Br], CAS: 104-73-4, >98%), 1-butyl-4-dimethylaminopyridinium chloride ([Py4-4NMe2][Cl], >98%), 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([IM18][$B(CN)_4$], CAS: 178631-04-4, >98%), trimethyloctylammonium bromide ([N1118][Br], CAS: 2083-68-3 98%), sodium trifluoromethanesulfonate ([Na][CF_3SO_3], CAS: 2926-30-9, >98%), and potassium trifluoromethanesulfonimide ([K][Tf_2N], CAS: 90076-67-8, 97%) were purchased

from Sigma-Aldrich (Poland). Sodium tricyanomethanide ([Na][C(CN)$_3$], CAS: 90076-67-8 , >98%) was purchased from TCI (Japan). Benzyldimethyldecylammonium chloride ([N11-10-Ph][Cl], CAS: 965-32-2, 98%), benzyldimethyldodecylammonium chloride ([N11-12-Ph][Cl], CAS: 139-07-1, 98%), benzyldimethyltetradecylammonium chloride ([N11-14-Ph][Cl], CAS: 139-08-2 (98%), 1-benzyl-3-methyl-3H-imidazolium chloride ([IM1-1Ph][Cl], CAS: 36443-80-8, 98%), 1-methyl-3-(2-phenylethyl)-3H-imidazolium chloride ([IM1-2Ph][Cl], 98%), potassium trifluorotris(pentafluoroethyl)phosphate ([K][$(C_2F_5)_3PF_3$], CAS: 123215-04-3, >98%), potassium 1,1,2,2,-tetrafluoroethanesulfonate ([K][H$(C_2F_4)SO_3$], >98%) and 1-octyl-3-methylimidazolium trifluoromethanesulfonimide ([IM18][Tf2N], CAS: 178631-04-4, >99%) were obtained from IoLiTec (Germany).

2.2 Analysis of membrane partitioning using SSLMs

The affinity of ionic liquids to SSLMs was determined using TRANSIL^{XL} Intestinal Absorption Kit microplate assays purchased from Sovicell GmbH (Germany). Each test plate consisted of 12 rows of wells filled with phosphate buffer (PBS) and SSLMs. SSLMs are 10 μm porous silica spheres with noncovalently bound egg yolk phosphatidylcholine (PC) that mimics a biological membrane.

While measuring the membrane partitioning of chemicals, the composition of the membrane may be important. As previously investigated, several properties, such as the length of an acyl chain, the degree of saturation, and the cholesterol content could be of significance (Kwon et al., 2007; Yamamoto and Liljestrand, 2004). However, the physical state of the membranes is crucial here, and when comparing different phospholipids in the physiologically relevant liquid crystalline phase, obtained results do not vary significantly (Yamamoto and Liljestrand, 2004). As confirmed by Xie et al. (2010), another ionic contaminant of concern, namely perfluorooctanesulfonic acid (PFOS), partitions into membrane bilayers independently of the lipid chain length, with minor differences in partitioning to 1,2-dimyristoyl-sn- glycero-

3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero- 3-phosphocholine (DSPC) membranes (Goh and Tero, 2021).

SSLMs used in our study consisting of egg yolk PCs are in the liquid crystalline phase under experimental conditions.

Eight wells were used to evaluate the affinity of each compound - six of them contained different concentrations of SSLMs (lipid concentration equal to: 261, 470, 846, 1523, 2741, 4934 μM), while two were controls and contained only buffer. To each of the 8 wells 15 μl of a solution of the IL in PBS buffer at a concentration of 800 μM was added, resulting in the final experimental concentration of 50 μM. The samples were then incubated by shaking them for 12 minutes at room temperature (20°C). After incubation, the samples were centrifuged for 10 minutes, and the supernatant (about 100 μl) was collected and subjected to chromatographic analysis using high-performance liquid chromatography with spectrophotometric detection (HPLC-DAD) or ion chromatography with conductometric detection (IC). Based on the concentration of ionic liquids determined in the unbound fraction analysed, isotherms were plotted, and the values of the logarithmic membrane-water partition coefficient (logMA [L kg^{-1}] were determined. The linear isotherms used to determine partition coefficients were obtained by plotting the ratio of total mass of ILs to concentration of ILs in supernatant against the volume of lipids. Next, a linear regression equation was calculated and the value of the partition coefficient was determined from the slope of the regression curve. An exemplary linear isotherm used to determine the partition coefficient is presented in Fig. 2.

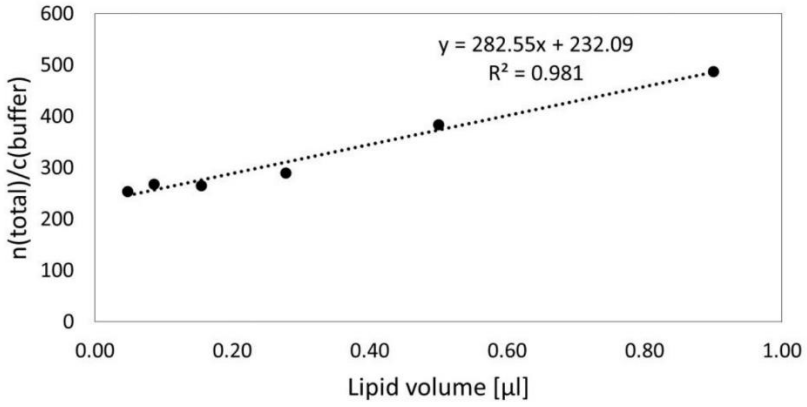


Figure 2. Linear isotherm of sorption determined for [IM18][TF2N] (cation only).

2.3. Instrumental analysis

All chromophore-containing cations were analysed using the HPLC-DAD system Shimadzu Nexera XR A. All anions, as well as cations without chromophores, were analysed by employing a Metrohm 881 compact IC system with a conductometric detector. Details of the individual analytical methods used in this work, including the chromatographic columns, the mobile phase gradient, as well as the validation parameters are summarized in the Supplementary information (Table S1.).

2.4 Modelling of membrane partitioning using COSMO*mic*

Structure files of the studied ILs both as connected ion pairs and as single ions were obtained using COSMOtherm software combined with TURBOMOLE. All calculations were based on BP density functional theory using triple-zeta valence polarization (TZVP) basis sets. Membrane partition coefficients were predicted via COSMO*mic* using a previously published 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) structure as the predominant PC in egg-PC. Bittermann et al., (2016) revealed that for charged species the dipole potential of the membrane plays a major role and has to be taken into account when performing COSMO*mic*

calculations. Therefore, corresponding correction terms were applied and an offset of 0.32 log units subtracted according to the suggestions of the mentioned publication.

3. Results and discussion

Table 1. Affinity of tested cations and anions of ionic liquids to membrane lipid (phosphatidylcholine) assessed by TRANSIL and predicted by COSMO*mic*

Tested compound	Assessed experimentally		Predicted by COSMO*mic*		
	LogMA of cation [L kg^{-1}]	LogMA of anion [L kg^{-1}]	Log MA of isolated cation [L kg^{-1}]	Log MA of isolated anion [L kg^{-1}]	Log MA of ion pair [L kg^{-1}]
Cations with various side chains					
[N11-10-Ph][Cl]	3.45 ± 0.06	-	3.86	-0.04	2.97
[N11-12-Ph][Cl]	4.26 ± 0.10	-	4.61	-0.04	4.25
[N11-14-Ph][Cl]	4.79 ± 0.06	-	5.60	-0.04	4.84
[Mor8][Cl]	1.72 ± 0.15	-	1.98	-0.04	0.27
[Mor12][Cl]	2.97 ± 0.14	-	3.93	-0.04	1.89
[IM-10-10-2Me][Cl]	4.58 ± 0.06	-	7.38	-0.04	4.83
[Py12][Br]	4.10 ± 0.06	-	4.19	-0.04	2.59
[Py4-4NMe2][Cl]	No affinity	-	0.81	-0.04	-
[N1118][Cl]	No affinity	-	2.06	-0.04	1.53
[P1118][Br]	2.15 ± 0.05	-	2.22	0.11	0.60
Anion and cation interaction					
[IM18][C(CN)$_3$]	4.23 ±0.06	No affinity	2.29	1.86	3.19
[IM18][B(CN)$_4$]	2.45 ±0.05	1.98 ±0.07	2.29	2.35	4.42
[IM18][TF$_2$N]	2.44 ±0.06	2.47 ±0.45	2.29	2.92	4.48
[IM1-12][H(C$_2$F$_4$)SO$_3$]	4.14 ± 0.04	-	4.32	1.76	5.00
[IM18][H(C$_2$F$_4$)SO$_3$]	2.50 ± 0.04	-	2.29	1.76	2.84
Cations with aromatic rings					
[IM1-1Ph][Cl]	No affinity	-	0.64	-0.04	0.23
[IM1-2Ph][Cl]	No affinity	-	1.19	-0.04	0.16
[P1-3Ph][Cl]	1.64 ± 0.03	-	1.77	-0.04	-
[IM1-1PhF$_5$][Cl]	1.18 ± 0.22	-	1.08	-0.04	0.17
Anions					
[Na][C(CN)$_3$]	-	No affinity	0.08	1.86	1.81
[Na][B(CN)$_4$]	-	1.87 ±0.07	0.08	2.35	2.16
[Na][TF$_2$N]	-	2.13 ±0.04	0.08	2.92	2.55
[Na][PF$_6$]	-	1.85 ± 0.17	0.08	2.78	2.34
[K][H((C$_2$F$_4$)SO$_3$]	-	No affinity	-0.23	1.76	1.33
[Na][CF$_3$SO$_3$]	-	1.27 ± 0.07	0.08	1.65	1.69

$[K][(C_2F_5)_3PF_3]$	-	3.02 ± 0.10	-0.23	4.00	3.55
$[K][(FSO_2)_2N]$	-	1.86 ± 0.23	-0.23	3.60	2.75

Understanding the structural and functional features influencing potential bioconcentration of chemicals is crucial in order to correctly predict their environmental fate. The uptake of charged species into cell membranes is influenced by electrostatic interactions with phospholipid head groups, highlighting the limitations of relying solely on log K_{ow} for predictions (Treu et al., 2015; Endo and Goss, 2013). Moreover, the absorption of ionic compounds into biological membranes depends on the location of the charge in the molecule, as well as its structural and steric properties (Escher et al., 2000; Armitage et al., 2013). Therefore, our work is meant to provide additional insights to better understand this complex process, based on high quality experimental data.

The results obtained in this work are summarized in Table 1. According to the data obtained, it may be concluded that the length of the side chain may be crucial for the affinity of IL cations for lipids that form cell membranes, which is supported by the results obtained for ammonium cations with different side chain lengths. An increase in logMA was observed from 3.45 ± 0.06 at 10 carbon atoms per chain to 4.79 ± 0.06 at 14 carbon atoms. High affinity was also observed for an imidazolium cation with two decyl chains – for which the logMA was 4.58. Subsequent studies confirmed that for imidazolium and pyridinium cations, side chain length can be crucial. The affinities of the pyridinium and imidazolium cations with single 12-carbon chains were 4.10 ± 0.06 and 4.14 ± 0.04, while the imidazolium cation with 8-carbon chain showed an affinity of 2.50 ± 0.04. This may provide further confirmation of observations from our previous research (Dolzonek et al., 2017), in which preliminary assessment of membrane partitioning was performed for several ILs, suggesting that the key factor responsible for the affinity of IL cations for membrane lipids is the presence of a long hydrophobic chain. The length of alkyl chain also plays crucial role in membrane affinity of other chemicals of concern, including ionic (Timmer and Droge, 2017; Droge, 2019) and non-ionic surfactants

(Muller et al., 1999). Therefore, based on numerous studies it is clear that for chemicals with long terminal alkyl chains, their length determines membrane affinity of entire molecule.

In fact, the huge effect of side chain length on the potential for bioconcentration of ionic liquids was also confirmed in our recent study (Maculewicz et al., 2022). The logarithmic bioconcentration factor (logBCF) value determined for $[IM1\text{-}12]^+$ in a 21-day *in vivo* test using *M. trossulus* mussels was 4.34 ± 0.07, which is very close to the logMA obtained for $[IM1\text{-}12]^+$ in the present study (4.14 ± 0.04). On the other hand, in the case of $[IM18]^+$ logBCF was as low as 1.73 ± 0.14, which also corresponds quite well to the value assessed by our SSLMs partitioning tests. However, it must be noted that phospholipids account for approximately 10% of mussel's dry weight (Lin et al., 2003), therefore such good agreement between data obtained *in vitro* and *in vivo* suggests significant sorption in other body compartments, especially proteins. Nevertheless, our data, both those presented here and those obtained earlier in an *in vivo* test (Maculewicz et al., 2022), demonstrated that not only imidazolium cations at side chains longer than 15 carbon atoms may accumulate in living tissues as it was presented before (Droge et al. 2021), but also less hydrophobic imidazolium ILs may strongly interact with membrane lipids and thereby affect potential bioconcentration.

Although the dependence of the logMA value on the side chain length is observed regardless of the head group of the cation, in the case of morpholinium cations this interaction with phospholipids is noticeably weaker (1.72 ± 0.15 and 2.97 ± 0.14 for 8 and 12 carbon atoms in the side chain, respectively). These results once again indicate that morpholinium cations may be a safer alternative to more classical ILs, such as imidazolium and pyridinium. Previous studies have shown that of the five different head groups tested, it is the morpholinium ones that show the least toxicity to bacteria and algae (Stolte et al., 2007). Admittedly, although morpholinium cations fare slightly worse in biodegradability, they are still more environmentally friendly than imidazolium cations in this regard (Beil et al., 2021).

Another aspect considered in our work, in addition to the effect of aliphatic side chains on the potential for bioconcentration of ionic liquids, was the presence of aromatic rings. However, the obtained results suggest that the presence of such structures has no significant effect on the affinity of the cations towards phospholipids. In the case of two imidazolium cations ([IM1-1Ph][Cl] and [IM1-2Ph][Cl]), no affinity for SSLMs was recorded, while a phosphonium cation with three phenyl rings ([P1-3Ph][Cl]) obtained a logMA of 1.64 ± 0.03. Lower affinity for lipids in the case of cations with aromatic rings may be due to their higher polarity compared to corresponding structures with long aliphatic chains. In a recent study by Kaur et al. (2022) it was shown that dicationic imidazolium ILs exhibit significantly lower affinity to lipid membranes than corresponding monocationic species. Moreover, it was suggested that lower ability to penetrate into the lipid bilayers may be responsible for significantly reduced toxicity of dicationic ILs. Interestingly, the imidazolium cation with an aromatic ring substituted with fluorine atoms ([IM1-1PhF5][Cl]) also showed no high binding to lipids, with a logMA of 1.18 ± 0.22. Therefore also steric hindrance might be the cause of weaker affinity to membrane lipid of cations containing aromatic rings.

Another important factor that can affect the affinity of cations towards membrane lipids may be the counterion used. LogMA of the cation $[IM18]^+$ was observed to be around 2.50 when combined with each of the three anions: $[B(CN)_4]^-$, $[H(C_2F_4)SO_3]^-$ or $[TF_2N]^-$. In contrast, when the same cation was paired with the anion $[C(CN)_3]^-$, its interaction with cell membrane-forming lipids is many times stronger, with a logMA of 4.23 ± 0.06. The results thus suggest that the interaction between anion and cation in some cases can lead to a significant increase in lipid bilayer affinity of the cation. However, logMA determined for $[C(CN)_3]^-$ in both cases, tested as $[IM18][C(CN)_3]$ or coupled with $[Na]^+$, indicates lack or very low interaction of this anion with PC. Although, previous studies have suggested that ion pair formation may be important for membrane interactions of imidazolium cations paired with the anion $[TF_2N]^-$ (Yoo

et al., 2014), this anion did not affect the affinity of $[IM18]^+$ to membrane lipids within our study. Nevertheless, it is worth noting that the influence of the $[TFN]^-$ anion on imidazolium cations has been observed for a cation with a shorter side chain (Yoo et al., 2014), for which electrostatic interactions, both with phospholipids and counterions may be stronger than in case of more hydrophobic ones.

In contrast, the interaction of the anions themselves with the SSLMs was noticeably weaker than that of the cations. The highest logMA value was observed for the anion $[(C_2F_5)_3F_3]^-$ and was 3.02 ± 0.10. A logMA value above 2 was also observed for the anions $[TF_2N]^-$ (2.13 ± 0.04) and $[(CF_3SO_2)_2N]^-$ (2.34 ± 0.05). The other tested anions did not show any significant interaction with SSLMs. Therefore, our data suggests that anions of ILs are less likely to bioconcentrate through the pathway related to interactions with phospholipids than cations. The only exception may be anions containing long fluorinated alkyl chains in their structure. These substances may behave like structurally similar perfluoroalkyl substances (PFAS), which are known for their ability to bioconcentrate (Armitage et al., 2012). In recent study by Droge (2019), several PFAS showed logMA more than 4. Moreover, it must be remembered that proteins can also have a significant influence on the bioconcentration of organic chemicals (Kowalska et al., 2021). Therefore, although lipid partitioning of IL anions is rather limited, their bioconcentration cannot be excluded.

Another property that can play significant role in the membrane partitioning of ILs is their polarizability. Polarizability is the ability of the charge distribution of a molecule to deform under the influence of an external electric field and thus form electric dipoles, which leads to the polarization (Putz et al., 2015; Cho et al., 2011; 2021). According to some studies, polarizability can be considered a measure of dispersive forces within a fluid (Freire et al., 2010; Shimizu et al., 2010). Furthermore, as indicated by Sivapragasam et al. (2018) the strength of polarizability and thus dispersive forces in ILs may be an indicator of their toxicity. It was

demonstrated that ILs with high dispersive forces strongly interact with bacterial cell walls and thus are highly toxic for them, as demonstrated for phosphonium and ammonium ILs coupled with different anions (Sivapragasam et al., 2018).

As far as the membrane affinity determined within the framework of this work and previously published data (Dołżonek et al. 2017) is concerned, a clear correlation can be observed between polarizability (Bica et al. 2013) and logMA coefficient for imidazolium ionic liquids (from the same homologous series). Polarizability is a key descriptor used in QSAR modelling for predicting BCF (Freitas et al. 2016; Papa et al. 2007). Nevertheless, it needs to be emphasized that polarizability may be a source of under or overestimation of predicted bioconcentration. Indeed, the results indicate that the cation $[Py4\text{-}4NMe2]^+$ has no affinity for the lipid membrane (Table 1), while its polarizability, which is amounts to 22.53 (Lacrămă et al. 2007) is comparable to the strongly binding $[IM1\text{-}10]^+$ cation (Dołżonek et al. 2017), for which polarizability is determined as 23.49 (Bica et al. 2013).

Nevertheless, currently there are no other studies that have directly investigated the effect of the polarizability of ILs on their bioconcentration potential *in vitro* or *in vivo*. However, some works have addressed this issue using specific computer models (Rehman et al., 2021; Cho et al., 2013), but they have focused more on toxicity potential than bioconcentration and still need to be improved. According to some works, the polarizability has the significant effect on the octanol-water partitioning (Cho et al., 2011; Rybińska et al., 2016; Bittermann et al., 2018), viscosity (Lacrămă et al., 2007; Putz et al., 2015; Deng, Yun 2011 thesis) and solvating activity of the anion group (Khodadoust et al., 2006; Lacrămă et al., 2007), each of them are important for determining the potential for bioconcentration and toxicity in organisms. Polarizability also affects the sorption potential of ILs in sediments, which in turn affects their bioavailability and potential toxicity (Macário et al., 2020; Pignatello and Xing, 2020). Binding to sediments often reduces bioavailability and toxic effects (Macário et al., 2020; Pignatello

and Xing, 2020). Not only the individual properties but also the interaction between them have an essential influence on how the ILs can behave in the environment and in living organisms (Khodadoust et al., 2006; Lacrămă et al., 2007; Cho et al., 2021; Pignatello and Xing, 2020). For example, Cho et al (2013) observed that interactions between dipolarity/polarizability and excess molar refraction have a small but considerable contribution to the prediction of ILs toxicity against *Scenedesmus vacuolatus.*

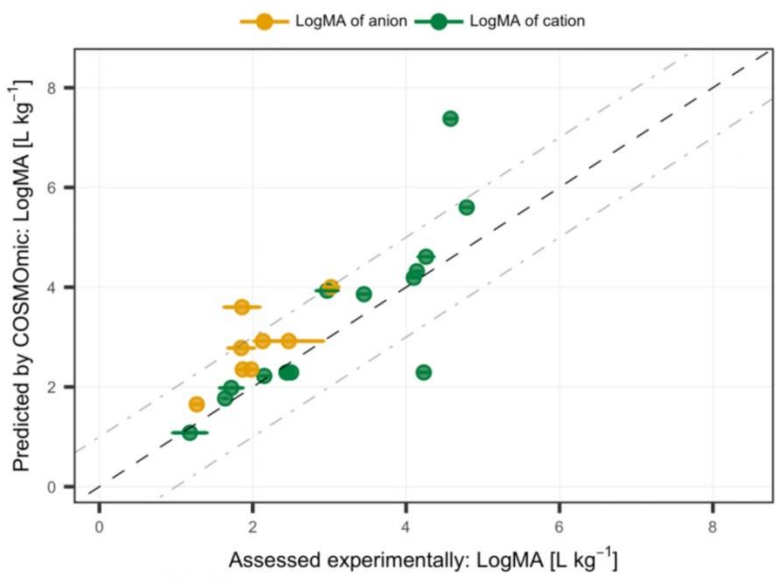


Figure 3. Plot of experimentally observed (Table 1, including error bar) versus estimated with the COSMO*mic* model values of logMA for isolated ions. The solid black line represents a best fit (i.e., perfect correlation). The grey dot-dash lines represent a ±1 logarithmic unit deviation from the experimentally determined logMA values.

Figure 3 illustrates a reasonable agreement between the experimentally observed and predicted with COSMO*mic* model affinities of isolated ions. As depicted in the above figure, the COSMO*mic* model-based predictions reveal considerable variations in the anticipated

logMA values for two cations, diverging by more than one logarithmic unit. Notably, substantial over- or underestimations were only evident for cations exhibiting a high logMA value (above 4.2). The reason for this phenomenon remains unclear, but it might be associated with the intricate relationship between the structure of ionic liquids and their lipid affinities. Specifically, the non-monotonic dependence of IL-lipid interactions suggests that within a specific range of logMA values, a linear relationship may prevail, while in another range, a non-linear relationship could predominate. It tallies well with our previous findings for 1-alkyl-3-methylimidazolium chlorides, when reduced increase in the logMA with elongation of the side chain was observed (Dołżonek et al. 2017).

A correlation analysis was performed to gain deeper insight into the strength and direction of the relationship between experimentally determined and COSMO*mic* model-predicted affinities of isolated cations, anions, and ion pairs. Analysis of the correlogram depicted in Figure 4 unveiled a relatively strong positive correlation ($r = 0.82$) between the experimentally observed and COSMO*mic* model-estimated logMA values for isolated anions, indicating their tendency to move in the same direction. Similarly, a comparable positive correlation ($r = 0.81$) was identified between the experimentally measured and theoretically estimated logMA values for isolated cations. Conversely, a significant negative correlation was apparent for a pair of experimentally determined membrane partitioning of isolated ions (i.e., anions-cations), with $r = -0.88$, as well as for a pair of theoretically determined logMA values for anions-cations ($r = -0.72$). Additionally, moderate positive correlations ($r = 0.56$ and 0.64) were observed between experimentally assessed logMA values for isolated anions or cations and predicted affinities of ion pairs by the COSMO*mic* model, respectively. This suggests that although an increase in the first variable is likely to increase the latter, the relationship is not particularly strong.

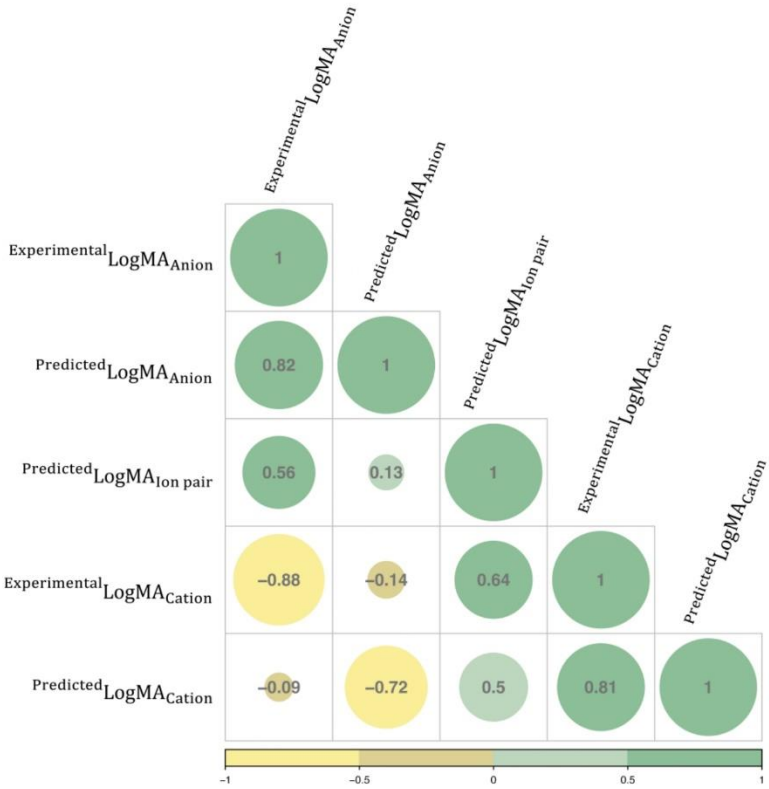


Figure 4. The correlogram visualizes the correlation coefficients between any pairs of variables. Each circle's colour and size illustrate the strength and direction of the linear association: green signifies positive correlations, whereas yellow indicates negative ones. Smaller circles denote weaker correlation coefficients, while larger circles represent stronger ones.

Nevertheless, there are several cases in which the assumption of ion pair partitioning generates values closer to the experimentally obtained. This is particularly noticeable in the case of the cation $[IM18]^+$ combined with various anions. While COSMO predicts logMA well for the cation $[IM18]^+$ with the anions $[B(CN)_4]^-$ and $[TF_2N]^-$ and $[H(C_2F_4)SO_3]^-$ (the determined values are 2.45±0.05, 2.44±0.06 and 2.50 ± 0.04, respectively, compared to the predicted 2.29), the discrepancy is very large for the anion $[C(CN_3)]^-$ (Table 1). The logMA value obtained is almost 2 orders of magnitude higher (4.23 ±0.06). However, it is worth noting

that although this result deviates significantly from that predicted for the isolated cation, it is closer to that obtained for the ion pair (3.19). Neutralising the positive charge by forming an ion pair can enhance binding to the lipid membrane by removing the repulsion between the imidazolium cation and the positively charged choline moiety of the phospholipids. Formation of ion pairs is suggested as one of the reasons for enhanced bioconcentration of perfluorinated compounds (Jeon et al., 2010) On the other hand, even if no ion pair formation occurs, the sorption of the $C(CN)_3$ anion itself may also prevent the repulsive interaction of $[IM18]^+$ with the positively charged choline moiety of the phospholipids.

To address whether there are significant differences between the results predicted by the COSMO*mic* model and those obtained through experimentation, a pairwise Student's *t*-test was employed. For cations, the computed *t*-test value (t=0.942) is lower than the critical *t*-value ($t_{critical}$=2.145 for α=0.05), and the associated *p*-value is not statistically significant (p=0.362 >0.05). This supports the hypothesis that no noteworthy difference exists between the experimentally observed logMA values and those predicted with the COSMO*mic* model. Meanwhile, examining the differences between experimental and predicted logMA values for anions reveals a significant disparity (p=0.0023 < 0.05). The sample size could potentially rationalize this difference; generally, as the sample size increases, so does the test's statistical power. Consequently, further investigation is imperative to validate the concordance between experimentally derived affinities of anions and those calculated using the COSMO*mic* model. It should be noted, however, that both the experimental data and the model unanimously confirm that the anion $[(C_2F_5)_3PF_3]^-$ has the highest affinity for membrane lipids among the tested anions what is in agreement with previous findings (Dołżonek et al. 2017).

As previously reported by Bittermann et al. (2014; 2016), among existing models, COSMO*mic* proves to be the most suitable one for estimation of membrane partitioning of charged chemicals. An important advantage of this model is the mechanistic approach, which

makes it possible to estimate the partitioning to lipids based on the structure of chemicals (Bittermann et al., 2014). However, as observed in our work, while in some cases the model is in a good agreement with the experimental data, there are several significant points of divergence. However, at this point is it not possible to undoubtedly point out the reason for unpredictable behaviour observed for several compounds. Therefore, more research is needed to fully understand the mechanism of membrane partitioning of permanently charged chemicals.

4. Conclusions

In conclusion, the results obtained in this study clearly indicate that ionic liquids can have high affinity for membrane lipids. An important contribution to a better understanding of the structure dependence of IL-lipid interactions is provided by the data presented in this paper. In the case of cations, our work further confirms that the key factor is the length of the side chain, which directly translates into the hydrophobicity of the compound. What is more, our studies suggest that the effect of anions on the affinity of cations also requires special attention. Although anions themselves mostly show weaker interactions with cell membrane-forming lipids than cations, their influence on the counter ion can be significant. Consequently, our data suggest that anions might affect the bioconcentration of cations.

Comparison of experimentally obtained data with predictions obtained using COSMO generally performs best when considering the affinity of isolated ions. However, while in some cases the model data are in good agreement with the experimental data, there are several significant points of divergence. Therefore, it should be emphasized that the large number of data provided on the affinity of chemically diverse ionic compounds for cell membrane-forming lipids may provide a basis for improving existing models or creating new, more reliable ones.

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Declaration of competing interest

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

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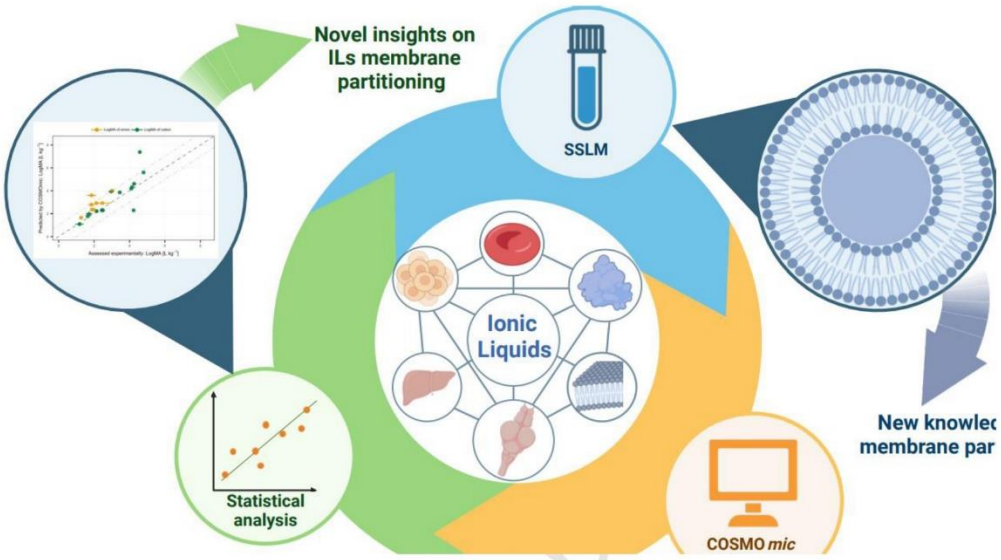
Piotr Stepnowski reports financial support was provided by National Science Centre Poland. Stefan Stolte reports financial support was provided by German Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical abstract



Highlights

- Ionic liquids can have high affinity for membrane lipids.
- In the case of cations the key factor is the length of the side chain.
- Anions influence on the counter ion affinity to membrane lipids can be significant.

Supplementary Material for:

Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMO*mic*

Jakub Maculewicz[a], Anna Białk-Bielińska[a], Dorota Kowalska[a] Piotr Stepnowski[a], Stefan Stolte[b], Stephan Beil[b], Agnieszka Gajewicz-Skretna[c], Joanna Dołżonek[a*]

[a]*Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland*

[b]*Institute of Water Chemistry, TU Dresden, 01062 Dresden, Germany*

[c] *Laboratory of Environmental Chemoinformatics, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland*

*corresponding author: joanna.dolzonek@ug.edu.pl

S1. Validation of analytical methods for final analysis of ILs by HPLC and IC.

For the purpose of method validation the standard solutions of each of ILs in PBS buffer were prepared at 9 concentration levels (1, 2, 5, 10, 20, 40, 60, 80, 100 µM) and analysed six times each. Validation parameters such as linearity, coefficient of determination (r^2), intra-day precision (expressed by RSD), accuracy (defined as the percentage of deviation from the known amount of analyte added to the sample and calculated value), instrumental limit of quantification (LOQ, calculated as the lowest concentration at which the method is linear with acceptable precision and accuracy) and instrumental detection limit (LOD, calculated as $\frac{LOQ}{3}$) were established according to standard procedures, as suggested by Eurachem (1998).

Table S1. Analytical methods used for determination of ILs.

Compound analyzed	Chromatographic column	Flow rate [ml/min]	Injection volume [µL]	Analysis time [min]	Retention time [min]	Wave length [nm]	Mobile phase
[IM1-1Ph]$^+$ [Cl]$^-$	Kinetex C-8 2.6 µm. 100 × 3 mm			5	1.5	205	Phase A 98%; Phase B 2%
[IM1-2Ph]$^+$ [Cl]$^-$				5	2.4	205	Phase A 98%; Phase B 2%
[Py4-NMe2]$^+$ [Cl]$^-$				5	2.2	285	Phase A 95%; Phase B 5%
[IM-10-10-2Me]$^+$ [Cl]$^-$	Luna PS C-18 3.0 µm. 100 × 3 mm			15	7.2	212	Phase A 58%; Phase B 42%
[N11-10-Ph]$^+$ [Cl]$^-$	Kinetex XB C-18 2.6 µm. 100 × 3 mm			10	6.2	215	Phase A 60%; Phase B 40%
[N11-12-Ph]$^+$ [Cl]$^-$				10	7.5	215	Phase A 50%; Phase B 50%
[N11-14-Ph]$^+$ [Cl]$^-$				8	5.2	215	Phase A 40%; Phase B 60%
[IM18]$^+$ [C(CN)$_3$]$^-$		0.5	10	7	4.2	212	Phase A 80%; Phase B 20%
[IM18]$^+$ [B(CN)$_4$]$^-$				7	2.9	212	Phase A 80%; Phase B 20%
[IM18]$^+$ [TF$_2$N]$^-$				7	3.2	212	Phase A 60%; Phase B 40%
[P1-3Ph]$^+$ [Cl]$^-$				10	5.8	208	Phase A 90%; Phase B 10%
[Py-12] [Br]				10	5.6	260	Phase A 60%; Phase B 40%
[IM18H(C$_2$F$_4$)SO$_3$]				9	4.1	212	Phase A 80%; Phase B 20%
[IM1-12H(C$_2$F$_4$)SO$_3$]				10	6.5	212	Phase A 60%; Phase B 40%
[Morph8][Cl]	Metrosep C4 5 µm. 150 × 4 mm	1.2	20	14	8.8	-	Phase A 50%; Phase B 50%
[Morph12][Cl]		1.2	20	14	8.1	-	Phase A 50%; Phase B 50%
[K]$^+$ [(C$_2$F$_5$)$_3$PF3]$^-$	Metrosep A Supp 5 µm 50 × 4 mm	1.2	20	17	12.2	-	Gradient elution: from 0 to 9 min: Phase A 100 %; from 9 to 17 min: Phase A 45%: Phase B 55%
[Na]$^+$ [PF$_6$]$^-$		1.2	20	17	13.1	-	Gradient elution: From 0 to 9 min: Phase A 100 %; From 9 to 17 min: Phase A 45%: Phase B 55%

$[K]^+$ $[H((C_2F_4)SO_3]^-$	1.2	20	17	12.9	-	Gradient elution: From 0 to 9 min: Phase A 100 %; From 9 to 17 min: Phase A 30%: Phase B 70%
$[Na]^+$ $[CF_3SO_3]^-$	1.2	20	17	11.7	-	Gradient elution: From 0 to 9 min: Phase A 100 %; From 9 to 17 min: Phase A 40%: Phase B 60%
$[K]^+$ $[(CF3SO_2)_2N]^-$	1.2	20	17	12.8	-	Gradient elution: From 0 to 9 min: Phase A 100 %; From 9 to 17 min: Phase A 45%: Phase B 55%
$[K]^+$ $[(FSO_2)_2N]^-$	1.2	20	17	12.7	-	Gradient elution: From 0 to 9 min: Phase A 100 %; From 9 to 17 min: 40% ACN; 60% IC Phase B
$[K]^+$ $[C(CN)_3]^-$	1.2	20	15	12.2	-	Gradient elution: From 0 to 5 min: Phase A 100 %; From 5 to 10 min: Phase A 65%: Phase B 45%; From 10 to 15 min: Phase A 70%: Phase B 30%
$[K]^+$ $[B(CN)_4]^-$	1.2	20	15	11.6	-	Gradient elution: From 0 to 5min: Phase A 100 %; From 5 to 10 min: Phase A 55%: Phase B 45%; From 10 to 15 min: Phase A 60%: Phase B 40%

HPLC

Phase A: 1 mM ammonium acetate buffer (8 mg/L ammonium acetate + 150 µL/L acetic acid)

Phase B: 100% acetonitrile

IC:

Phase A (anions): carbonate buffer (5 mM $NaHCO_3$ + 5mM Na_2CO_3). supressor phase: sulfuric acid (0.1 M)

Phase A (cations): nitric acid buffer (5 mM nitric acid + 5 mM dipicolinic acid)

Phase B: 100% acetonitrile

Table S2. Validation parameters of analytical methods used for determination of ILs.

Compound analyzed	LOQ [µM]	LOD [µM]	Accuracy [%]	Precision RSD [%]	R^2
[IM1-1Ph]$^+$ [Cl]$^-$	2.5	0.83	98.4-115.1	0.68-1.51	0.9997
[IM1-2Ph]$^+$ [Cl]$^-$	2.5	0.83	97.1-123.5	0.51-3.83	0.9996
[Py4-NMe2]$^+$ [Cl]$^-$	1	0.33	99.1-117.2	0.16-3.09	1.0000
[IM-10-10-2Me]$^+$ [Cl]$^-$	2.5	0.83	78.3-104.9	1.29-8.90	0.9985
[N11-10-Ph]$^+$ [Cl]$^-$	2.5	0.83	90.6-102.9	0.20-2.69	0.9995
[N11-12-Ph]$^+$ [Cl]$^-$	5	1.67	90.2-108.3	0.24-1.56	0.9996
[N11-14-Ph]$^+$ [Cl]$^-$	5	1.67	91.9-102.2	0.72-2.33	0.9997
[IM18]$^+$ [C(CN)$_3$]$^-$	5	1.67	90.6-100.9	0.57-3.67	0.9998
[IM18]$^+$ [B(CN)$_4$]$^-$	1	0.33	89.8-105.6	0.71-3.26	0.9996
[IM18]$^+$ [TF$_2$N]$^-$	2.5	0.83	91.5-103.5	0.79-3.85	0.9998
[P1-3Ph]$^+$ [Cl]$^-$	1	0.33	92.1-102.6	0.13-1.79	0.9998
[Py-12] [Br]	5	1.67	98.5-105.5	0.75-3.29	0.9999
[IM18H(C$_2$F$_4$)SO$_3$]	2.5	0.83	96.3-104.1	0.62-3.01	0.9997
[IM1-12H(C$_2$F$_4$)SO$_3$]	2.5	0.83	84.1-102.7	0.66-2.94	0.9992
[Morph12][Cl]	5	1.67	90.8-105.7	0.68-4.91	0.9998
[K]$^+$ [(C$_2$F$_5$)$_3$PF3]$^-$	10	3.33	98.9-101.2	0.24-2.76	0.9999
[Na]$^+$ [PF$_6$]$^-$	10	3.33	93.1-111.6	0.29-3.62	0.9957
[K]$^+$ [H((C$_2$F$_4$)SO$_3$]$^-$	5	1.67	95.3-101.6	0.19-1.47	0.9999
[Na]$^+$ [CF$_3$SO$_3$]$^-$	5	1.67	98.4-105.9	0.16-1.37	0.9995
[K]$^+$ [(CF3SO2)2N]$^-$	5	1.67	97.2-107.2	0.30-2.24	0.9994
[K]$^+$ [(FSO$_2$)$_2$N]$^-$	10	3.33	95.8-103.5	0.74-2.98	0.9986
[K]$^+$ [C(CN)3]$^-$	5	1.67	89.7-105.6	0.92-3.39	0.9998
[K]$^+$ [B(CN)4]$^-$	5	1.67	95.2-132.9	0.50-2.77	0.9983

S2. Literature

1. Eurachem (1998) The fitness for purpose of analytical methods: a laboratory guide to method validation and related topic. LGC Teddington Ltd, UK (First English Edition 1.0)

Publikacja 5

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Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: A preliminary study based on in vitro assessment

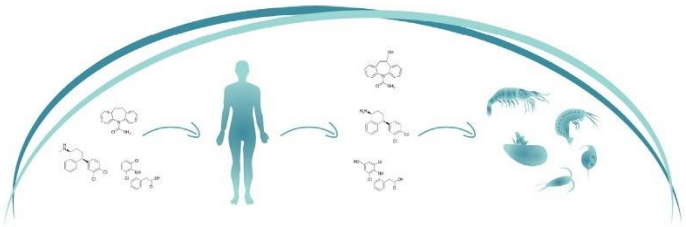
Dorota Kowalska, Jakub Maculewicz *, Piotr Stepnowski, Joanna Dołżonek

Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland

HIGHLIGHTS

- Metabolites of pharmaceuticals may exhibit "non-classical" bioaccumulation behavior.
- The affinity of drug metabolites for proteins is stronger than for lipids.
- Drug metabolites may pose a threat to the environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceuticals pose a real threat to the environment, which has been proven in many studies to date. However, still little is known about the transformation products (TPs) of these compounds, which can also interact with organisms, causing adverse effects like noticeable toxicity or bioconcentration. Many recent works confirm that metabolites of pharmaceuticals are present in the environment, and preliminary studies suggest that they may be equally dangerous to or even more so than their parent compounds. Additionally, it has been proven that some of them have high hydrolytic stability, thus they may be persistent in the environment. This property also increases the likelihood that these compounds will be uptaken and accumulated in the tissues of organisms. Therefore, the aim of the present study was to preliminarily estimate the affinity of the transformation products of selected drugs for blood proteins and cell membrane-forming lipids, considered as important sorption phases during distribution in a living organism. In this study, it was shown that although the examined metabolites do not have a strong affinity for membrane lipids, they exhibit relatively strong binding to proteins, which may considerably affect the distribution of TPs in an organism and may indicate a non-classical process of bioconcentration. The results obtained confirm that the TPs of pharmaceuticals should be given much more attention and their potential for bioconcentration should be further determined.

1. Introduction

Awareness of the presence of pharmaceuticals in the environment and their effects on organisms is already widespread in the scientific community (Desbiolles et al., 2018; Zhou et al., 2018; Puckowski et al., 2016; Świacka et al., 2020). In recent years, however, attention has been drawn to the fact that this problem may be much deeper and concern not only the already wide range of compounds used in medicine and veterinary pharmacotherapy. It appears that pharmaceuticals may be dangerous not only in their primary form, but also as numerous transformation products (TPs) - both those excreted by humans and those formed directly in the environment during abiotic degradation or transformation within organisms. According to numerous works,

* Corresponding author.
E-mail address: jakub.maculewicz@phdstud.ug.edu.pl (J. Maculewicz).

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pharmaceuticals can be metabolised and excreted in the form of derivatives by aquatic animals, such as fish or mussels, but also plants and microorganisms (Burkina et al., 2015; Świacka et al., 2019; Ojemaye et al., 2019). Therefore, in the case of TPs, the problem may be even more complex, because many of them are far less understood than the original compounds, and their adverse effects on the environment may be more difficult to predict.

The presence of TPs in the environment has so far been confirmed to a great extent. A study by Bueno et al. (2016) showed that seawater off the coast of France is polluted not only by the parent compound – carbamazepine, but also by its major metabolites, such as 10,11-dihydro-10,11-trans-dihydroxycarbamazepine and 10-hydroxy-10,11 dihydrocarbamazepine. In the work conducted by Alygizakis et al. (2016), one of the most frequently detected compounds in samples collected from the coast of Greece was norvenlafaxine – the main metabolite of venlafaxine. Interestingly, in this study the parent compound was not detected at all. The analysis of hospital effluent in Japan showed that the metabolites of acetaminophen – acetaminophen glucuronide and acetaminophen sulphate, reached the highest concentrations among analyzed compounds, and their levels were as high as 24 μg/L (Azuma et al., 2016), which was about one order of magnitude higher than the concentration of the parent pharmaceutical. A number of researchers have also observed the presence of metabolites of the popular proton-pump inhibitor, omeprazole, in the environment (Gracia-Lor et al., 2014; Boleda et al., 2013; Boix et al., 2015). According to data presented by Petrie et al. (2015a), up to 20% of emerging contaminants recorded in United Kingdom waters so far are metabolic products. According to a review paper published by Zhou et al. (2019), 66 compounds that are metabolites or transformation products of pharmaceuticals have been recorded in European surface waters to date.

Interestingly, an analysis by Han and Lee (2017) showed that for most of the analyzed common drug compounds, the amount of metabolites excreted through urine and faeces is much higher than that of native compounds. Therefore, it was concluded that metabolites of pharmaceuticals may pose a greater threat to the environment than their native compounds, because a significant amount of them may enter the environment, affecting various organisms, especially due to toxicity (Han and Lee, 2017). For example, norfluoxetine, the main human metabolite of fluoxetine, is 50% more toxic than its parent compound (Nałęcz-Jawecki, 2007). Also in the work by Česen et al. (2016), it was shown that one of the metabolites of cyclophosphamide, carboxycyclophosphamide, shows much greater toxicity to aquatic organisms than its parent compound. In turn, the study by Trovó et al. (2009) showed that the phototransformation products of sulfomethoxazole can be more toxic than the drug itself.

Bioaccumulation is an essential part of risk assessment, because it could be an important link between the chemicals which exist in the environment and in humans (Nendza et al., 2018). The European regulation on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), for all the compounds which are produced or imported in amounts of more than 10 t stipulates that chemicals have to be screened and evaluated for their overall Persistence (P), Bioaccumulation (B) and Toxic (T) (PBT) behavior. The PBT characteristic provides an opportunity to assess the potential risk that the chemical poses to the environment and to living organisms (Article 14 of REACH, 2006). Although so far the metabolites of pharmaceuticals have not been found to qualify for PBT assessment under REACH, their great resistance to hydrolytic degradation indicates a high probability of their presence in the environment (Toński et al., 2019), which in turn may pose the risk of their bioaccumulation in living organisms. Therefore, a proactive approach should be considered for this group of compounds.

Although there are only a few papers in the literature addressing the potential bioconcentration of drug metabolites, they clearly indicate that this phenomenon is possible. A study conducted by Brooks et al. (2005) showed that tissues of fish collected from streams in North Texas, in addition to parent pharmaceuticals, also contained their metabolites: norfluoxetine and desmethylsertraline. Lajeunesse et al. (2011) observed that *N*-desmethyl metabolites of selective serotonin reuptake inhibitors (SSRI) exhibited greater bioaccumulation potential than their parent molecules. Also, Fu et al. (2020) observed significantly higher bioconcentration of the methyl ester metabolite of diclofenac in comparison to the parent compound. Those results indicate that methylation, leading to an increase in the hydrophobicity of metabolites, may enhance bioaccumulation across different non-target species.

Bioaccumulation tests using living organisms, i.e., in vivo methods, seem to be the best reflection of environmental conditions on a laboratory scale. However, they have a number of disadvantages such as high cost and being time-consuming as well as the necessity of using many organisms to evaluate one chemical substance. For this reason, bioconcentration assessment via laboratory experiments without the participation of living organisms, i.e., in vitro or in silico methods, are recommended (REACH, 2006). In general, many models for predicting the bioaccumulation factor (BAF) are based on the octanol-water partition coefficient (Kow), which is a good reflection of partitioning to lipids in living organisms. However, it is important to keep in mind that for ionic or ionogenic compounds, relying only on the Kow may lead to some underestimation (Klamt et al., 2008). This is due to the fact that this model does not account for the interactions of ions with the charged components of lipid membranes. A notable example is a group of compounds named ionic liquids, for which the potential for bioaccumulation determined by partitioning to lipids far exceeded estimations based on the Kow (Dołżonek et al., 2017). Therefore, for ionic compounds, it is essential to use procedures that allow more reliable estimates of bioaccumulation potential (e.g. solid-supported lipid membranes, liposomes or Immobilized Artificial Membrane (IAM) Chromatography) (Tsopelas et al., 2017). Furthermore, it is important to note that other biomolecules may also be involved in the bioaccumulation process. There are increasing literature reports that binding to proteins may be also very important (Armitage et al., 2017). This aspect has been thoroughly investigated in the case of PFAS (perfluoroalkyl substances), for which it has been shown that their high bioaccumulation potential is mainly due to interactions with proteins (Forsthuber et al., 2020; Ng and Hungerbühler, 2013). The non-classical model of bioaccumulation is based on the fact that it results from a series of physiological and physicochemical processes: absorption, distribution, metabolism and extraction (ADME), in which blood proteins have important functions in both humans and fish. The internal distribution of chemicals in the body depends, among others, on the rate at which they are delivered to the tissues with the blood (Armitage et al., 2017). In addition, proteins play a role in the elimination process because chemicals bound to plasma components can be desorbed and thus become available to membrane transport proteins. Thus, blood proteins can provide an important sorption phase for ionic and ionogenic compounds (Henneberger et al., 2016).

Therefore, the aim of the present study was to investigate whether binding to proteins may contribute significantly to the potential bioaccumulation process, in comparison to membrane lipids, for selected metabolites of commonly used pharmaceuticals. Albumin (HSA) and α-1-acid glycoprotein (AGP) were chosen as model proteins due to their different binding properties. AGP predominantly binds neutral and basic compounds, whereas HSA preferentially binds acidic compounds (Fasano et al., 2005). However, due to the various moieties in the structure of chosen analytes, different types of interaction may occur, and partitioning to these proteins may be based on a 'mixed-mode' mechanism. As a representative of membrane lipids, phosphatidylcholine was chosen. The investigated metabolites are a representative group of ionogenic compounds because they are not permanently charged but have functional groups that can dissociate or attach a proton, as a result of which they become charged. Therefore, it is extremely important to

assess their affinity for proteins, as protein binding may be driven not only by the charge, but also may be influenced by the spatial structure and relocation of the charge.

For this reason, determining partitioning to plasma proteins, as well as lipids, may be helpful for the structure-binding relationship and hence important for the future prediction of their potential threat to the environment with regard to bioaccumulation.

2. Materials and methods

2.1. Chemicals used in this study

O-Desmethylnaproxen (des-NPX), 2-hydroxyibuprofen (2-OH-IBU), 4-hydroxydiclofenac (4-OH-DIC, ≥98%), *O*-desmethyltramadol (O-DMTRA, >97%), *N*-acetyl sulfamethoxazole (ac-SMX, >98.5%) and ammonium acetate (ACS reagent, ≥ 97%) were purchased from Sigma Aldrich (Germany). 10,11-dihydro-10-hydroxy carbamazepine (10-OH-CBZ) and carbamazepine-10,11-epoxide (CBZ-Ep) were purchased from TRC (Canada). Organic solvents, such as methanol, acetonitrile and DMSO (all HPLC grade) were purchased from POCH (Poland). Potassium dihydrogen phosphate (KH_2PO_4, ≥99.5%), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$, >99%),potassium chloride (KCl, >99%), formic acid (>98%) were purchased from POCH (Poland). Sodium chloride (NaCl, >99%) was purchased from STANLAB (Poland).

2.2. Affinity for proteins

In order to confirm the binding properties of HSA and AGP towards the investigated compounds, the association constant (K_a) values were determined for the selected metabolites using commercially available test kits: TRANSIL^{XL} HSA Binding Kit and TRANSIL^{XL} AGP Binding Kit, purchased from Sovicell GmbH (Germany). The experiments were carried out according to the protocol attached to the test kits. To put it briefly, the plate after thawing was spun and then the solution of the studied compound was added to each of eight wells of a single column. Two of them were treated as reference samples due to a lack of protein, while other wells included HSA or AGP at different concentrations (in the range of 0.0074 mM–0.14 mM for HSA and 0.004 mM–0.03 mM for AGP) immobilized on silica beads. In the next step, the plate was incubated for 20 min and 2 min for HSA and AGP, respectively, and subsequently centrifuged. Finally, the supernatant was collected and analyzed by HPLC-DAD (see Section 2.4 Instrumental analysis).

2.3. Membrane partitioning

In order to assess the affinity of the investigated compounds for membrane lipids, the membrane-water partition coefficient (log MA) values were determined using commercially available test kits: TRANSIL^{XL} Intestinal Binding Kit, purchased from Sovicell GmbH (Germany). The experiments were carried out according to the protocol attached to the test kits. Briefly, after thawing and centrifuging the multi-well plate, each of the tested compounds was added to eight wells. Six wells contained different concentrations of solid supported lipid membranes, while two wells contained only a buffer and served as controls. In the next step, the plate was incubated for 12 min and subsequently centrifuged. Finally, the supernatants were collected and analyzed by HPLC-DAD (see Section 2.4 Instrumental analysis).

2.4. Instrumental analysis

All the metabolites were analyzed by the HPLC-DAD system with a Gemini® NX-C18 chromatographic column - 5 μm 50 × 2 mm, purchased from Phenomenex, which was used in the case of all of the analytes. All analyses were carried out at 30 °C, the volume of injection was 20 μL and the flow rate was 1 ml/min. For most of the tested compounds, the analytical methods were developed by our team in previous studies (Tońki et al., 2019) and adapted to the work within this study. The validation of the analytical method was carried out for des-NPX. In order to validate the method, des-NPX solutions with concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50 μg/ml were analyzed in six repetitions each. The results obtained made it possible to plot a calibration curve and to determine the corresponding validation parameters. The developed method is characterized by very good linearity, confirmed by a correlation coefficient value greater than 0.99. The method accuracy was in an acceptable range from 90 to 120%, and the value of the coefficient of variation (<3%) confirmed very good precision of the applied analytical method. The limit of quantification (LOQ), determined as the lowest concentration of standard curve, was 1.56 μM and the limit of detection (LOD), determined as LOQ/3, was 0.52 μM. The conditions of analysis for all the tested metabolites are presented in Table 1.

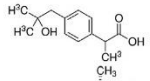
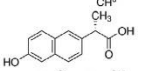
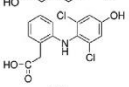
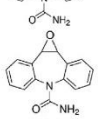
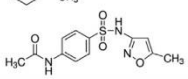
3. Results and discussion

The association constant (K_a) was determined for selected metabolites of pharmaceuticals and blood proteins by using the commercially available TRANSIL^{XL} HSA or AGP Binding Kits. These tests allow the K_a values to be determined by titrating different subphysiological concentrations of HSA/AGP. The obtained values for the tested compounds are presented in Table 2. The results obtained are consistent with the binding properties of proteins. 2-hydroxyibuprofen, *O*-desmethylnaproxen and 4-hydroxydiclofenac exhibit the strongest binding to HSA due to their relatively low values of pKa (4.12, 4.87, 4.17, respectively) under the experimental conditions (pH ~ 7.4), hence their carboxylic group is deprotonated, which results in a negative charge (Rossini et al., 2016). In addition, there is a visible increase in the binding as the hydrophobicity of the compound increases. The presence of two phenyl rings in *O*-desmethylnaproxen and 4-hydroxydiclofenac makes them more hydrophobic than 2-hydroxyibuprofen (log Kow = 3.9, 4.05, 2.37 respectively), which results in a higher log Ka value. For PFAS a close relationship was also observed between the hydrophobicity and binding to HSA (Zhong et al., 2019). Among these three compounds, only *O*-desmethylnaproxen binds to AGP, but the log Ka value is clearly lower than for HSA. Lipophilicity has an impact on the interaction of compounds with both tested proteins (Chrysanthakopoulos et al., 2014, 2010). Therefore, given the binding properties of AGP and the lipophilic nature of O-desNPX, hydrophobic interactions might have a large contribution to this partitioning. For *N*-acetylsulfamethoxazole, affinity for both of the proteins was observed. The presence of the sulfonamide moiety, which is characterized as weak organic acid (pKa in the case of ac-SMX = 5) and can lose its proton relatively easily, explains its

Table 1
Parameters of the HPLC-DAD methods used for the analysis of the metabolites.

Analyte	Detection wavelength [nm]	Mobile phase A	Mobile phase B	Composition of the mobile phase (V/V)	Retention time [min]
CBZ-Ep	209	Acetonitrile	H_2O	30:70	4.45
10-OH-CBZ	237	Acetonitrile	H_2O	25:75	8.79
2-OH-IBU	218	Acetonitrile	0.1% HCOOH	30:70	5.48
O-DMTRA	272	Acetonitrile	0.1% HCOOH	8:92	4.69
des-NPX	232	Acetonitrile	5 mM CH_3COONH_4 + 5% acetonitrile	30:70	4.39
4-OH-DIC	221	Acetonitrile	5 mM CH_3COONH_4 + 5% acetonitrile	35:65	5.05
ac-SMX	263	Acetonitrile	5 mM CH_3COONH_4 + 5% acetonitrile	20:80	6.76

Table 2
Logarithmic values of the association constant (log K_a) for selected metabolites of pharmaceuticals and human serum albumin (HSA), and α-1-acid glycoprotein (AGP), as well as their membrane affinity (log MA).

Analyte	Structure	log $K_{a\ HSA}$ [L/mol]	log $K_{a\ HSA}$ [L/kg protein]	log $K_{a\ AGP}$ [L/mol]	log $K_{a\ AGP}$ [L/kg protein]	log MA [L/kg]	Log Kow
2-OH-IBU		3.59 ± 0.12	1.76	n.b.	n.b.	1.39 ± 0.13	2.37[a]
des-NPX		4.36 ± 0.18	2.54	2.65 ± 0.43	1.06	n.b.	3.9[e]
4-OH-DIC		4.80 ± 0.29	2.98	n.b.	n.b.	2.17 ± 0.11	4.05[b]
10-OH-CBZ		2.72 ± 0.30	0.89	3.76 ± 0.70	1.93	1.79 ± 0.02	1.26[c]
CBZ-Ep		2.75 ± 0.61	0.93	3.83 ± 0.25	2.01	1.62 ± 0.01	1.97[e]
O-DMTRA		n.b.	n.b.	3.87 ± 0.33	2.05	1.59 ± 0.07	2.26[c]
ac-SMX		3.36 ± 0.33	1.53	3.83 ± 0.07	2.01	n.b.	0.86[d]

n.b. – no binding.
[a] Ferrando-Climent et al. (2012).
[b] Ohyama et al. (2014).
[c] Wojsławski et al. (2019).
[d] Lee et al. (2014).
[e] Flores-Mangual et al. (2020).

binding to HSA (Białk-Bielińska et al., 2012; Remko, 2010). However, the amides groups are very strong bases (e.g., pKa for acetamide equal to 15.1) so *N*-acetylsulfamethoxazole also binds to AGP (Milletti et al., 2010). Moreover, studies of the hydrolysis of sulphonamides showed that at pH ~ 7 a decrease in the anionic form and an increase in the neutral and cationic form was observed, which could be explained by the higher log Ka value for AGP than HSA (Białk-Bielińska et al., 2012). Given the relatively low value of log Kow (0.86) and thus the low lipophilicity of this compound, it is likely that the contribution of hydrophobic interactions in binding was negligible. *O*-Desmethyltramadol is an organic base with a pKa value of 9.13 (Tzvetkov et al., 2011), hence in the case of this compound, affinity only for AGP was observed. Both metabolites of carbamazepine showed a similar affinity for proteins. Due to the high pKa values (12.8 and 16 for 10,11-dihydro-10-hydroxy carbamazepine and carbamazepine-10,11- epoxide, respectively), both compounds are considered as bases, thus their stronger affinity for AGP than for HSA is justified. However, due to the presence of three heterocyclic rings in the structure of 10-OH-CBZ and CBZ-EP, these metabolites exhibit moderate lipophilicity (log Kow = 1.26, 1.97 for 10-OH-CBZ and CBZ-EP, respectively), and, thus interaction with HSA was also observed (Malvar et al., 2020).

According to Henneberger et al. (2020), the logarithmic values of plasma/water distribution ratios were significantly higher for diclofenac, ibuprofen and naproxen (3.87, 3.60, 4.36 [$L/kg_{proteins+lipids}$], respectively) than the affinity of their metabolites for HSA, which has been determined in the present study (Table 2). The results for 2-OH-IBU, des-NPX and 4-OH-DIC obtained in our study indicate that these metabolites might have a relatively small affinity for plasma compared to the parent compounds. However, the distribution ratios calculated by Henneberger et al. (2020) related to the total mass of proteins and lipids in the plasma sample, thus the results from both studies could not be compared in a simple way. On the basis of the results obtained for AGP and membrane lipids (Table 2), there is a high probability that the affinity of these metabolites (2-OH-IBU, *des*-NPX and 4-OH-DIC) for plasma (taking into account both protein and lipid fractions, not HSA alone) could have been significantly higher. The log K_a values obtained for ac-SMX (3.36 ± 0.33 [L/mol]) and HSA are slightly lower than those for the parent compound, which, depending on the temperature, was in the range of 3.64–4.07 [L/mol] (Naik et al., 2015a). Nevertheless, the affinity of ac-SMX for HSA still may be considered as significant. For CBZ-Ep, the literature presents a relatively high level of protein binding (47.86%) (Fortuna et al., 2013). However, the carbamazepine metabolites tested in our study showed lower binding to AGP when compared to the literature values for the parent compound, for which the log K_a value at 37 °C equals 5 [L/mol] (Xuan et al., 2010).

A higher affinity of acidic metabolites than basic ones for HSA was also observed, which is related with the presence of high-affinity binding sites for organic acids onto albumin (Henneberger et al., 2020; Fasano et al., 2005).

In contrast, the affinity of selected metabolites for membrane lipids was much weaker than in the case of proteins. The highest affinity was observed in the case of 4-hydroxydiclofenac, for which the log MA value was 2.17 ± 0.11 [L/kg]. However, this means that the affinity of this metabolite for cell membranes can be up to two orders of magnitude lower than that of the parent compound. In the study of Balon et al. (1999), by use of liposomes, the log MA was determined for diclofenac to be 4.3 [L/kg]. Other compounds analyzed in our study showed very low affinity for phospholipids, below 2 on the logarithmic scale. Moreover, no affinity was noted for *O*-desmethylnaproxen and *N*-acetyl sulfamethoxazole.

Regarding interactions with cell membranes, the results obtained in the present study are in good agreement with the key concepts of

pharmacokinetics. In general, high hydrophobicity is a factor that greatly facilitates drug absorption and retention in the body. Consequently, during metabolic processes, drugs are usually transformed into derivatives with much more hydrophilic properties, which ultimately facilitates their excretion from the body (Hacker et al., 2009). Thus our study confirms that for all the metabolites analyzed the affinity for membrane lipids is significantly weaker than for their parent compounds. However, it does not mean that these compounds will not be absorbed and distributed within organisms. It must be emphasized that hydrophobicity and affinity for lipids constitute just one factor in the bioconcentration phenomenon (Maculewicz et al., 2020). According to the current state of knowledge, it is clear that also other biomolecules can significantly affect this process, which is further confirmed by the results we obtained for proteins.

4. Conclusions

The results of our study showed that pharmaceutical metabolites have an affinity for blood proteins, which results from their structure and ability to dissociate or attach a proton. Taking into account the fact that all of them showed higher affinity for proteins than for lipids, it can be assumed that these biomolecules may have a crucial impact on their bioaccumulation process. Thus, metabolites of pharmaceuticals are another group of compounds whose behavior confirms the existence of a non-classical model of bioaccumulation that relies mainly on interactions with proteins. Therefore, it is very important not to overlook this aspect because, as can be seen from our results, the assessment of bioaccumulation potential based only on the partitioning to lipids is a mistake. In the case of ionogenic compounds, this can lead to large underestimations or even to the incorrect conclusion that a given group has no bioaccumulation potential.

Moreover, some of the tested compounds showed a higher affinity for proteins than parent compounds, which may be related to their higher bioaccumulation potential. Therefore, it is extremely important to conduct further studies using a broader group of compounds to elucidate these relationships and assess to what extent their affinity for blood proteins translates into their potential for bioaccumulation.

CRediT authorship contribution statement

Dorota Kowalska: Conceptualization, Investigation, Writing – original draft. **Jakub Maculewicz:** Conceptualization, Investigation, Writing – original draft. **Piotr Stepnowski:** Supervision. **Joanna Dołżonek:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

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Analysis of imidazolium ionic liquids in biological matrices: A novel procedure for the determination of trace amounts in marine mussels

Jakub Maculewicz *, Piotr Stepnowski, Joanna Dołżonek, Anna Białk-Bielińska

Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308, Gdańsk, Poland

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ABSTRACT

Imidazolium ionic liquids (ILs) are chemical compounds beginning to be used on a mass scale. Although their presence in the environment is usually treated as only potential threat, there are already first evidences that this has become a real case. Taking into account their increasing use it might be expected that this problem will also increase in the nearest future. Given that some of the imidazolium cations exhibit high potential for bioconcentration, it is likely that they will accumulate in the tissues of wild organisms. Thus, there is no doubt that monitoring the presence of these compounds in organisms from potentially contaminated waters will be needed. Therefore, the aim of our study was to develop and fully validate a novel and reliable analytical procedure for the determination of the mixture of imidazolium ILs in *Mytilus trossulus* mussels. For this purpose, different extraction techniques were tested such as: microwave-assisted extraction (MAE), accelerated-solvent extraction (ASE) and bullet-blender homogenization (HOMO). Finally, the proposed procedure is based on the application of MAE technique for the extraction of imidazolium cations and SPE technique using Oasis HLB cartridges for the purification of the obtained extracts and LC-MS/MS technique with QqQ analyzer for their final determination. Absolute recoveries of the proposed analytical procedure reached 71–90%. The developed procedure is characterized with low limits of quantification, at 50–100 ng g^{-1} dry tissue and allows for reliable determination of trace amounts of the tested compounds in complex biological matrix. Matrix effects obtained for the optimized procedure ranged from 7.8 to 37.5%. As a result, this is the first study presenting the analytical procedure for the analysis of imidazolium ILs in aquatic animal tissues.

1. Introduction

Over the last two decades, ionic liquids (ILs) have begun to gain interest from the scientific community at an extremely rapid pace [1,2]. Among many different types of ILs designed up to date, one of the most widely studied groups of these chemicals are those based on imidazolium cations. Most imidazolium ILs consist of a cation with a methyl group and a hydrophobic chain (usually from 2 to over a dozen carbon atoms) attached to the imidazolium ring. As counterions, both simple, inorganic ones such as chloride or bromide, but also more complex inorganic anions, often containing fluorine atoms in their structure, as well as organic anions, are used. This design makes it possible to create an enormous number of combinations of anions and cations with different properties, produced for specific purposes. As a result, dozens of imidazolium ILs are already commercially available and some of them are already being produced in tons. However, this is just the beginning, and it is clear that this market will continue to grow in the next years [3].

Like any other chemical compounds that are beginning to be used on a large scale, imidazolium ionic liquids will require the assessment of the possible risk they might cause after being introduced into the environment [4]. Although these compounds were designed to be a safer alternative to many classical chemicals, there is now increasing doubt about this claim. Firstly, due to their high stability, both photo- and hydrolytic, they are not expected to degrade once they enter the environment [5–9]. Numerous papers have also addressed the potential biodegradation of imidazolium ILs, reaching conclusions that most of them cannot be classified as readily biodegradable [10–12]. What is more, already over a decade ago, a considerable number of papers were published treating the toxicity of these compounds, especially towards aquatic organisms. These papers sowed the first doubts by showing that imidazolium ILs can exhibit relatively high toxicity (often higher than, for example, methanol or dichloromethane, which are "classic" solvents) to organisms such as bacteria [13] algae [14–17] or plants [18]. On the other hand, studies by El-Harbawi [19] showed that some of commonly

* Corresponding author.
E-mail address: jakub.maculewicz@phdstud.ug.edu.pl (J. Maculewicz).

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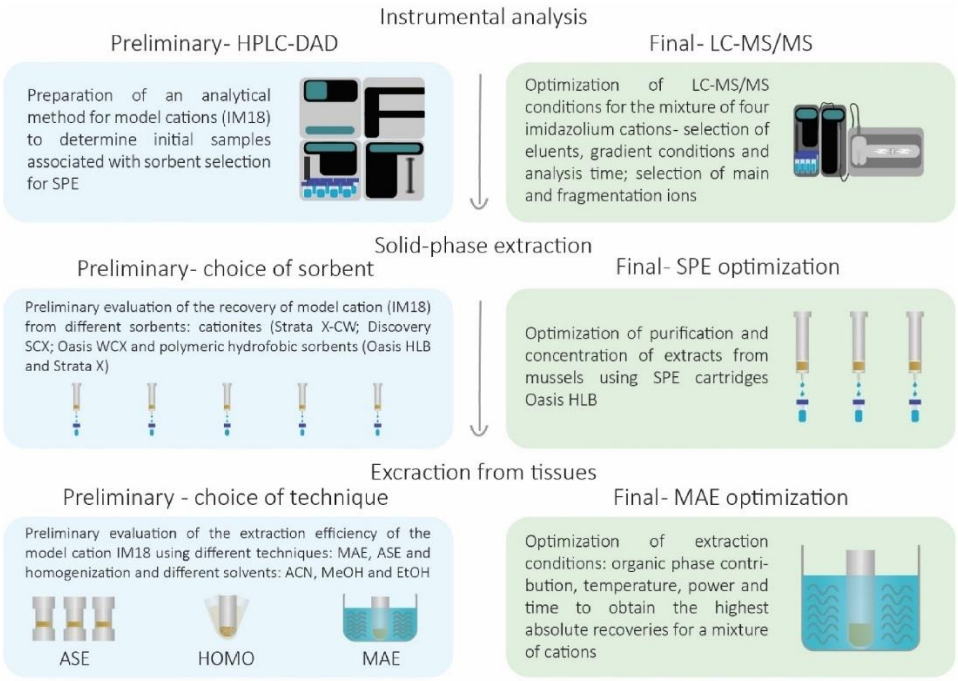

Fig. 1. Conceptual approach of this study.

used imidazolium ILs exhibit very low toxicity towards fish.

Even more importantly, while it seemed that the possible presence of ILs in the environment is more of a problem yet to come, research in recent years shows that it is already a reality. Two recently published papers have demonstrated the presence of 1-octyl-3-methyl imidazolium cation in both soil and human blood samples, further establishing that this compound may be a direct cause of one of the autoimmune diseases [20,21,]. A screening study by Neuwald et al. [22,23,], who looked for new contaminants in German rivers also provided further relevant information on this topic. An important group of compounds detected within this work were precisely ILs, both anions (especially fluorinated ones, like BF_4 or PF_6 often used in association with imidazolium cations) and cations.

Although the problem of environmental contamination by ILs is already starting to become apparent, despite their only fledgling use on a mass scale, very little work has been done so far on providing methods to analyze them in environmental samples. Imidazolium-based compounds are among the most widely commercially used ILs, but only a few simple methods for their determination in environmental matrices have been proposed so far [24–28], which have been revised in detail in our recent work [29]. Importantly, none of these methods involve determination of imidazolium ILs in organisms that could accumulate these compounds from water.

Although some important aspects related to potential environmental fate (stability) and impact (toxicity) have already been fairly well understood, there is a gap in the form of a very limited number of studies on the possible bioconcentration of imidazolium ILs. This is due to the fact that until recently it was widely assumed that charged chemicals do not carry a risk of bioconcentration. However, as shown in recent years, this is not the case, and ionic compounds including imidazolium ILs can also significantly bind to particular bioconstituents such as membrane lipids [30] or blood proteins [31]. These findings clearly indicate a risk that ILs, when released into the environment, may accumulate in the tissues of organisms. However, there is still a lack of conclusive studies that could confirm or deny the possible bioconcentration of imidazolium ionic liquids.

Therefore, the aim of this study was to develop and fully validate a novel methodology for the determination of imidazolium ILs cations in marine invertebrate tissues. Bivalves of the family Mytilidae were selected as model invertebrate organisms. These mussels, due to widespread occurrence in seas and oceans, sedentary lifestyle, and feeding through filtration, are one of the primary biomarkers in environmental studies. Four imidazolium compounds with different side chain lengths ranging from 6 to 12 carbon atoms were used as model ILs cations. Moreover, the utility of three different techniques, namely microwave-assisted extraction (MAE), accelerated-solvent extraction (ASE) and bullet-blender homogenization (HOMO), was evaluated in the extraction of imidazolium cations from mussel tissues. The present work aimed to fill an important gap by providing a new and reliable procedure to allow the determination of trace amounts of these compounds in complex environmental matrix.

2. Material and methods

2.1. Chemicals

Acetonitrile and methanol (LC-MS grade), as well as hexane, dimethyl sulfoxide and isopronanol (pure for analysis grade) were purchased from POCH (Poland). Ammonium acetate, acetic acid, ammonium formate and formic acid (all of LC-MS grade) were purchased from Sigma-Aldrich (Germany). ILs standards, namely: 1-hexyl-3-methylimidazolium chloride (IM16 Cl, purity ≥98), 1-octyl-3-methylimidazolium chloride (IM18C(CN)$_3$, purity ≥97%), 1-decyl-3-methylimidazolium chloride (IM1-10 Cl, purity ≥95.5%) and 1-dodecyl-3-

methylimidazolium bromide (IM1-12 Br, purity ≥98%) were purchased from Iolitec (Germany).

2.2. Conceptual approach

The conceptual approach of this study is presented in Fig. 1. It must be explained that during the development of this new method for the analysis of imidazolium cations from mussel tissues, one model compound, IM18, was used in the first two steps, to select a suitable sorbent for SPE and the most efficient extraction technique. At the sorbent selection stage, HPLC-DAD technique was used for the final analyses due to the lack of complicated matrix interfering with the analytes. However, in subsequent steps, samples were analyzed using LC-MS/MS technique. Once the extraction technique was selected for this model compound, further steps involving the selection of final conditions of the extraction and purification method were conducted using the target mixture of four imidazolium cations (IM16, IM18, IM1-10 and IM1-12).

2.3. Mussels collection and preparation

M. trossulus mussels were collected from an unpolluted area of the Gulf of Gdansk during a cruise on the RV Oceania owned by the Institute of Oceanology, Polish Academy of Sciences. The organisms were collected in Spring 2021 and immediately frozen (at −20 °C) and then lyophilized. After lyophilization, the resulting dry tissues were homogenized using porcelain mortar to obtain homogenized dry mass. The tissues thus prepared were stored in a dark and cool place.

2.4. Sample preparation for extraction tests

Three types of samples were prepared each time during the extraction tests: spiked tissues (with analytes), not spiked tissues (blanks) and controls (samples without tissue but containing appropriate amount of analytes). Spiking of tissues with ILs solutions was done each time before the scheduled extraction tests following the same procedure. The procedure consisted of weighing the required amount of dry matter and transferring it to a glass beaker. The tissues were then spiked with a 1-to-1 ratio of methanolic ILs solution (1 mL of solution per 1 g of dry tissue) and thoroughly mixed so that the methanolic solution evenly combined with the tissues. Finally, the spiked tissues were incubated at 50 °C overnight to completely evaporate the methanol. The tissues prepared in this way were subjected to extraction process on the same day after preparation. During optimization process, mussels were spiked at concentration of 10 μg g^{-1} dry mass. For the final validation of optimized method, tissues were spiked at 6 concentration levels, each in triplicate: 50, 100, 150, 200, 300, 500 ng·g^{-1}dry mass.

Controls were prepared by spiking the extraction tubes with the same amount of ILs solution as in the tests and then evaporated to dryness under a stream of nitrogen. For blanks, the same mass (0.5 g) of unspiked tissue was subjected to the entire sample preparation process.

2.5. SPE

SPE was performed using a Baker SPE 12G system (Avantor Performance Materials, Poland). Initial selection of a suitable sorbent was made for deionized water (without mussel-derived matrix) with the model compound, IM18, added. Analyte recovery from sorbents based on ionic interactions (Oasis WCX, Strata X–CW, Discovery SCX) and hydrophobic interactions (Strata X and Oasis HLB) was analyzed during the tests.

The finally selected optimal method utilized Oasis HLB cartirdges (6 mL, 200 mg; Waters, US). In the first step, the columns were flushed with 6 mL of methanol and then conditioned with 6 mL of deionized water with gravity flow. The dilluted sample (as described below), preadjusted to pH = 3 was then loaded using a vacuum pump, ensuring that the flow rate through the cartridge did not exceed 5 mL per minute. After sample loading, the columns were washed with 6 mL of deionized water and dried under vacuum. The final step was washing with hexane and drying again. The columns were stored in a refrigerator until elution. Analytes were eluted with 2 × 3 mL of methanol with gravity flow. During preselection of extraction technique and method selection steps, samples were diluted tenfold prior to LC-MS/MS analysis. During final validation of method, samples were evaporated under nitrogen and reconstituted in 0.6 mL of MeOH, resulting in tenfold concentration.

Table 1
Parameters used for preselection of the extraction technique.

Extraction technique	Temperature [°C]	Pressure [psi]	Power [W]	Time [min]	Number of cycles
MAE	100	–	400	20	1
ASE	80	1500	–	15	1
Homogenization	20	–	–	1	3

2.6. Extraction

In this study, three different extraction techniques were tested, namely MAE, ASE and HOMO. Additionally, during the initial selection of the extraction technique, three combinations of solvents were used as the extraction mixture: MeOH:H_2O 1:1 (v/v), EtOH:H_2O 1:1 (v/v), and ACN:H_2O 1:1 (v/v). The applied conditions of the tested techniques are presented in Table 1.

2.6.1. ASE

ASE extraction was performed on a DIONEX ASE 350 apparatus (Dionex Corporation, US). Initially, 19.8 mm cellulose filter was placed at the bottom of a 33 mL stainless steel extraction chamber. Then 3 g of diatomaceous earth, 0.5 g of extraction tissue, and another portion of diatomaceous earth were placed in the cell. Extraction was carried out at 80 °C, at 1500 psi (Table 1). The total extraction time per sample was 15 min, and the extract volume was approximately 27 mL. From the obtained extract, 10 mL was taken and diluted so that the proportion of organic phase did not exceed 5%, and then the samples were purified by SPE.

2.6.2. HOMO

HOMO extraction was performed on a Bead Ruptor Elite homogenizer (Omni International, US). After weighing 0.5 g of dry tissues into tubes, 10 mL of extraction solution, 20 porcelain beads, and 1 spatula (approximately 50 mg) of porcelain microbeads were added. The samples thus prepared were shaken for 1 min at 5000 rpm at room temperature. To avoid overheating of the samples, extraction was carried out in three cycles of 1 min each. Several minutes were waited between each cycle to cool the samples. Finally, samples were transferred to centrifuge tubes and centrifuged for 10 min at 5000 g. 6 mL of supernatant was then collected, diluted so that the proportion of organic phase did not exceed 5%, and purified by SPE.

2.6.3. MAE

MAE extraction was performed using a MarsXpress microwave system purchased from CEM Corporation (UK), equipped with 8 PTFE vessel carousel and internal temperature control. First, 0.5 g of weighed tissue was placed in the extraction tube and then 10 mL of extraction solution was poured. During extraction, samples were mixed using magnetic stirrers placed inside the tubes. The initial conditions used during the preselection of extraction technique were as follows: 2 min of temperature rise to 100 °C, then holding the temperature for 20 min with a constant microwave power set at 400 W (Table 1). In further development steps, the extraction conditions were modified according to the data presented in Table 2. Finally, samples were transferred to centrifuge tubes and centrifuged for 10 min at 5000 g. 6 mL of supernatant was then collected, diluted so that the proportion of organic

Table 2
Parameters of MAE and SPE tested during the selection of ILs extraction conditions from mussels.

Method	MAE				SPE					
					Activation and conditioning		Washing			Elution
	Temperature [°C]	Time [min]	Power [W]	% MeOH	MeOH	H_2O	5% MeOH	H_2O	Hexane	MeOH
MAE1	100	20	400	50	6 mL	6 mL	6 mL	–	6 mL	6 mL
MAE2	100	10	400	50	6 mL	6 mL	6 mL	–	6 mL	6 mL
MAE3	100	20	800	50	6 mL	6 mL	6 mL	–	6 mL	6 mL
MAE4	120	20	400	50	6 mL	6 mL	6 mL	–	6 mL	6 mL
MAE5	120	20	800	50	6 mL	6 mL	6 mL	–	6 mL	6 mL
MAE6	120	20	800	70	6 mL	6 mL	–	6 mL	6 mL	6 mL
MAE7	120	20	800	90	6 mL	6 mL	–	6 mL	6 mL	6 mL

Table 3
Selected parameters of analyzed cations in the developed method for their final determination.

Compound	Retention time [min]	Precursor ion [m/z]	Fragment ions [m/z]	Collision energy/eV
IM16	1.293	167.1	**83.1**; 43.1	−21; −23
IM18	1.723	195.1	**83.1**; 43.1	−22; −27
IM1-10	2.660	223.3	**83.2;** 57.2	−23; −22
IM1-12	3.723	251.0	**83.1;** 57.2	−25; −29

phase did not exceed 5%, and purified by SPE.

2.7. HPLC-DAD analysis

During the preliminary selection of SPE condition, IM18 cation was analyzed using HPLC-DAD system: Shimadzu Nexera XR. A Kinetex 2.6 μm XB-C18 100 Å, 100 × 3 mm LC column (Phenomenex, Canada) was used. The mobile phase was composed of acetonitrile (HPLC grade) and 10 mM ammonium acetate buffer (pH = 3.8). The analytical wavelength was 212 nm. The flow rate was 0.5 mL min^{-1}. The temperature of oven was set to 30 °C. This method for the final determination of the IM18 was fully validated in terms of its: precision, accuracy, linearity, instrumental limit of detection (LOD) and instrumental limit of quantification (LOD). For this purpose the standard solutions of the IM18 in MeOH were prepared at 8 concentration levels (5, 10, 20, 40, 60, 80 μM) and analyzed six times each.

2.8. LC-MS/MS analysis

Instrumental analyses of tissue extracts were performed using UHPLC system including LC-40B X3 solvent delivery module, SIL-40C X3 autosampler and CTO-40C column oven, coupled to a triple quadrupole mass spectrometer (LCMS-8050) equipped with an electrospray ionization (ESI) source (Shimadzu, Japan). A Kinetex 2.6 μm XB-C18 100 Å, 100 × 3 mm LC column (Phenomenex, Canada) was used for the chromatographic separation of the tested compounds. The optimized mobile phases consisted of A: acetonitrile and B: 10 mM ammonium acetate buffer. The flow rate of the mobile phases was 0.4 mL min^{-1}. The oven temperature during the analyses was set to 35 °C. The following gradient elution was used in the chromatographic separation: 50% A (0–1 min), linearly increased to 95% A (4 min), isocratic mode for 1 min and finally decreased to 50% A, with total analysis time of 6 min. The optimized MS/MS parameters were as follows: gas pressure: 270 kPa, nebulizing gas flow rate: 3.0 L min^{-1}; dry gas flow rate: 10 L min^{-1}; desolvation temperature: 526 °C; DL line temperature: 250 °C; heat block temperature: 400 °C. The capillary voltage was 3.0 kV. The transitions in multiple reaction monitoring mode (MRM) for the qualitative and quantitative analysis of imidazolium cations were selected, and are presented in Table 3. For the quantitative analysis, MRM transitions of the highest intensity were selected. The compounds were identified based on retention time and selected characteristic MRM transitions.

This method for the final determination of the mixture of four imidazolium ILs was also fully validated in terms of its: precision, accuracy, linearity, instrumental limit of detection (LOD) and instrumental limit of quantification (LOQ. For this purpose the standard solutions of the mixture of ILs in MeOH were prepared at 8 concentration levels (2, 10, 25, 50, 75, 100, 150, 250 ng mL^{-1}) and analyzed six times each.

2.9. Calculations

2.9.1. Absolute recovery

Absolute recovery of selected in this study analyte were calculated using the following equation:

$$AR(\%) = \frac{C - D}{A} \times 100$$

Where C refers to peak area in spiked samples of mussels processed with MAE and SPE, while A refers to peak area of standard dissolved in pure solvent, freshly prepared before analysis at the concentration level referring to 100% of AR of each analyte, while D refers to peak area in blank sample [32].

2.9.2. Matrix effects

Matrix effects (ME) were calculated according to the following equation:

$$ME(\%) = \left(\frac{B - D}{A} - 1\right) \times 100$$

Where A refers to peak area of standard dissolved in pure solvent, freshly prepared before analysis, B refers to peak area of standard dissolved in blank sample extract at the same concentration, while D refers to peak area in blank sample. Values above 0% indicate an ionization enhancement and below 0% a suppression [32].

2.9.3. Instrumental validation

Validation parameters such as linearity, coefficient of determination (r^2), intra-day precision (expressed by RSD), accuracy, LOQ (calculated as the lowest concentration at which the method is linear with acceptable precision and accuracy) and instrumental detection limit (LOD, calculated as $\frac{LOQ}{3}$) were established according to standard procedures [33].

2.9.4. Validation of the developed analytical procedure

In order to validate the entire proposed analytical procedure, tissues were spiked at 6 concentration levels, each in triplicate (50, 100, 150, 200, 300, 500 ng mL^{-1} dry mass) and subjected to the entire sample preparation procedure. Blank samples were also prepared in triplicate. Each sample was instrumentally analyzed in triplicate, resulting in ninefold analysis of each concentration. Validation parameters were calculated according to the information presented in Section 2.8.1. However, in this case limit of detection (LOD) and limit of quantification (LOQ)

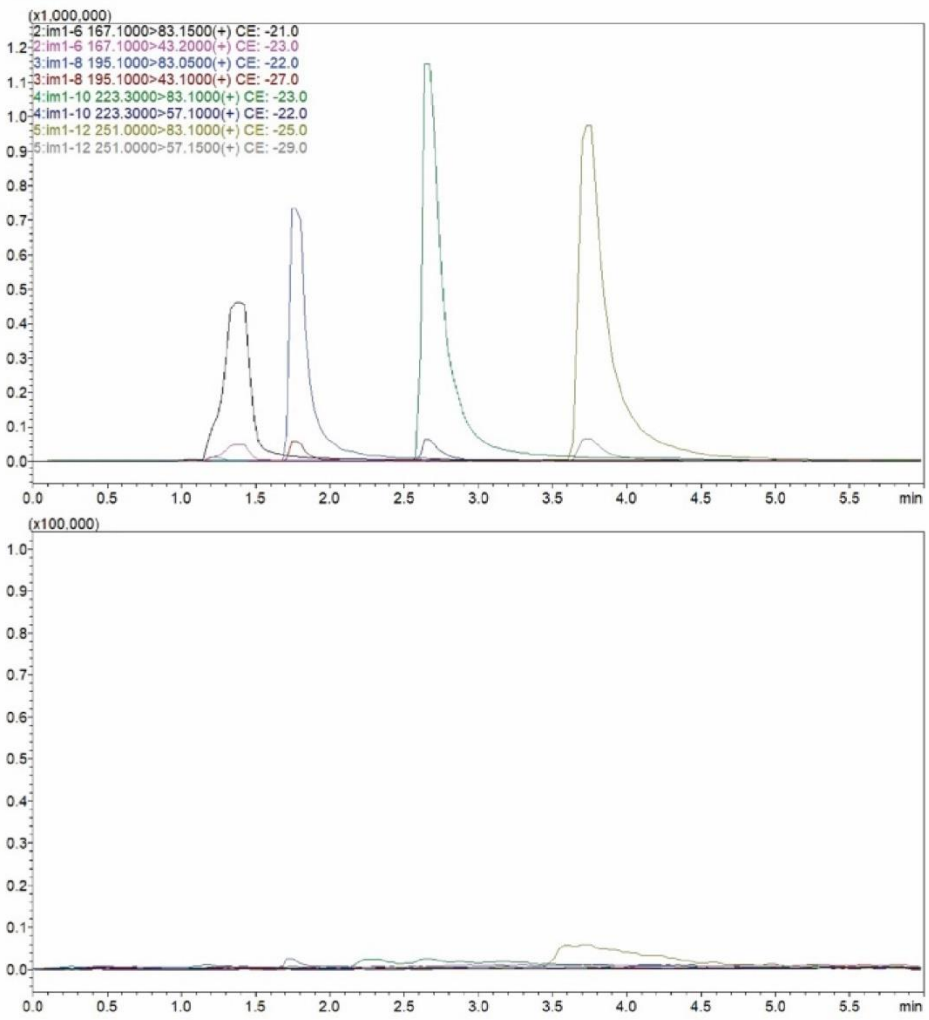

Fig. 2. Selected chromatograms obtained during the analysis of the investigated mixture of imidazolium cations (at the concentration of 50 ng mL^{-1} in MeOH) and blank sample (pure MeOH) in the developed LC-MS/MS method.

was determined for the whole method.

3. Results and discussion

3.1. Developed LC-MS/MS method for the final determination of imidazolium ILs

In general for hydrophobic organic cations, significant challenges are often encountered during analyses using LC-MS or LC-MS/MS technique. In our experience, the main problem with the analysis of imidazolium ILs was the fact that they sorb significantly on many components of the LC system, especially the thin capillaries associated with the dosing unit. Due to their nature, imidazolium cations with long side chains can be strongly sorbed both on metallic parts of the device, where electrostatic interactions occur, but also on different kinds of plastics due to hydrophobic interactions [34]. The effect of this phenomenon can be either a gradual disappearance of signals as the sorption proceeds and most of the analyte is deposited on its way to the mass spectrometer, eventually not reaching it. A second observed effect can be the presence of small but reproducible and difficult to discard signals in blank samples, which we have actually encountered during the method development. In an attempt to minimize it, various options for rinsing the dosing port were tested, including pure solvents such as MeOH, ACN, DMSO, isopropanol or their mixtures with acetate buffer. Eventually it was possible to reduce blank peaks to an acceptable level by using a rinsing mixture made of 20% DMSO in isopropanol, as well as conditioning the system with a high enough proportion of the organic phase (at least 25% of ACN as initial conditions in gradient method). Regarding chromatographic separation, due to the different side chain lengths, the analyzed cations were separated well using the XB-C18 column, even with a gradient elution starting at 50% MeOH (Fig. 2). Thus, the developed instrumental method allows the separation of the tested compounds during only 6 min of analysis.

The instrumental method obtained by us is characterized by low LOQ

Table 4
Determined instrumental validation parameters of the developed LC-MS/MS method for the final determination of four imidazolium cations.

Cation	Linear equation	r^2	LOD [ng·mL^{-1}]	LOQ [ng·mL^{-1}]	Linearity range	Precision [%, RSD]	Accuracy [%]
IM16	$y = 13{\cdot}10^4x + 10{\cdot}10^5$	0.9987	6.66	20	20–500	0.4–3.7	90.8–122.7
IM18	$y = 12{\cdot}10^4x + 30{\cdot}10^4$	0.9989	3.33	10	10–500	1.6–6.3	94.9–109.0
IM1-10	$y = 23{\cdot}10^4x + 54{\cdot}10^4$	0.9996	3.33	10	10–500	0.8–4.6	92.1–103.3
IM1-12	$y = 23{\cdot}10^4x + 36{\cdot}10^5$	0.9990	3.33	10	10–500	1.1–3.3	94.4–130.3

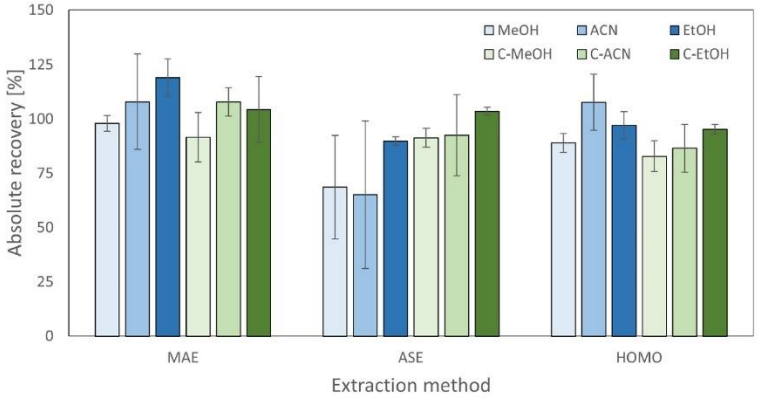

Fig. 3. Absolute recoveries of IM18 cation from *M. trossulus* tissues (blue color) and control sample (green color) in the tested methods and techniques. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(10–20 μg/L) values and a wide range of linearity (10–500 μg/L) (Table 4). The application of the MS/MS technique to the analysis of imidazolium cations allows the determination of these compounds at concentrations orders of magnitude lower than other techniques. For example, the work of Zhang et al. [35] analyzed ionic liquid cations by ion chromatography, achieving LOQs of a few mg/L. Similar limits were also achieved in the work of Fan et al. [36,37,] using indirect UV/Vis detection. Our instrumental method allows quantification of imidazolium cations at concentrations as low as 10 μg/L, confirming the efficiency of the MS/MS technique in this type of analysis.

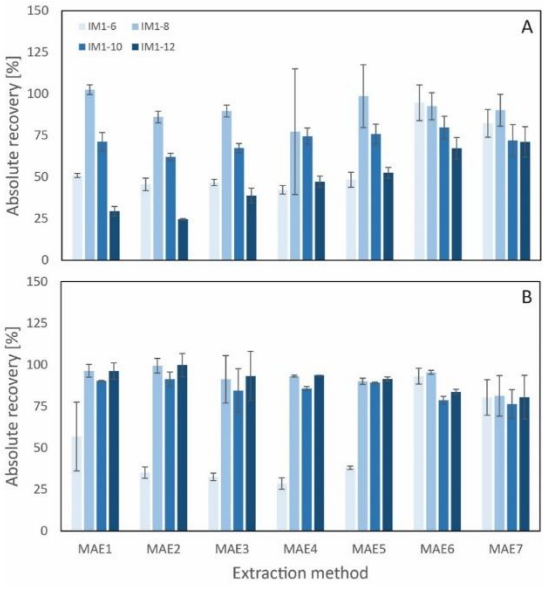

Fig. 4. Absolute recovery of imidazolium cations mixture from spiked tissues (A) and control (matrix-free samples; B) using MAE technique.

3.2. Preselection of extraction technique

MAE, ASE, and homogenization were tested during the pre-selection of techniques for the extraction of imidazolium ILs from mussel tissues. Within each of these techniques, three solvent mixtures were used, namely 50% aqueous solutions of MeOH, ACN or EtOH. Additionally, only one cation was selected as the model compound for the initial technique selection, which was 1-methyl-3-octylimidazolium (IM18). The absolute recovery of this compound in the different extraction variants tested is shown in Fig. 3. Based on the obtained results, it was decided to disqualify the ASE technique due to its low reproducibility and the lowest absolute recovery values, not exceeding 90%. In contrast, both of the other two techniques showed both very good absolute recoveries, at around 100%, and low variation between replicates. Ultimately, it was decided that the MAE technique would be selected for further work to develop an extraction method of the mixture of ILs from mussel tissues. This decision was motivated by the fact that within this technique there is much more options for the optimization, including potential increasing power (up to 1200 W) or temperature (up to 150 °C). In the case of homogenization, on the other hand, the possibility of adjusting the method is actually limited only to modifying the extraction mixture, the time and intensity of shaking. However, the results obtained in preliminary tests confirm that microbead homogenization, which is a relatively modern and economical method, can be a valuable alternative even for extraction of such problematic compounds as ILs from biological matrices.

Among the previously proposed methods for the extraction of imidazolium ILs from solid environmental matrices, only one [27] deals with biological (plant) samples, while the others treat soils and sediments [21,24–26]. The work of Nichthauser et al. [27] used a different approach than here, testing only one simple ultrasound-assisted

Table 5
Matrix effects assessed for the evaluated extraction methods (values presented in [%]).

Cation	MAE1	MAE2	MAE3	MAE4	MAE5	MAE6	MAE7
IM16	−9.9	−6.1	−9.5	−12.5	−15.3	−17.1	−17.8
IM18	−18.6	−14.5	−20.0	−18.5	−20.4	−24.4	−20.0
IM1-10	31.7	−5.1	1.2	−8.7	−16.2	−0.1	−7.8
IM1-12	−42.5	−32.0	−39.5	−39.8	−36.9	−38.4	−37.5

extraction technique, additionally analyzing the effect of different mineral and organic acids on the efficiency of the process. This work used imidazolium cations with shorter side chains than in our study, and tests were conducted individually instead of in a mixture. The recoveries obtained for biological samples (potato, grass, rice) ranged from 70.2% to 83.5%. In our work, we obtained higher recoveries by the application of both MAE and HOMO techniques, confirming that the proposed techniques may be more efficient for the extraction of imidazolium cations from biological matrices. Furthermore, to date, all works involving the analysis of imidazolium cations in soil and sediment samples have also considered only the application of ultrasound-assisted extraction [24,25,38]. Therefore, there is no doubt that the experiments performed in this study on the efficiency of three other extraction techniques represent an important addition to the knowledge on the extraction of imidazolium ILs from environmental matrices.

3.3. Selection of optimal MAE conditions and SPE purification steps

Based on the obtained results from the preselection study, as it was described above, for the extraction of the mixture of four imidazolium cations differing in their physico-chemical properties the MAE technique was further investigated. The conditions that worked best for the extraction of IM18 alone (called as MAE1) were chosen as the starting point for extraction of the mixture. Although in the case of IM18 and IM1-10 these conditions resulted in high absolute recovery (103% and 70% respectively), efficient extraction of the most polar (IM16) and the most hydrophobic (IM1-12) among tested compounds required different approach (Fig. 4). It is worth noting that simply increasing the extraction temperature from 100 to 120 °C and increasing the power from 400 to 800 W did not yield sufficient results, increasing the absolute recovery of these two cations only to about 50%. Therefore, for MAE6 and MAE7 methods, the proportion of MeOH in the extractant was increased to 70% and 90%, respectively. In addition, in the purification step, a 5% MeOH rinse was replaced with a deionized water rinse, prompted by the concern that the low recovery for the relatively polar IM16 cation might be due to its leaching from the Oasis HLB sorbent during washing step. Such modifications ultimately led to a satisfactory extraction and purification method, achieving absolute recoveries above 70% for all analytes tested in the case of MAE6 and MAE7 methods.

In the work by Pati et al. [24], relative recoveries of imidazolium cations from spiked sediments were below 20% for cations with 2 and 4 carbon atoms in the side chain, but above 100% for cations with 6, 8, and 10 carbon atoms. The simultaneous extraction and analysis of cations with such different properties, from hydrophilic to highly hydrophobic, can be challenging due to the easy loss of one of the extreme fractions of the compounds during the sample preparation procedure. Considerable success in this regard was achieved in the work of Lu et al. [38], where recoveries above 70% were obtained for all 11 imidazolium cations tested. However, it is worth noting that this work does not report whether relative or absolute recoveries were used and how they were determined.

It is important to emphasize that in our work we have taken an approach to accurately and fully assess the performance of our method by evaluating, absolute recoveries (AR) for spiked samples and controls (Figs. 3 and 4), as well as matrix effects (ME) (Table 5) according to the procedure described previously by our research group [32]. This

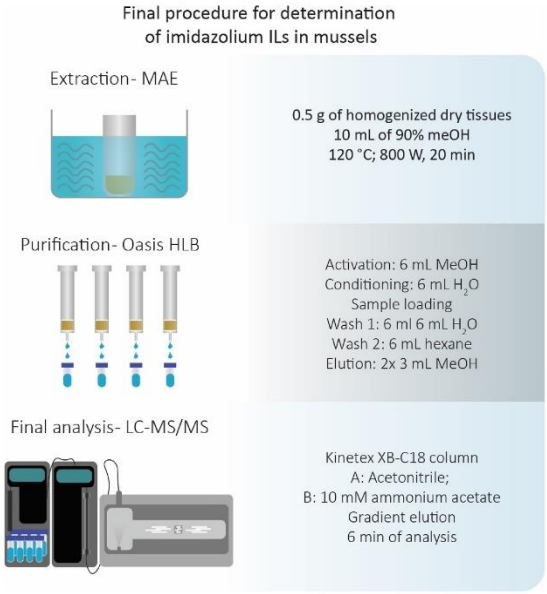

Fig. 5. Final conditions of the developed analytical procedure for the extraction and analysis of imidazolium ILs from bivalve tissues.

approach allows to get a complete picture on this how efficient the developed method is and get a better insight at which stages there are possible losses of analytes, and how to prevent them from affecting the reliability of the obtained results.

In Table 5 the data on the determined ME in each tested extraction method is presented. Taking into account this data it might be concluded that despite the high complexity of the matrix analyzed, the MEs were at low levels in each of the proposed extraction conditions, not exceeding 20% in most cases, reaching a maximum of −42.48% (ion suppression) for IM1-12 in the MAE1 method. Also for the most effective extraction methods such as MAE6 and MAE7, the determined MEs varied from −0.05 (IM1-10 in MAE6) to −38.40 (IM1-12 in MAE6). Such relatively low values clearly confirm that the coextracted interferents in the present method have little effect on the ionization of the tested compounds or they were removed efficiently enough during the purification step. Finally, based on the presented results, the MAE7 method has been selected for further proceeding. The summary of whole analytical procedure has been presented in Fig. 5.

It should be mentioned that in the available literature concerning the analysis of imidazolium cations in different matrices no information on ME in any of the other analytical works could be found. Although ILs cations, as compounds bearing a permanent charge, generally exhibit strong signals in MS/MS analysis, it would certainly be valuable to compare MEs obtained in assays with different environmental matrices treated with various types of extraction and purification techniques. However, as indicated by scientists involved in the analytics of various environmental trace pollutants, the lack of unification in the validation of analytical methods results in a wide variation in the data presented in individual methodological papers [32].

3.4. Validation of the developed analytical procedure

After the selection of the best extraction conditions for four imidazolium cations the developed analytical procedure was fully validated. For this purpose *M. trossulus* tissues were spiked at the following concentration levels 50, 100, 150, 200, 300 and 500 ng g^{-1}, each in

Table 6
Validation parameters of the developed analytical procedure.

Cation	Linear equation	r^2	MDL [ng·g^{-1}]	MQL [ng·g^{-1}]	Linearity range	Precision [%RSD]	Accuracy [%]
IM16	$y = 30{\cdot}10^2x + 65{\cdot}10^3$	0.9983	17	50	50–500	5.3–22.9	93.5–106.4
IM18	$y = 23{\cdot}10^3x + 18{\cdot}10^5$	0.9952	17	50	50–500	1.9–11.6	80.9–114.7
IM1-10	$y = 64{\cdot}10^3x + 12{\cdot}10^6$	0.9942	17	50	50–500	1.0–7.0	79.4–110.8
IM1-12	$y = 78{\cdot}10^3x + 50{\cdot}10^4$	0.9972	33	100	100–500	1.1–5.6	96.1–107.8

triplicate, and then subjected to the whole analytical procedure. For all tested compounds, except IM1-12, the method was linear over the entire concentration range, achieving a coefficient of determination r^2 from 0.9942 to 0.9982. For the cation with the longest side chain, the proposed analytical method was linear over the range 100–500 ng g^{-1}. Good linearity was also supported with residuals plots and response factor plots (Fig. S5-S8). The complete validation data is summarized in Table 6. The MDL, which was determined at 16.67–50 ng g^{-1} for all tested compounds, and the MQL in the range of 50–100 ng g^{-1}, make this procedure suitable for future environmental analyses.

Analytical work treating the determination of imidazolium ILs in animal tissues is lacking in the literature. However, ammonium surfactants, which are also permanently charged compounds with long hydrophobic chains, were determined in the works by Kierkegaard et al. [39,40,]. MQL values obtained in fish tissue analyses were similar those presented in our study, oscillating between a few and several hundred ng·g^{-1}. In the work by Wolecki et al. [41] an analytical procedure was developed for the determination of several pharmaceuticals from *M. trossulus* tissues, achieving MQLs of 3–22 ng g^{-1} utilizing gas chromatography coupled to mass spectrometry (GC-MS) for the final determinations. Apart from the very limited number of works allowing direct comparison of proposed analytical procedure, based on analyses of ionic or ionizable compounds from similar matrices, it should be considered that it complies with current analytical standards.

4. Conclusions

In the present work, to the best of our knowledge, the first comprehensive procedure to determine the concentration of a mixture of imidazolium cations in marine invertebrate tissues has been developed and fully validated. The presented methodology allows the determination of these compounds at trace concentrations (at the level of ng·g^{-1}) and may provide a good basis for environmental studies. Bivalves, as sedentary filter-feeding organisms, are among the animals particularly exposed to the bioconcentration phenomenon and are therefore excellent indicators of environmental pollution. Therefore, the developed procedure may find viable applications in future research.

Reports from recent years confirm that imidazolium cations may already be appearing in the environment. However, still growing production and consumption of these compounds make it reasonable to expect that this problem is yet to come. With more frequent appearance of such contaminants, including them in future monitoring studies could be necessary. Therefore, to meet the upcoming needs, we are already providing a tool for the determination of these compounds in bivalve tissues.

CRediT authorship contribution statement

Jakub Maculewicz: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. **Piotr Stepnowski:** Supervision. **Joanna Dołżonek:** Conceptualization, Supervision, Writing – review & editing. **Anna Białk-Bielińska:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123790.

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Supplementary Material for:

Analysis of imidazolium ionic liquids in biological matrices: a novel procedure for the determination of trace amounts in marine mussels

Jakub Maculewicz[a*], Piotr Stepnowski[a], Joanna Dołżonek[a], Anna Białk-Bielińska[a]

[a]*Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland*

[*]*Corresponding author. E-mail address: jakub.maculewicz@phdstud.ug.edu.pl*

Content: 4 Figures

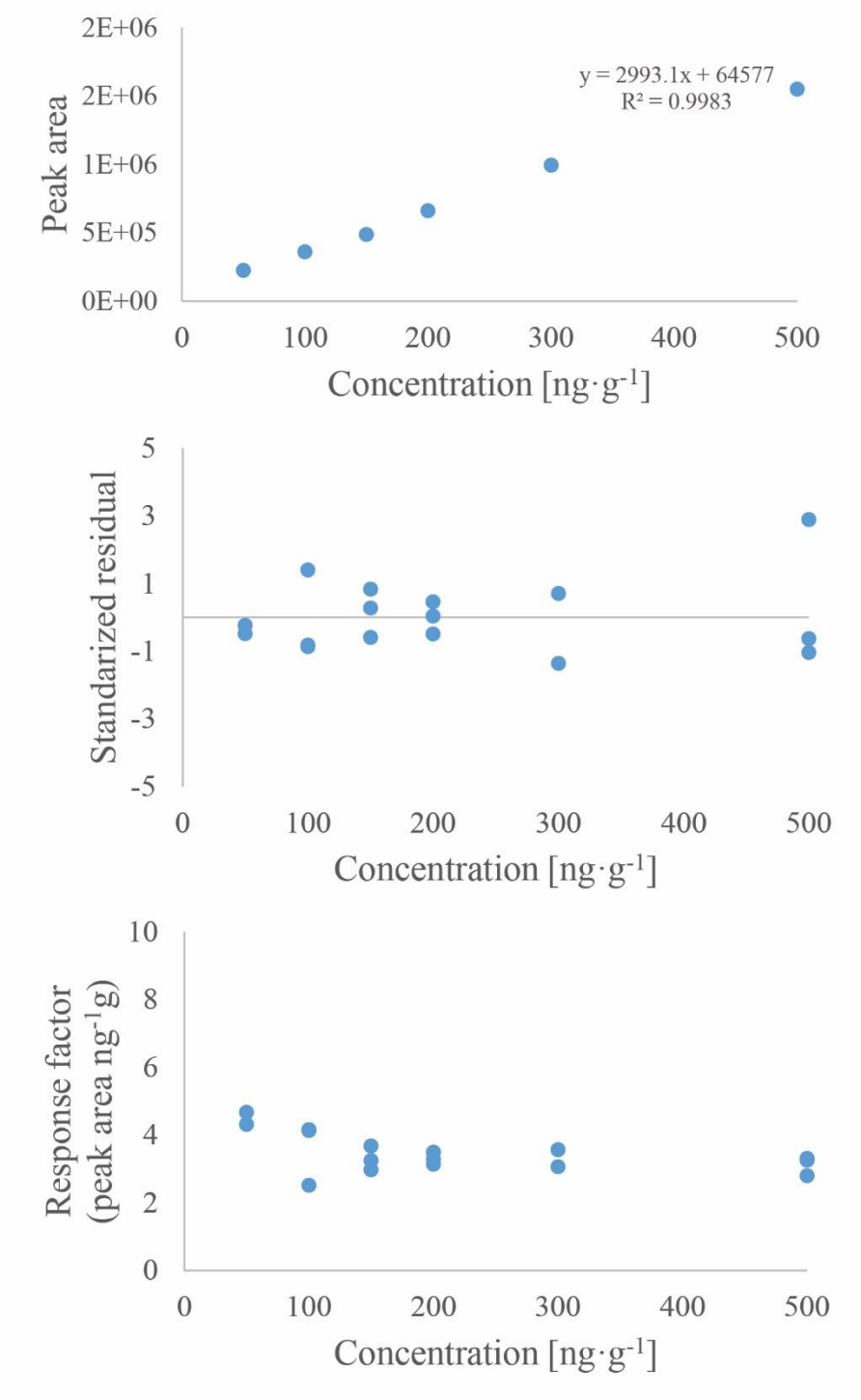

Figure S1. Calibration curve, standarized residuals plot and response factor plot for IM16 analysis in *M. trossulus* tissues

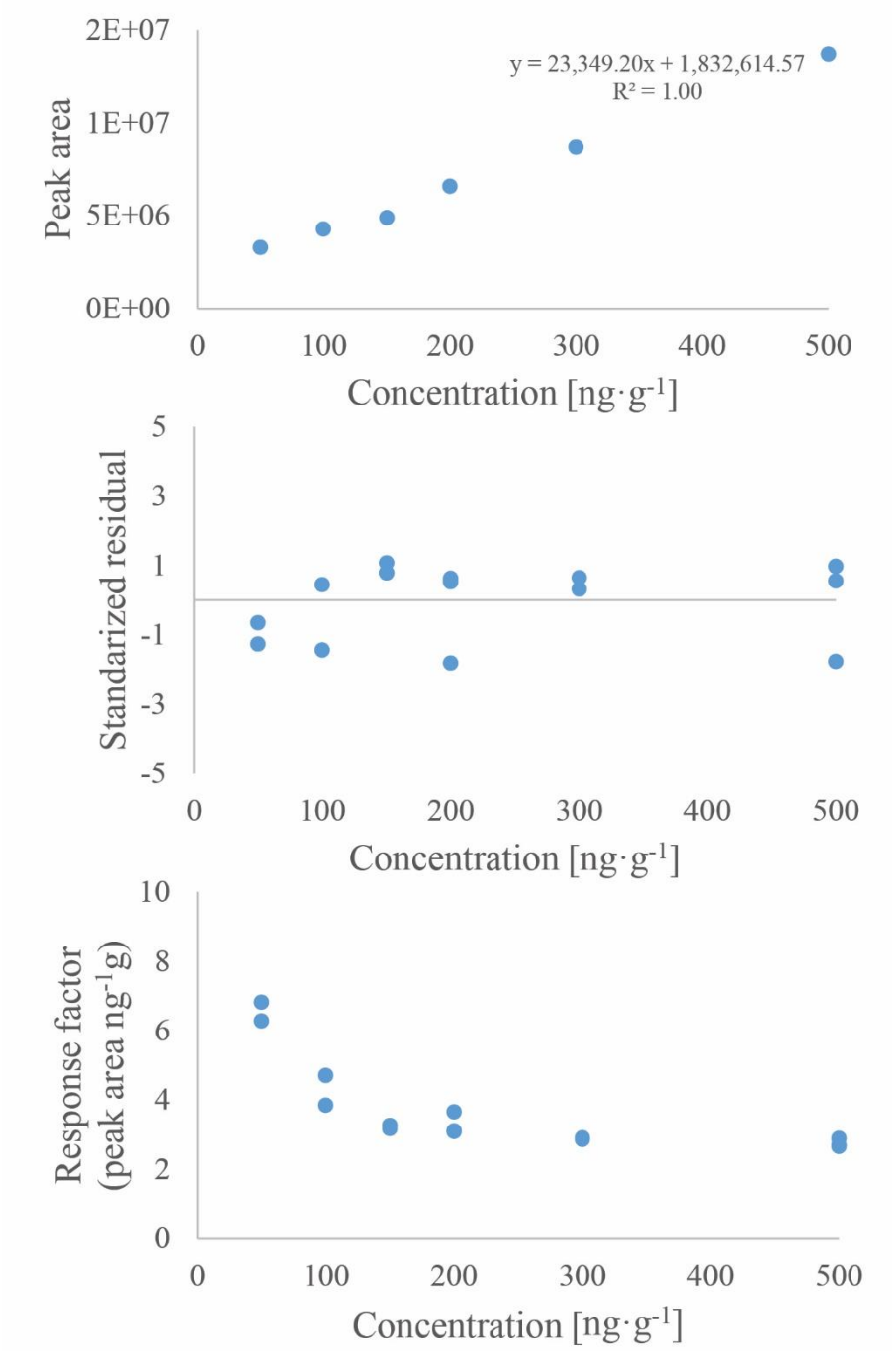

Figure S2. Calibration curve, standarized residuals plot and response factor plot for IM18 analysis in *M. trossulus* tissues

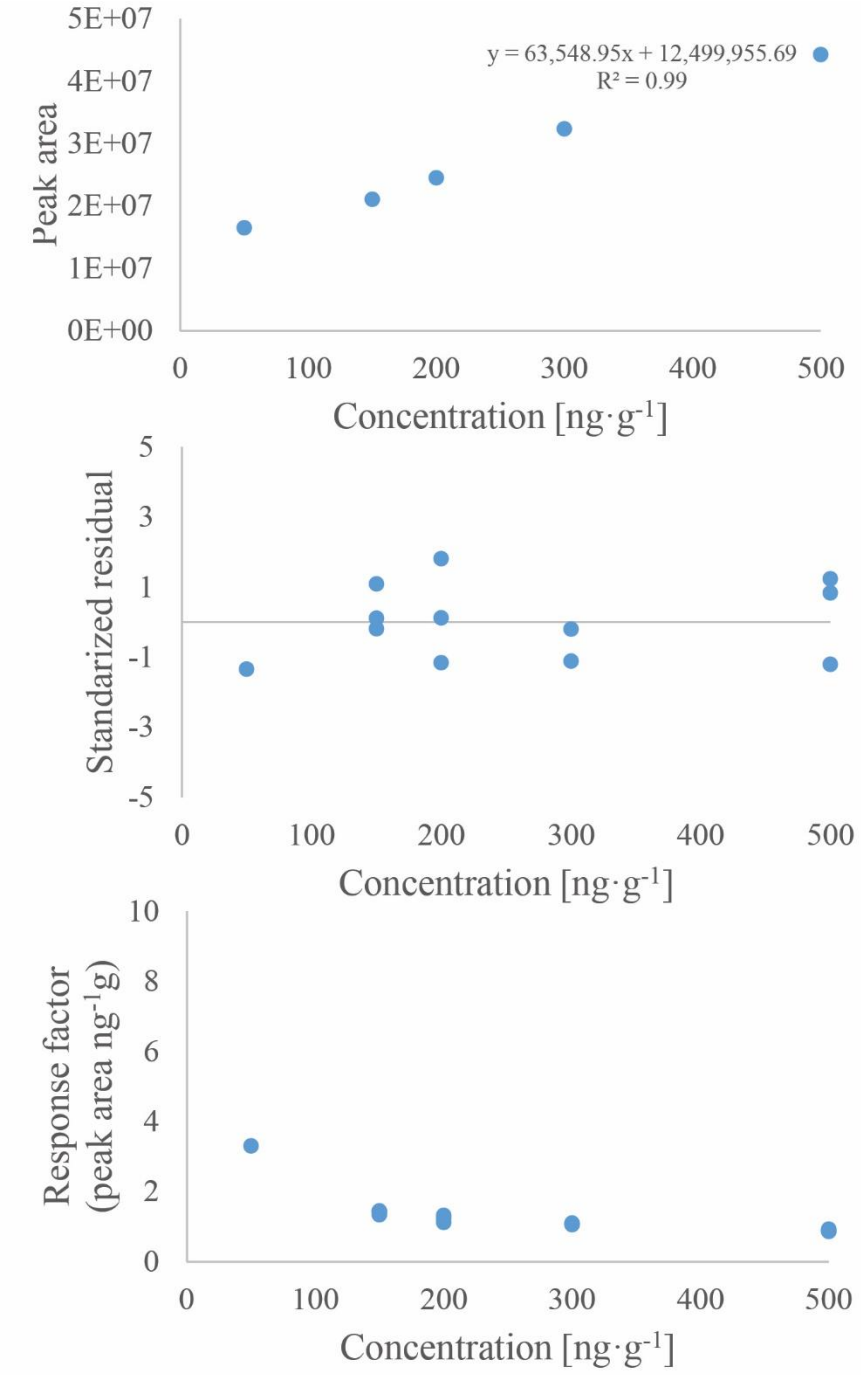

Figure S3. Calibration curve, standarized residuals plot and response factor plot for IM1-10 analysis in *M. trossulus* tissues

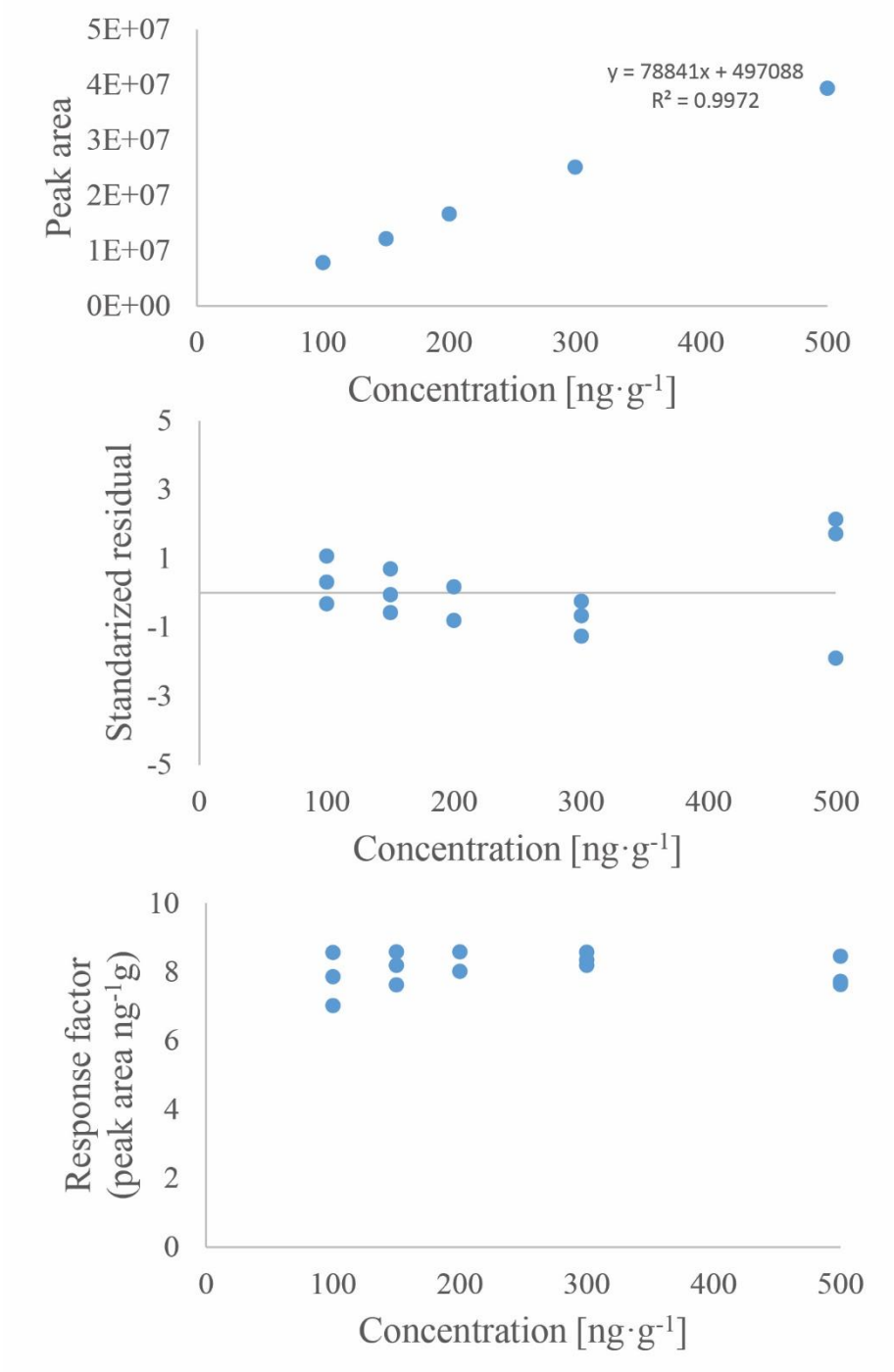

Figure S4. Calibration curve, standarized residuals plot and response factor plot for IM1-12 analysis in *M. trossulus* tissues

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Bioconcentration of imidazolium ionic liquids: *In vivo* evaluation in marine mussels *Mytilus trossulus*

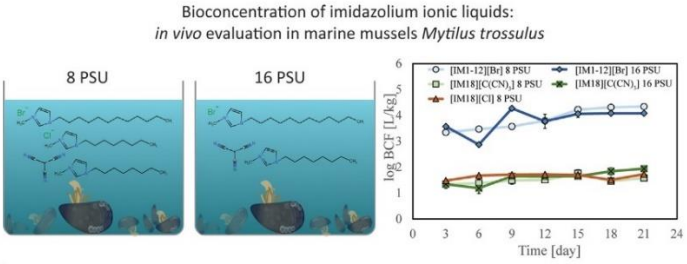
Jakub Maculewicz [a], Joanna Dołżonek [a,*], Lilianna Sharma [b], Anna Białk-Bielińska [a], Piotr Stepnowski [a], Ksenia Pazdro [b]

[a] Department of Environmental Analysis, Faculty of Chemistry, University of Gdańsk, Wita Stwosza 63, 80-308 Gdańsk, Poland
[b] Institute of Oceanology, Polish Academy of Sciences, ul. Powstańców Warszawy 55, 81-712 Sopot, Poland

HIGHLIGHTS

- Imidazolium ionic liquids can bioconcentrate in marine invertebrates tissues.
- $[IM1–12]^+$ can be considered as highly bioaccumulative compound.
- $[IM18]^+$ has little potential for bioconcentration.
- LC_{50} reached 0.68 mg/L for [IM1–12][Br], and 11.66 mg/L for $[IM18][C(CN)_3]$.

GRAPHICAL ABSTRACT



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ABSTRACT

Although imidazolium ionic liquids (ILs) are beginning to be used more widely in many industrial fields *e.g.*, as reaction media, electrolytes, stationary phases in gas chromatography), there is still little information about their potential environmental fate. Among the uncertainties regarding the risks associated with these compounds, bioconcentration is one of the key issues, about which many doubts have been raised in recent years. While *in vitro* data suggest that permanently charged compounds can also bioconcentrate, conclusive evidence in the form of studies on organisms, at least for selected compounds, is needed. Therefore, the main objective of this work was to determine whether imidazolium cations of ILs, namely 1-methyl-3-octylimidazolium ($[IM18]^+$) and 1-methyl-3-dodecylimidazolium ($[IM1–12]^+$), can bioconcentrate in marine invertebrates tissues. During 21-day experiments, *Mytilus trossulus* mussels were exposed to these cations individually, at a concentration of 10 μg/L. In our study, it has been demonstrated for the first time during *in vivo* study, that long-chain imidazolium ionic liquids can bioconcentrate. The determined BCF value for $[IM1–12]^+$ of 21,901 ± 3400 L/kg makes this compound to be considered highly bioaccumulative according to commonly accepted criteria. However, the obtained BCF for $[IM18]^+$ (with the value below 100) suggests that this cation has little potential for bioconcentration. On the other hand, no salinity or anion influence on the bioconcentration of the tested cations was observed. Our tests also confirm that imidazolium ILs exhibit acute toxicity only at relatively high concentration levels, as LC_{50} reached 0.68 mg/L for [IM1–12][Br], and 11.66 mg/L for $[IM18][C(CN)_3]$. This further confirms that the risks associated with the potential presence of these compounds in the environment should be attributed to their high persistence and potential bioconcentration, rather than acute toxicity.

1. Introduction

Ionic liquids (ILs) are compounds that represent a promising solution in many chemical industries (Beil et al., 2021). Among the different groups of

* Corresponding author.
E-mail address: joanna.dolzonek@ug.edu.pl (J. Dołżonek).

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ILs that have been developed in recent years, the classical ones, based on imidazolium cations, are still the most common (Singh and Savoy, 2020; Miao et al., 2021; Aghaie et al., 2018; Greer et al., 2020). The number of their potential applications is constantly growing and their use is finally being transferred from the laboratory to the industrial scale. Although several imidazolium ILs are already available on the market with production measured in tons, these quantities are expected to definitely increase in the near future (Greer et al., 2020). Therefore, in the view of proactive hazard identification, the potential risks that may arise with the use of these compounds on a mass scale, should be assessed promptly.

Although imidazolium ILs were originally classified as "green solvents", this concept has been repeatedly questioned (Bystrzanowska et al., 2019). Undoubtedly, these compounds have a number of favourable properties, *e.g.* negligible vapor pressure, which prevents their release into the atmosphere, but they also have characteristics that would lead one to consider them as a potential threat (Kowalska et al., 2021). Due to their good solubility in water, ILs may pose a potential threat to aquatic organisms (Bubalo et al., 2017; Flieger and Flieger, 2020), which was recently confirmed by Neuwald et al. (2020,2021) who has detected these compounds in German river systems. Moreover, their amphiphilic character, may favour their accumulation in soils and sediments (Amde et al., 2015).

In terms of acute toxicity, imidazolium ionic liquids have already been quite well characterized (Romero et al., 2008; Biczak et al., 2016; Kulacki and Lamberti, 2008; Docherty and Kulpa, 2005; Studzińska and Buszewski, 2009; Tsarpali et al., 2015; Stolte et al., 2007; Matzke et al., 2007, 2008, 2009). The conclusions of these studies put imidazolium ILs rather in good light, since their toxicity, although slightly higher than classical solvents such as methanol or acetonitrile, does not reach particularly alarming values. It is worth noting that, in addition to numerous studies on the acute toxicity of these compounds, there are also some works on their possible chronic effects. For example Ma et al. (2018) proved that exposure to 1-methyl-3-octylimidazolium ($[IM18]^+$) alters haematological parameters of silver carp, additionally causing oxidative stress and inflammatory reaction in the liver. Chronic toxicity of imidazolium ILs was also assessed in *Daphnia magna* demonstrating that long exposure can result in many negative effects, including reproductive disorders (Bernot et al., 2005a).

An important property of imidazolium ILs is their high resistance to degradation processes, both biotic (Gathergood et al., 2004; Stolte et al., 2012; Romero et al., 2008) and abiotic (Thuy Pham et al., 2010; Gomez-Herrero et al., 2018; Gomez-Herrero et al., 2020; Fabiańska et al., 2012). Although such properties may be desired from the view of industrial purposes, they are certainly a disadvantage when faced with a potential environmental fate (Kowalska et al., 2021).

As a consequence, another significant environmental issue with regard to imidazolium ILs may be their accumulation in aquatic organisms. It was previously thought that this phenomenon would not occur in the case of ionic compounds, which was supported by *in vitro* studies based on the octanol-water partition coefficient (Ropel et al., 2005). However, it turns out that the conclusions made using this tool were misleading, and the application of more advanced methods yielded different results (Hodges et al., 2019; Niu et al., 2021). Recent works analyzing the potential bioconcentration of ILs with membrane lipids (Dołżonek et al., 2017) and proteins (Kowalska et al., 2022) has revealed that imidazolium cations have a high affinity for them. This raises renewed questions about the potential bioconcentration of imidazolium ILs, the resolution of which requires comparison of the data obtained by *in vitro* methods with actual observations in experiments involving organisms.

Therefore, the main objective of this study was to determine whether and to what extent imidazolium cations of ILs, namely $[IM18]^+$ and $[IM1–12]^+$, can bioconcentrate in soft tissues of marine invertebrates. In addition, to determine the non-lethal concentrations of the ionic liquids tested, acute toxicity was also assessed in preliminary investigation. For this purpose, laboratory tests were conducted in which marine mussels *M. trossulus* were exposed to these compounds for 21 days. *M. trossulus* mussels are commonly used in both biomonitoring studies and ecotoxicological experimental works, due to their favourable characteristics such as filter feeding and sedentary lifestyle, as well as high resistance and easy breeding (Świacka et al., 2019). Although reports in recent years have suggested that ILs may accumulate in organisms, this has not yet been confirmed by *in vivo* studies. Thus, the presented results are crucial in terms of the future assessment of the potential environmental fate of imidazolium ILs. Moreover, the effect of the salinity on the bioconcentration phenomenon of imidazolium ILs by tested mussels was also investigated as salinity can have a dual effect on bioconcentration because it can influence both the properties of the test compound in water and also the physiology of organisms living in the aquatic environment. Furthermore, to evaluate the effect of anion on bioconcentration, the bioconcentration of the $[IM18]^+$ when paired with a simple inorganic chloride anion and a more complex tricyanomethanide anion was compared.

2. Materials and methods

2.1. Conceptual approach

In general, the whole study was divided into two main research sections such as: *(i)* preliminary assessment of acute toxicity of investigated compounds toward test organisms; *(ii)* performance of the main *in vivo* bioconcentration tests in order to determine the bioconcentration factor – BCF of the selected ILs cations, including dependence of water salinity (8 PSU (practical salinity unit) and 16 PSU) (Fig. 1). In order to perform these experiments appropriate analytical procedures and techniques were applied, which were previously optimized and fully validated in our previous paper (Maculewicz et al., 2022, 2023). Details of each stage of the study are described below.

2.2. Chemicals

Acetonitrile and methanol (LC-MS grade), as well as hexane, dimethyl sulfoxide and isopropranol (pure for analysis grade) were purchased from POCH (Poland). Ammonium acetate, acetic acid, ammonium formate and formic acid (all LC-MS grade) were purchased from Sigma-Aldrich (Germany). ILs standards, namely: 1-octyl-3-methylimidazolium chloride ([IM18][Cl], ≥97 %), powder, 1-octyl-3-methylimidazolium tricyanomethanide ([IM18][$C(CN)_3$], ≥98 %, liquid) and 1-dodecyl-3-methylimidazolium bromide ([IM1–12][Br], ≥98 %, powder) were purchased from Iolitec (Germany).

2.3. Mussel collection and acclimatization

Mytilus trossulus mussels were collected by benthic dredge from the Gulf of Gdańsk (the southern Baltic Sea, coordinates 54°36 W, 18°35E) in September 2020, October 2021, and November 2021 during the 'Oceania' research vessel cruise. Immediately after collection, selected individuals with shell lengths ranging from 2.7 to 4.5 cm were transferred to the tanks with aerated seawater to depurate for 24 h.

Next, mussels were transported to the laboratory, carefully cleaned from epiphytes and acclimatized in thermostatic tanks filled with artificial seawater (8 PSU and 16 PSU) applying environmental parameters (T = 10 °C, pH = 7.4, dim light) for 14 days prior to use in the experiment. Aquaforest® Reef Salt and Milli-Q water were used to prepare seawater with a final salinity of 8 and 16 PSU. During acclimatization and experiment mussels were daily fed with 20 mL (5×10^5 cells/mL) of suspension containing the green algae *Chlorella vulgaris* A1–76 (Culture Collection IO PAN, Sopot; isolated from the Baltic Sea).

2.4. Toxicity evaluation

Due to the lack of data on the lethal levels of both [IM18][$C(CN)_3$] and [IM1–12][Br] ionic liquids, the mortality test was first performed to estimate the range of each concentration of IL in which no mortality occurs. The mussels were kept in saline water (8 PSU) and exposed for 21 days to 5 different final concentrations of the target ILs: 0.01, 0.1 0.5; 5 and

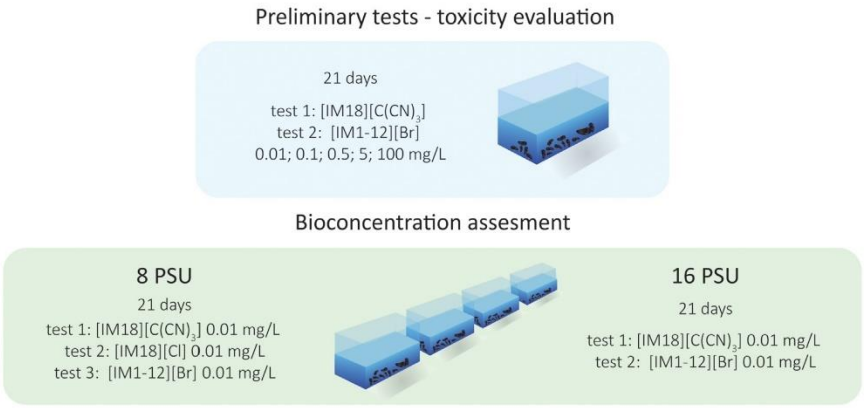

Fig. 1. Conceptual approach of the present study.

100 mg/L. The preliminary mortality experiments were conducted in a similar way to the *in vivo* test with the same setup and conditions, which are described in detail below (Section 2.5).

2.5. Bioconcentration assessment

The experimental design of this part of study is schematically presented in the Fig. 2.

In general, four aquaria filled with 6 L of artificial seawater were placed in a thermostatic system ($T = 10$ °C) with dim lighting. A stock solution of the target analyte was added to three tanks to achieve the final concentration of 10 μg/L, while the fourth aquarium served as a control. Mussels (50 individuals) were placed in each exposure tank and exposed for 21 days to the target ionic liquid: [IM18][$C(CN)_3$], [IM1–12][Br] or [IM18][Cl]. Another group of 50 mussels was kept as a control in ILs-free artificial seawater. The tanks were continuously aerated during the experiment. Half of the total seawater volume in the aquaria was changed every 3 days (3 L out of 6 L were replaced) and new equivalent amounts of ILs were added to each tank to ensure relatively stable concentration of chemical in the exposure chambers. The water subsamples were collected daily throughout the experiment to assess the concentration of the target analytes. To assess bioconcentration, five individuals of *M. trossulus* were collected every 72 h, transferred to 1 L static tanks, and left in ionic liquid-free seawater for 6 h before further steps of analysis to clean their digestive system and avoid further contamination. Next, tissues were dissected and the wet mass of each individual was weighed and the length of the shell was measured. Finally, mussel and water samples collected for chemical analysis were frozen and stored at −20 °C prior to analysis.

2.6. Sample preparation and LC-MS/MS analysis

The mussel tissues were lyophilized (FreeZone 6 Liter Benchtop Freeze Dry System, collector $T = -51$ °C, $P = 0{,}055$ mBar) and the dry mass of tissues was determined. Subsequently, lyophilized tissues from the experiments were homogenized using a porcelain mortar and then weighed (0.5 g for each replicate). The tissues thus prepared were extracted, purified and analyzed according to the procedure described in our previous work (Maculewicz et al., 2022, 2023). Briefly, the extraction of investigated ILs

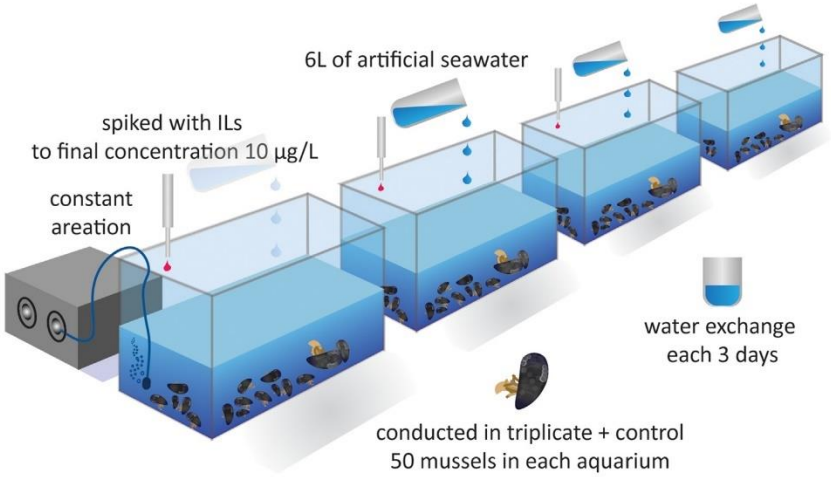

Fig. 2. Experimental design of bioconcentration tests.

cations from the mussel tissues was performed using microwave assisted extraction (MAE) technique using a MarsXpress system (CEM Corporation, UK). The conditions used during the extraction of $[IM18]^+$ were as follows: 2 min of temperature rise to 100 °C, then holding the temperature for 20 min with constant microwave power set at 400 W. For extraction of $[IM1–12]^+$ conditions were set at 120 °C and 800 W (Maculewicz et al., 2022, 2023).

After extraction, the samples were subjected to the purification process by solid-phase extraction (SPE) technique. First, the samples were centrifuged for 10 min at 5000*g*. 6 mL of supernatant was then collected, diluted so that the organic phase proportion did not exceed 5 %, and loaded to Oasis HLB cartridges (6 mL, 200 mg; Waters, US). The analytes were eluted with 2 × 3 mL of methanol under gravity flow. In the case of $[IM18]^+$, before final LC-MS/MS analysis, samples were evaporated under the stream of nitrogen and reconstituted in 0.6 mL of MeOH, resulting in tenfold concentration, whereas in the case of $[IM1–12]^+$ samples were diluted tenfold in MeOH before LC-MS/MS analysis.

For the extraction of analytes from water samples after bioconcentration tests SPE purification procedure, described above, was applied. Before LC-MS/MS analysis samples were evaporated under the stream of nitrogen and reconstituted in 0.25 mL of MeOH, resulting in fourfold concentration.

Instrumental analyses of tissue extracts as well as water samples collected from *in vivo* bioconcentration tests were performed using UHPLC system including LC-40B X3 solvent delivery module, SIL-40C X3 autosampler and CTO-40C column oven, coupled to a triple quadrupole mass spectrometer (LCMS-8050) equipped with an electrospray ionisation (ESI) source (Shimadzu, Japan). A Kinetex 2.6 μm XB-C18 100 Å, 100 × 3 mm LC column (Phenomenex, Canada) was used for the chromatographic separation of the compounds tested. The mobile phases consisted of A: acetonitrile and B: 10 mM acetate buffer. The flow rate of the mobile phases was 0.4 mL/min. The oven temperature during the analyses was set at 35 °C. The following gradient elution was used in the chromatographic separation: 50 % A (0–1 min), linearly increased to 95 % A (4 min), isocratic elution step at 95 % of A for 1 min with a total analysis time of 6 min (Maculewicz et al., 2022, 2023).

Although the entire analytical procedure was validated in our previous work (Maculewicz et al., 2022, 2023), during bioconcentration tests, for the accurate quantification of each analyte's concentration in the tissues, matrix matched calibration curves (Fig. S1) were freshly prepared before analyzing samples from each test. For this purpose, spiking of pure lyophilized mussel tissues with ILs solutions was done at 4 concentration levels: 50, 100, 200, 500 ng/g dry mass, each time before the scheduled extraction tests following the same procedure. The procedure consisted of weighing the required amount of dry soft tissue and transferring it to a glass beaker. The tissues were then spiked with a 1-to-1 ratio of methanolic ILs solution (1 mL of solution per 1 g of dry tissue) and thoroughly mixed so that the methanolic solution evenly combined with the tissues. Finally, the spiked tissues were incubated at 50 °C overnight to completely evaporate the methanol. The tissues prepared in this way were subjected to an extraction process, according to the procedure described above, on the same day after preparation.

2.7. Calculations

BCF was calculated according to the standard equation (ECHA, 2017):

$$BCF = \frac{C_T}{C_w}$$

where: C_T refers to the concentration of cation in the tissues [μg/kg], while C_w refers to the concentration of cation in water [μg/L] measured at the same time.

LC_{50} values were calculated using the Quest Graph™ EC_{50} Calculator online tool (AAT Bioquest Inc., 2022).

3. Results and discussion

3.1. Toxicity assessment

During the initial experiments, mussel mortality was analyzed for 21 days to determine the non-acute toxicity concentration at which bioconcentration could be evaluated. Detailed data on mussel mortality on each test day are summarized in Fig. 3. For both the control and the two lowest concentrations tested (0.01 and 0.1 mg/L), no mortality was observed. The lowest concentration causing mortality in bivalves (LOEC) was 0.5 mg/L, but for both ILs this was not observed until the last week of the test and was ultimately 10 % and 35 % for $[IM18][C(CN)_3]$, [IM1–12][Br], respectively. The highest concentration tested, 100 mg/L caused rapid death of all *M. trossulus* individuals for $[IM18][C(CN)_3]$, (100 % mortality after 5 days), while progressively leading to the death of 90 % of individuals for [IM1–12][Br]. Additionally, the LC_{50} (21 d) value was determined based on toxicity tests, which was 0.68 mg/L for [IM1–12][Br], and 11.66 mg/L for $[IM18][C(CN)_3]$. These values are in good agreement with the range of LC_{50} data reported in the literature. In a paper by Huang et al. (2013) the LC_{50} (14 days) value of [IM18][Cl] in a 14-day experiment on earthworms was determined to be 150.35 mg/kg. On the other hand, Bernot et al. (2005b) determined the LC_{50} (72 h) of [IM18][Br] for a freshwater snail of 8.2 mg/L. In general, according to the literature, the toxicity of $[IM1–12]^+$, is higher than that of $[IM18]^+$, although most studies assess these cations as paired with an inorganic anion. As shown in invertebrates studies, the difference in LC_{50} values between these two compounds is even greater than order of magnitude (Wu et al., 2013; Li et al., 2014). In our work, the cation with the longer side chain was shown to be more toxic, with the LC_{50} almost 20 times higher.

Based on the preliminary mortality tests, it was decided that a concentration of 10 μg/L will be used for all bioconcentration tests. This value is an order of magnitude lower than the concentration at which no mortality was observed in preliminary tests. It is also >3 orders of magnitude smaller

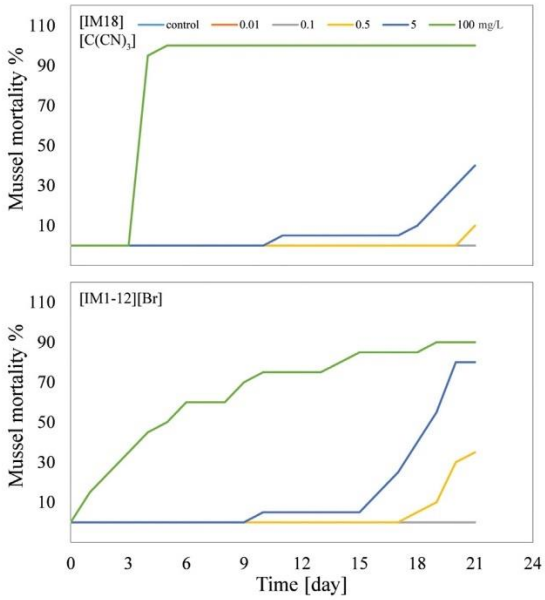

Fig. 3. Mortality of *M. trossulus* mussels during 21-day preliminary test. Mussels were exposed to ILs individually at concentrations of 0.01; 0.1; 0.5; 5 and 100 mg/L.

than the determined LC_{50} for [IM1–12][Br] and nearly 2 orders of magnitude lower than LC_{50} for [IM18][$C(CN)_3$].

3.2. Bioconcentration assessment

3.2.1. Determination of ILs in water

The concentration of $[IM18]^+$ was close to the nominal concentration in all three tests. The concentration of $[IM18]^+$ oscillated between 5.28 and 8.61 μg/L in 8 PSU and 5.22–8.84 μg/L in 16 PSU for water spiked with [IM18][$C(CN)_3$] while $[IM18]^+$ concentration in water spiked with [IM18][Cl] ranged between 7.96 and 10.84 μg/L (Fig. 4). The high agreement between the measured and nominal concentrations indicates that the determined $[IM18]^+$ exhibits high stability in water under experimental conditions and is highly soluble in water. On the contrary, a >10-fold loss from the nominal concentration for $[IM1–12]^+$ was observed in both tests after only 72 h of experimentation. A further loss of this compound was observed to a lesser extent during the first 9 days, followed by stabilization, with concentrations fluctuating between 0.18 and 0.50 μg/L by the end of the test (Fig. 4). Such a high decrease in $[IM1–12]^+$ concentration in water has already been an indication of its high accumulation in bivalve tissues, however it might also be partly the result of deposition of the compound on the aquarium walls, as well as accumulation at the phase boundary near the water surface. It has been confirmed by an abiotic test, which also shows a significant decrease in $[IM1–12]^+$ concentration. However, despite this decrease and such large differences between the tested cations, in both cases the water concentration analyses suggest that equilibrium has been achieved in the aquaria (Fig. 4) and the available amount of analytes was sufficient to be measured in bivalves tissues.

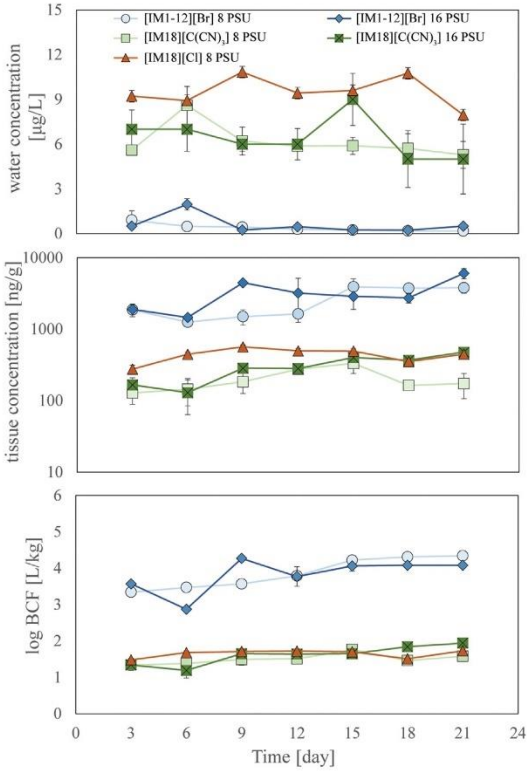

Fig. 4. Measured water concentration, tissue concentration and log BCF calculated for subsequent days of the experiment.

3.2.2. Determination of ILs in mussel tissues and evaluation of bioconcentration

In the case of all bioconcentration tests performed, already after 3 days of the exposure the concentration of imidazolium cations in mussel tissues was high enough to be measured in the extracts. On the following days of the experiment, a slight increase in tissue concentrations was observed up to day 9, while then they mainly fluctuated without a clear trend. This indicates that the uptake and most efficient concentration of imidazolium cations by bivalves occurs during the first days of exposure. For the 8 PSU salinity experiments, $[IM1–12]^+$ concentrations in mussel tissues increased from 1867 ng/g to 3793 ng/g between days 3 and 21 of the experiment. For [IM18][$C(CN)_3$], tissue concentrations ranged from 128 ng/g on day 3 to 332 ng/g on day 15, and for [IM18][Cl] from 276 ng/g on day 3 to 564 ng/g on day 9.

According to the criteria adopted under REACH (EC 1907/2006: Registration, Evaluation, Authorisation and restriction of CHemicals), a BCF above 2000 L/kg classifies a chemical as bioaccumulative and above 5000 L/kg as very bioaccumulative. Slightly less restrictive level of BCF has been established by United States Environmental Protection Agency (EPA), which consider substance as bioaccumulative when BCF factor is equal or exceed 1000 for fish (EPA, 2012). The BCF values obtained in our work after 21 days of exposure indicate that $[IM1–12]^+$ should be considered a compound with a very high potential for bioaccumulation (BCF = 21,901 ± 3400 L/kg). In addition, the actual bioconcentration of the $[IM1–12]^+$ observed in our test is significantly higher than the values estimated from *in vitro* studies. The value of the logarithmic membrane-water partition coefficient for this compound was determined to be 3.76 ± 0.04 (Dołżonek et al., 2017), while the logBCF obtained in our work was 4.34 ± 0.10 L/kg (8 PSU). According to the estimates of Droge et al. (2021), for imidazolium cations, only for alkyl chain lengths above 15 carbon atoms a BCF above 2000 L/kg should be expected. In contrast, our experiments on mussels show that already a 12-carbon side chain contributed to values that allowed the $[IM1–12]^+$ to be classified as very bioaccumulative with BCF of 21,901 ± 3400 L/kg. In contrast, the $[IM18]^+$ does not exhibit a high potential for bioaccumulation. The BCF value was 40 ± 11 L/kg (log BCF = 1.58 ± 0.12 L/kg (8 PSU). Previous *in vitro* estimations, based on membrane lipid partitioning (log K_{MW} = 2.06 ± 0.15), cannot be directly compared, due to the simplified *in vitro* system, which does not take into account metabolism, protein binding *etc.* However, the bioconcentration potential of the $[IM18]^+$ estimated using both *in vivo* and *in vitro* methods is rather low.

Moreover, no significant influence of anion on the bioconcentration of cation ($[IM18]^+$) was observed in our tests. Only minor bioconcentration of $[IM18]^+$ (as [IM18][Cl]) was observed in mussel tissues and the log BCF was 1.73 ± 0.14 L/kg, which is similar to the value observed for $[IM18]^+$ tested as [IM18][$C(CN)_3$] (1.58 ± 0.12) (Table 1).

So far only one work on the *in vivo* bioconcentration of imidazolium ILs has been published (Nędzi et al., 2013). However, only a cation with short, butyl side chain was analyzed, showing very low bioconcentration in both tested organisms (BCF < 10 L/kg) including *M. trossulus* and crustacean *Amphibalanus improvisus*. Although there is a lack of comparable studies on the *in vivo* bioconcentration of imidazolium compounds, recent work of Kierkegaard et al. (2021), who analyzed the bioconcentration of cationic surfactants, including quaternary ammonium compounds and alkylamines, in fish tissues, also observed a low potential for bioconcentration (BCF < 100 L/kg) of permanently charged quaternary cations with 10- and

Table 1
BCF and log BCF calculated for the last (21) day of the experiment.

Salinity [PSU]	Tested compound	BCF of cation [L/kg]	Log BCF of cation [L/kg]
8	[IM1–12][Br]	21,901 ± 3400	4.34 ± 0.10
	[IM18][$C(CN)_3$]	40 ± 11	1.58 ± 0.12
	[IM18][Cl]	57 ± 16	1.73 ± 0.14
16	[IM1–12][Br]	12,108 ± 1900	4.08 ± 0.10
	[IM18][$C(CN)_3$]	88.2 ± 6.6	1.94 ± 0.10

14-carbon aliphatic chains. Moreover, although little bioconcentration of permanently charged compounds was observed, a structurally similar tertiary cation with a 14-carbon chain showed a very high potential for bioconcentration, reaching a BCF of nearly 10,000 L/kg (Kierkegaard et al., 2021). In general, it was observed that the tertiary cations are >2 orders of magnitude more bioaccumulated than the corresponding quaternary cations (Kierkegaard et al., 2021). The observed phenomenon was linked to the presence of a small fraction of the neutral amine form (about 0.2 %), whose uptake was much higher than that of the cations. In contrast, results obtained in our work suggest that in the case of imidazolium cations, the lack of a neutral form is not an obstacle to accumulation in the tissues of aquatic organisms. Moreover, the BCF values obtained for the $[IM1–12]^+$ are higher than for the ammonium cations even with 14- and 16-carbon side chains. It must be however remembered that in the work of Kierkegaard et al. (2021) fish were used, while our tests were conducted on bivalves, so the observed differences may be also linked to interspecies differences. Nevertheless, our data undoubtedly suggest that permanently ionized cations can also strongly bioconcentrate in aquatic organisms.

3.2.3. The effect of salinity on bioconcentration

The effect of salinity on the bioconcentration phenomenon was observed in our work, but it varied depending on the tested compound. For the $[IM18]^+$, an increase in bioconcentration was observed with increasing salinity, and BCF after 21 days of exposure was 40 ± 11 L/kg for 8 PSU and 88.2 ± 6.6 L/kg for 16 PSU, respectively. In contrast, the opposite effect was observed for the $[IM1–12]^+$, where the BCF was 21,901 ± 3400 L/kg in 8 PSU and almost twice lower, 12,108 ± 1900 L/kg in 16 PSU.

Salinity is an important factor when considering the effects of contaminants on aquatic organisms because it can affect both the properties and bioavailability of chemical compounds as well as the physiology of organisms (Jeon et al., 2010b). The work of Chen et al. (2017) analyzed the effect of salinity on the bioconcentration of antibiotic, sulfamethoxazole in zebrafish *Danio rerio*. In this work, salinities of 10 and 20 PSU higher than the natural salinity for this species were tested, in both cases resulting in a decrease in bioconcentration compared to environmental salinity. In our tests, for $[IM1–12]^+$ it was observed that with increased salinity the BCF was lower than in native salinity for *M. trossulus*. On the other hand, Chen et al. (2017) observed that the increase in salinity affected the higher affinity of the test compound for the sediment, which also had a significant effect on the bioavailability of the drug to fish. However, in our work, mussels were kept in sediment-free aquaria and no effect of salinity was observed on the stability of the tested compounds in water. Therefore, the observed influence of salinity on BCF cannot be attributed to this phenomenon.

In the work of Jeon et al. (2010a), the effect of salinity on the bioconcentration of organic contaminants in fish was also observed, this time for perfluorinated compounds. The observed salinity effect may have been due to the influence of the osmolality gradient on the diffusion of compounds between water and fish tissues. However, different results were observed in the work of Scott et al. (2019), where no effect of salinity changes on the bioconcentration of organic ionizable contaminants in fish was demonstrated. Perfluorinated contaminants have also been studied for bioaccumulation in Pacific oysters (Jeon et al., 2010b), testing this phenomenon at native salinity (34 PSU) and lower salinities (25, 17.5, and 10 PSU). In this study, a complex effect of salinity on the bioaccumulation phenomenon was observed, which in the case of native salinity was mainly due to the dietary intake of pollutants, while with increasing osmotic stress it was mainly dependent on bioconcentration. Finally, an increase in salinity caused an increase in the uptake of contaminants from the water, but at the same time it also led to an accelerated purification phase. However, in general, the increase in uptake was higher, leading to higher BCFs at higher salinities. A similar effect may have occurred in our study for the $[IM18]^+$, for which a nearly two-fold higher BCF was observed on day 21 in higher salinity.

On the other hand, the work of Weinstein (2003) examined the effect of salinity on fluoranthene bioconcentration in two aquatic invertebrates, the shrimp *Palaemonetes pugio* and the polychaete *Monopylephorus rubroniveus*. In the case of shrimp, no effect on the bioconcentration of the test compound was observed, but for the polychaete, with increasing salinity, a linear decrease in the bioconcentration coefficient was demonstrated, which for 7 PSU was almost 20,000 L/kg, while for 28 PSU was only slightly over 5000 L/kg. The effect of salinity on bioconcentration may be particularly important in the case of osmocomformers, *i.e.* organisms that maintain an isotonic internal environment in relation to their environment. As suggested (Weinstein, 2003), reduced salinity may increase the volume of internal fluids and thus cell surface area. In turn, increased lipid membrane surface area may result in increased bioconcentration of lipophilic compounds. In our work, it was observed that for the more lipophilic cation: $[IM1–12]^+$, the BCF value was higher in lower salinity. Therefore, the slight effect of salinity on bioconcentration of this compound in *M. trossulus* mussels, which are also osmocomformers, could be caused by the same mechanism.

4. Conclusions

The present study demonstrates for the first time that imidazolium ILs can undergo bioconcentration in marine invertebrates. *In vivo* tests showed that the bioconcentration of imidazolium cations may be highly related to the side chain length. However, more experimental data are needed to definitively confirm this assumption and its possible application to other similar compounds. On the other hand, no salinity or anion influence on the bioconcentration of the tested cations was observed. Our tests also confirm that imidazolium ILs exhibit acute toxicity only at relatively high concentration levels. This further confirms that the risks associated with the potential presence of these compounds in the environment should be attributed to their high persistence and potential bioconcentration, rather than acute toxicity.

The BCF value of over 5000 L/kg determined for $[IM1–12]^+$ (21,901 ± 3400 L/kg) indicates that it is a very bioaccumulative compound according to the established criteria (REACH and EPA). Therefore, our data clearly indicate that some ILs, especially those with long side chains, require special attention in terms of potential accumulation in living organisms.

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CRediT authorship contribution statement

Jakub Maculewicz: Investigation, Writing – original draft, Visualization. **Joanna Dołżonek:** Conceptualization, Project administration, Writing – review & editing. **Lilianna Sharma:** Investigation, Writing – original draft. **Anna Białk-Bielińska:** Writing – review & editing. **Piotr Stepnowski:** Funding acquisition, Resources. **Ksenia Pazdro:** Conceptualization, Resources, Supervision, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

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9. Oświadczenia współautorów

Jakub Maculewicz
ul. Towarowa 25
84-120 Władysławowo

03.04.2024, Christchurch

Oświadczenie

Oświadczam, że w poniżej wymienionych publikacjach:

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mój wkład polegał na uczestniczeniu w zaplanowaniu zakresu tematycznego manuskryptu, analizie danych literaturowych dotyczących metod in vitro wykorzystywanych w ocenie potencjału do biokoncentrajci związków chemicznych, przygotowaniu rysunków i napisaniu wszystkich rozdziałów manuskryptu oraz korespondencji z edytorem i przygotowaniu odpowiedzi na recenzje

Maculewicz, J., Kowalska, D.*, Świacka, K., Toński, M., Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco) toxicity and bioaccumulation potential. Science of The Total Environment, 802, 149916.

mój wkład polegał na zaplanowaniu tematyki manuskryptu, współudziale przy sporządzeniu koncepcji pracy, analizie danych literaturowych dotyczących toksyczności i potencjału do biokoncentracji produktów transformacji leków, przygotowaniu rysunków i napisaniu wstępu oraz części manuskryptu dotyczącej toksyczności i potencjału do biokoncentracji produktów transformacji leków przeciwnowotworowych oraz antydepresantów, a także końcowej edycji manuskryptu

Maculewicz, J.*, Świacka, K., Stepnowski, P., Dołżonek, J., & Białk-Bielińska, A. (2022). Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments. Journal of Hazardous Materials, 437, 129353.

mój udział polegał na uczestniczeniu w zaplanowaniu zakresu tematycznego manuskryptu, analizie danych literaturowych dotyczących cieczy jonowych jako zagrożenia dla środowiska, ich obecności w środowisku oraz metod analitycznych wykorzystywanych do ich oznaczania, przygotowaniu rysunków i napisaniu wszystkich rozdziałów manuskryptu oraz korespondencji z edytorem i przygotowaniu odpowiedzi na recenzje

Maculewicz, J.*, Stepnowski, P., Dołżonek, J., & Białk-Bielińska, A. (2023). Analysis of imidazolium ionic liquids in biological matrices: a novel procedure for the determination of trace amounts in marine mussels. Talanta, 252, 123790.

mój udział polegał na uczestniczeniu w zaplanowaniu prac eksperymentalnych, przeprowadzeniu badań laboratoryjnych, opracowaniu wyników, przygotowaniu rysunków oraz napisaniu manuskryptu a także korespondencji z edytorem i przygotowaniu odpowiedzi na recenzje

Maculewicz, J., Dołżonek, J.*, Sharma, L., Białk-Bielińska, A., Stepnowski, P., & Pazdro, K. (2023). Bioconcentration of imidazolium ionic liquids: *In vivo* evaluation in marine mussels *Mytilus trossulus*. Science of the Total Environment, 858, 159388.

mój udział polegał na wykonaniu ekstrakcji próbek uzyskanych w teście in vivo, przeprowadzeniu analiz LC-MS i opracowaniu uzyskanych wyników, przygotowaniu rysunków oraz napisaniu manuskryptu, a także współuczestniczeniu w przygotowaniu odpowiedzi na recenzje

Maculewicz J., Białk-Bielińska A., Kowalska D., Stepnowski P., Stolte S., Beil S., Dołżonek J.* (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (w recenzji)

mój udział polegał na przeprowadzeniu eksperymentów podziału cieczy jonowych do membrany lipidowej, oznaczeń końcowych z wykorzystaniem HPLC-DAD i IC (w tym celu opracowałem i przeprowadziłem walidację metod analitycznych cieczy jonowych z wykorzystaniem techniki HPLC-DAD), opracowaniu uzyskanych wyników, przygotowaniu rysunków oraz napisaniu manuskryptu, a także współuczestniczeniu w przygotowaniu odpowiedzi na recenzje

Kowalska, D., **Maculewicz, J.***, Stepnowski, P., & Dołżonek, J. (2021). Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: A preliminary study based on *in vitro* assessment. Science of the Total Environment, 783, 146987.

mój udział polegał na uczestniczeniu w zaplanowaniu oraz przeprowadzeniu eksperymentów dotyczących podziału produktów transformacji farmaceutyków (TPs) do membrany lipidowej, opracowaniu wyników uzyskanych w toku tych eksperymentów, przygotowaniu rysunków oraz napisaniu części manuskryptu dotyczącej podziału TPs do sztucznej błony biologicznej, a także korespondencji z edytorem i przygotowaniu odpowiedzi na recenzje.

Jakub Maculewicz

Uniwersytet Gdański

dr hab. inż. Anna Białk-Bielińska, prof. UG
Katedra Analizy Środowiska
Wydział Chemii

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Oświadczenie

Oświadczam, że w poniżej wymienionych publikacjach:

- Maculewicz, J., Kowalska, D.*, Świacka, K., Tański, M., Stepnowski, P., **Białk-Bielińska, A.,** & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: Their fate,(eco) toxicity and bioaccumulation potential. Science of The Total Environment, 802, 149916.

mój udział polegał na współudziale w zaplanowaniu koncepcji artykułu, nadzorowaniu przebiegu prac, opiece merytorycznej i zredagowaniu manuskryptu oraz współuczestnictwie w przygotowaniu odpowiedzi na recenzje.

- Maculewicz, J.*, Świacka, K., Stepnowski, P., Dołżonek, J., & **Białk-Bielińska, A.** (2022). Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments. Journal of Hazardous Materials, 437, 129353.

mój udział polegał na zaplanowaniu tematyki publikacji, nadzorowaniu przebiegu prac i ostatecznej redakcji manuskryptu oraz współuczestnictwie w przygotowaniu odpowiedzi na recenzje.

- Maculewicz, J.*, Stepnowski, P., Dołżonek, J., & **Białk-Bielińska, A.** (2023). Analysis of imidazolium ionic liquids in biological matrices: a novel procedure for the determination of trace amounts in marine mussels. Talanta, 252, 123790.

mój udział polegał na zaplanowaniu i koordynowaniu prac badawczych, opiece merytorycznej i zredagowaniu manuskryptu oraz współuczestnictwie w przygotowaniu odpowiedzi na recenzje.

- Maculewicz, J., Dołżonek, J.*, Sharma, L., **Białk-Bielińska, A.,** Stepnowski, P., & Pazdro, K. (2023). Bioconcentration of imidazolium ionic liquids: *In vivo* evaluation in marine mussels *Mytilus trossulus*. Science of The Total Environment, 858, 159388.

mój wkład polegał na opiece merytorycznej, nadzorowaniu prac eksperymentalnych związanych z analityką cieczy jonowych w próbkach uzyskanych w teście in vivo, a także współudziale w zredagowaniu manuskryptu.

Wydział Chemii
Katedra Analizy Środowiska
tel. +48 58 523 52 07, fax. +48 58 523 50 12
e-mail: anna.bialk-bielinska@ug.edu.pl
ul. Wita Stwosza 63
80-308 Gdańsk
www.ug.edu.pl

Uniwersytet Gdański

Maculewicz J., **Białk-Bielińska A.,** Kowalska D., Stepnowski P., Stolte S., Beil S., Dołżonek J.* (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (w recenzji)

mój udział polegał na opiece merytorycznej i współuczestnictwie w zredagowaniu manuskryptu.

Białk-Bielińska

dr hab. inż. Anna Białk-Bielińska, prof. UG

Wydział Chemii
Katedra Analizy Środowiska

tel. +48 58 523 52 07, fax. +48 58 523 50 12
e-mail: anna.bialk-bielinska@ug.edu.pl

ul. Wita Stwosza 63
80-308 Gdańsk

www.ug.edu.pl

Dr Joanna Dołżonek
Adiunkt
Katedra Analizy Środowiska
Wydział Chemii

2.04.2024, Gdańsk

Oświadczenie

Oświadczam, że w poniżej wymienionych publikacjach mój wkład polegał na:

Maculewicz, J.*, Świacka, K., Kowalska, D., Stepnowski, P., Stolte, S., & **Dołżonek, J.** (2020). *In vitro* methods for predicting the bioconcentration of xenobiotics in aquatic organisms. Science of The Total Environment, 739, 140261.

zaplanowaniu tematyki manuskryptu, opiece merytorycznej i zredagowaniu manuskryptu oraz współuczestnictwie w przygotowaniu odpowiedzi na recenzje;

Maculewicz, J., Kowalska, D.*, Świacka, K., Toński, M., Stepnowski, P., Białk-Bielińska, A., & **Dołżonek, J.** (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco) toxicity and bioaccumulation potential. Science of The Total Environment, 802, 149916.

uczestniczeniu w zaplanowaniu tematyki manuskryptu, opiece merytorycznej i współudziale w zredagowaniu manuskryptu;

Maculewicz, J.*, Świacka, K., Stepnowski, P., **Dołżonek, J.,** & Białk-Bielińska, A. (2022). Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments. Journal of Hazardous Materials, 437, 129353.

opiece merytorycznej i współudziale w zredagowaniu manuskryptu oraz współuczestnictwie w przygotowaniu odpowiedzi na recenzje;

Maculewicz, J.*, Stepnowski, P., **Dołżonek, J.,** & Białk-Bielińska, A. (2023). Analysis of imidazolium ionic liquids in biological matrices: a novel procedure for the determination of trace amounts in marine mussels. Talanta, 252, 123790.

uczestniczeniu w zaplanowaniu badań, opiece merytorycznej, współudziale w zredagowaniu manuskryptu oraz administrowaniu projektem w ramach, którego zrealizowano zadania badawcze;

Wydział Chemii
Katedra Analizy Środowiska
tel. +48 58 523 5207
e-mail: joanna.dolzonek@ug.edu.pl
ul. Wita Stwosza 63
80-308 Gdańsk
www.ug.edu.pl

Uniwersytet Gdański | Związek Uczelni Fahrenheita

Maculewicz, J., **Dołżonek, J.***, Sharma, L., Białk-Bielińska, A., Stepnowski, P., & Pazdro, K. (2023). Bioconcentration of imidazolium ionic liquids: In vivo evaluation in marine mussels *Mytilus trossulus*. Science of the Total Environment, 858, 159388.

współudziale w zaplanowaniu i koordynowaniu prac badawczych, opiece merytorycznej, uczestniczeniu w analizie i interpretacji uzyskanych wyników, zredagowaniu manuskryptu, przygotowaniu odpowiedzi na recenzje i ostatecznej korekty manuskryptu, korespondencji z edytorem oraz administrowaniu projektem w ramach, którego zrealizowano zadania badawcze;

Maculewicz J., Białk-Bielińska A., Kowalska D., Stepnowski P., Stolte S., Beil S., **Dołżonek J.*** (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (w recenzji)

zaplanowaniu i koordynowaniu prac eksperymentalnych, opiece merytorycznej, uczestniczeniu w analizie i interpretacji uzyskanych wyników, współudziale w zredagowaniu manuskryptu, przygotowaniu odpowiedzi na recenzję i ostatecznej korekty manuskryptu, korespondencji z edytorem oraz administrowaniu projektem w ramach, którego zrealizowano zadania badawcze;

Kowalska, D., Maculewicz, J.*, Stepnowski, P., & **Dołżonek, J.** (2021). Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: A preliminary study based on in vitro assessment. Science of the Total Environment, 783, 146987.

zaplanowaniu prac badawczych, koordynowaniu przebiegu badań, opiece merytorycznej, uczestniczeniu w analizie i interpretacji uzyskanych wyników i zredagowaniu manuskryptu oraz współuczestnictwie w przygotowaniu odpowiedzi na recenzje.

Dołżonek

Wydział Chemii
Katedra Analizy Środowiska

tel. +48 58 523 5207
e-mail: joanna.dolzonek@ug.edu.pl

ul. Wita Stwosza 63
80-308 Gdańsk

www.ug.edu.pl

Uniwersytet Gdański

prof. dr hab. Piotr Stepnowski
Katedra Analizy Środowiska
Wydział Chemii UG

02.04.2024, Gdańsk

Oświadczenie

Oświadczam, że w poniżej wymienionych publikacjach:

1. Maculewicz, J., Świacka, K., Kowalska, D., **Stepnowski, P.**, Stolte, S., & Dołżonek, J. (2020). *In vitro* methods for predicting the bioconcentration of xenobiotics in aquatic organisms. Science of The Total Environment, 739, 140261.

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników

2. Maculewicz, J., Kowalska, D., Świacka, K., Toński, M., **Stepnowski, P.**, Białk-Bielińska, A., & Dołżonek,
J. (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco) toxicity and bioaccumulation potential. Science of the Total Environment, 802, 149916.

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników

3. Maculewicz, J., Świacka, K., **Stepnowski, P.**, Dołżonek, J., & Białk-Bielińska, A. (2022). Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments. Journal of Hazardous Materials, 437, 129353.

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników

4. Maculewicz, J., **Stepnowski, P.**, Dołżonek, J., & Białk-Bielińska, A. (2023). Analysis of imidazolium ionic liquids in biological matrices: a novel procedure for the determination of trace amounts in marine mussels. Talanta, 252, 123790.

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników oraz organizacji finansowania badań ze środków projektu (NCN/2016/23/G/ST5/04245), którego byłem kierownikiem

5. Maculewicz, J., Dołżonek, J., Sharma, L., Białk-Bielińska, A., **Stepnowski, P.**, & Pazdro, K. (2023). Bioconcentration of imidazolium ionic liquids: *In vivo* evaluation in marine mussels *Mytilus trossulus*. Science of The Total Environment, 858, 159388.

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników oraz organizacji finansowania badań ze środków projektu (NCN/2016/23/G/ST5/04245), którego byłem kierownikiem.

Wydział Chemii
Katedra Analizy Środowiska

tel. +48 58 523 52 05, fax. +48 58 523 50 12
e-mail: piotr.stepnowski@ug.edu.pl

ul. Wita Stwosza 63
80-308 Gdańsk

www.ug.edu.pl

Uniwersytet Gdański

6. Maculewicz J., Białk-Bielińska A., Kowalska D., **Stepnowski P.**, Stolte S., Beil S., Dołżonek J. (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (zaakceptowana do druku)

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników oraz organizacji finansowania badań ze środków projektu (NCN/2016/23/G/ST5/04245), którego byłem kierownikiem.

7. Kowalska, D., Maculewicz, J., **Stepnowski, P.**, & Dołżonek, J. (2021). Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: A preliminary study based on *in vitro* assessment. Science of the Total Environment, 783, 146987.

mój udział polegał na wsparciu merytorycznym koncepcji badań i dyskusji wyników

Wydział Chemii
Katedra Analizy Środowiska

tel. +48 58 523 52 05, fax. +48 58 523 50 12
e-mail: piotr.stepnowski@ug.edu.pl

ul. Wita Stwosza 63
80-308 Gdańsk

www.ug.edu.pl

Klaudia Świacka
ul. Towarowa 25
84-120 Władysławowo

04.04.2024, Christchurch

Oświadczenie

Oświadczam, że w poniżej wymienionych publikacjach:

Maculewicz, J., **Świacka, K.**, Kowalska, D., Stepnowski, P., Stolte, S., & Dołżonek, J. (2020). *In vitro* methods for predicting the bioconcentration of xenobiotics in aquatic organisms. Science of The Total Environment, 739, 140261.

mój udział polegał na analizie części danych literaturowych

Maculewicz, J., Kowalska, D., **Świacka, K.**, Toński, M., Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco) toxicity and bioaccumulation potential. Science of the total environment, 802, 149916.

mój udział polegał na analizie danych literaturowych i napisaniu części manuskryptu dotyczącej toksyczności i potencjału do biokoncentracji produktów transformacji leków opioidowych oraz niesteroidowych leków przeciwzapalnych

Maculewicz, J., **Świacka, K.**, Stepnowski, P., Dołżonek, J., & Białk-Bielińska, A. (2022). Ionic liquids as potentially hazardous pollutants: Evidences of their presence in the environment and recent analytical developments. Journal of Hazardous Materials, 437, 129353.

mój udział polegał na analizie części danych literaturowych

Klaudia Świacka

Dorota Kowalska

ul. Baczyńskiego 6A/11

80-410 Gdańsk

04.04.2024, Gdańsk

Oświadczenie

Oświadczam, że w poniżej wymienionych publikacjach:

Maculewicz, J., Świacka, K., **Kowalska, D.**, Stepnowski, P., Stolte, S., & Dołżonek, J. (2020). *In vitro* methods for predicting the bioconcentration of xenobiotics in aquatic organisms. Science of The Total Environment, 739, 140261.

mój wkład polegał na współudziale w zredagowaniu manuskryptu

Maculewicz, J., **Kowalska, D.**, Świacka, K., Toński, M., Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco) toxicity and bioaccumulation potential. Science of the total environment, 802, 149916.

mój wkład polegał na zaplanowaniu tematyki manuskryptu, współudziale przy sporządzeniu koncepcji pracy, analizie danych literaturowych i napisaniu części manuskryptu dotyczącej toksyczności i potencjału do biokoncentracji produktów transformacji antybiotyków oraz leków przeciwpadaczkowych, a także współudziale w przygotowaniu odpowiedzi na recenzje oraz korespondencji z edytorem

Maculewicz J., Białk-Bielińska A., **Kowalska D.**, Stepnowski P., Stolte S., Beil S., Dołżonek J. (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (w recenzji)

mój udział polegał na opracowaniu i walidacji metod instrumentalnych cieczy jonowych z wykorzystaniem chromatografu jonowego

Kowalska, D., Maculewicz, J., Stepnowski, P., & Dołżonek, J. (2021). Interaction of pharmaceutical metabolites with blood proteins and membrane lipids in the view of bioconcentration: A preliminary study based on in vitro assessment. Science of the Total Environment, 783, 146987.

mój udział polegał na uczestniczeniu w zaplanowaniu oraz przeprowadzeniu eksperymentów dotyczących podziału produktów transformacji farmaceutyków (TPs) do białek, opracowaniu wyników uzyskanych w toku tych eksperymentów oraz napisaniu wstępu i części manuskryptu dotyczącej podziału TPs do białek, a także współudziale w przygotowaniu odpowiedzi na recenzje.

Kowalska

TECHNISCHE UNIVERSITÄT DRESDEN

Faculty of Environmental Sciences Institute of Water Chemistry

Technische Universität Dresden, 01062 Dresden

Jakub Maculewicz
University of Gdańsk Faculty of Chemistry

ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

Prof. Dr. habil. Stefan Stolte
Chair of Hydrochemistry and Water Technology
Director of the Institute of Water Chemistry
Telefon: 0351 463-32759
Telefax: 0351 463-37271
E-Mail: Stefan.Stolte@tu-dresden.de
AZ: StSt

Statement of co-authorship

I hereby declare that in the following publications:

Maculewicz, J., Świacka, K., Kowalska, D., Stepnowski, P., Stolte, S., Dołżonek, J. (2020). In vitro methods for predicting the bioconcentration of xenobiotics in aquatic organisms. Science of The Total Environment, 739, 140261.

My contribution included critical revisions of manuscript.

Maculewicz J., Białk-Bielińska A., Kowalska D., Stepnowski P., Stolte S., Beil S., Dołżonek J. (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (accepted for publication).

My contribution included minor contribution in outlining the paper and its critical revision.

Prof. Dr. Stefan Stolte

Postadresse (Briefe)
TU Dresden, 01062 Dresden
Postadresse (Pakete u. ä.)
TU Dresden
Bergstraße 66
01069 Dresden

Besucheradresse
Sekretariat:
Neubau Chemie,
Bergstr. 66,
2. Etage,
Zimmer 261

Steuernummer
(Inland)
203/149/02549
Umsatzsteuer Id-Nr.
(Ausland)
DE 188 369 991

Bankverbindung
Konto-Nr.: 08 004 004 00
BLZ: 850 400 00
Commerzbank AG, Dresden
IBAN-Code:
DE52 8504 0000 0800 4004 00
Swift-Code: COBADEFF850
Währung: EUR

Zufahrt
Helmholtzstr.
zum Neubau Chemie in den Innenhof
http://tu-dresden.de

TECHNISCHE UNIVERSITÄT DRESDEN

Faculty of Environmental Sciences Institute of Water Chemistry

Technische Universität Dresden, 01062 Dresden

Jakub Maculewicz
Uniwersytet Gdański | Wydział Chemii
Katedra Analizy Środowiska
ul. Wita Stwosza 63
80-308 Gdańsk

Contact: Dr. rer. nat. Stephan Beil
Research Associate / Coordinator CFEA
Tel.: +49 (0) 351 463-40658
Fax: +49 (0) 351 463-37271
Email: Stephan.Beil@tu-dresden.de

Dresden, 02/04/2024

Statement of co-authorship

Dear Mr. Maculewicz,

I hereby declare that I acted as a co-author in the following publication:

Maculewicz J., Białk-Bielińska A., Kowalska D., Stepnowski P., Stolte S., Beil S., Dołżonek J. (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes

my contribution included

conducting thermodynamic predictions using COSMOmic, contributions to paper drafting (sections concerning COSMOmic calculations)

Furthermore, I confirm that the mentioned publication was mainly drafted by you as first author and that I am fully aware of and consent to its usage as part of your PhD thesis.

Best regards

St. Beil

Stephan Beil

Postal address (letters)
TU Dresden
01062 Dresden
Germany

Visitor address
Mommsenstraße 11 (University Executive Board)
01069 Dresden, Germany

Tax number (Germany)
203/149/02549

Bank details
Commerzbank AG,
Dresden Branch

Postal address (packages)
TU Dresden, Helmholtzstraße 10
01069 Dresden, Germany
Internet www.tu-dresden.de

Accessible entrance
via side entrance,
designated parking spaces
in the courtyard

VAT number
(international)
DE 188 369 991

IBAN
DE52 8504 0000 0800 4004 00
BIC
COBADEFF850

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Uniwersytet Gdański | Związek Uczelni Fahrenheita

Gdańsk, 20.03.2024 r.

dr hab. AGNIESZKA GAJEWICZ-SKRĘTNA
Uniwersytet Gdański, Wydział Chemii
Katedra Chemii i Radiochemii Środowiska
Pracownia Chemoinformatyki Środowiska

Oświadczenie o udziale w publikacji

Oświadczam, że mój indywidualny wkład w publikację stanowiącą część rozprawy doktorskiej Pana mgra Jakuba Maculewicz:

> *Maculewicz J., Białk-Bielińska A., Kowalska D., Stepnowski P., Stolte S., Beil S.,* ***Gajewicz-Skrętna A****., Dołżonek J. (2023). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA) – Biomembranes (w recenzji)*

Polegał na przeprowadzeniu analiz statystycznych, napisaniu części dyskusji dotyczącej porównania statystycznego wyników eksperymentalnych i uzyskanych za pomocą modelu COSMOmic, a także przygotowaniu odpowiedzi na komentarze recenzentów związanych z tą częścią pracy.

Agnieszka Gajewicz-Skrętna

Uniwersytet Gdański, Wydział Chemii,
Katedra Chemii i Radiochemii Środowiska
Pracownia Chemoinformatyki Środowiska
ul. Wita Stwosza 63
80-308 Gdańsk

e-mail: agnieszka.gajewiczi@ug.edu.pl

www.ug.edu.pl

CHEMIA UG

POLSKA AKADEMIA NAUK
INSTYTUT OCEANOLOGII
Zakład Chemii i Biochemii Morza

Powstańców Warszawy 55,
81-712 Sopot

tel. (48 58) 7311939

prof. dr hab. Ksenia Pazdro
pazdro@iopan.pl

Sopot, 29.03.2024

Oświadczenie

Oświadczam, że w poniższej publikacji:

Maculewicz, J., Dołżonek, J., Sharma, L., Białk-Bielińska, A., Stepnowski, P., & **Pazdro, K.** (2023). Bioconcentration of imidazolium ionic liquids: *In vivo* evaluation in marine mussels *Mytilus trossulus*. Science of the Total Environment, 858, 159388.

mój wkład polegał na współudziale w zaplanowaniu i koordynowaniu prac badawczych związanych z przeprowadzeniem eksperymentu na małżach M. trossulus, opiece merytorycznej i współudziale w zredagowaniu manuskryptu

Lilianna Sharma

ul. Parkowa 20/7

81-726, Sopot

20.03.2024, Gdańsk

Oświadczenie

Oświadczam, że w poniższej publikacji:

Maculewicz, J., Dołżonek, J., **Sharma, L.**, Białk-Bielińska, A., Stepnowski, P., & Pazdro, K. (2023). Bioconcentration of imidazolium ionic liquids: *In vivo* evaluation in marine mussels *Mytilus trossulus*. Science of the Total Environment, 858, 159388.

mój udział polegał na zbiorze i hodowli małży, przeprowadzeniu testów laboratoryjnych związanych z ekspozycją M. trossulus na ciecze jonowe, przygotowaniu materiału w postaci próbek wody i tkanek do badań analitycznych oraz napisaniu części metodycznej dotyczącej wykonanych przeze mnie prac.

Lilianna Sharma

Michał Toński

ul. Myśliwska 22a/37

80-162 Gdańsk

24.03.2024, Gdańsk

Oświadczenie

Oświadczam, że w poniższej publikacji:

Maculewicz, J., Kowalska, D., Świacka, K., **Toński, M.**, Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential. Science of the Total Environment, 802, 149916.

mój udział polegał na przeglądzie oraz analizie danych literaturowych dotyczących losu środowiskowego produktów transformacji farmaceutyków oraz napisaniu części manuskryptu dotyczącej tych aspektów.

10. Dorobek naukowy

Publikacje

1. Maculewicz J., Białk-Bielińska A., Kowalska D., Stepnowski P., Stolte S., Beil S., Dołżonek J. (2024). Bioconcentration potential of ionic liquids: new data on membrane partitioning and its comparison with predictions obtained by COSMOmic. Biochimica et Biophysica Acta (BBA)-Biomembranes (w druku)

2. Świacka, K., Maculewicz, J., Kowalska, D., & Grace, M. R. (2023). Do pharmaceuticals affect microbial communities in aquatic environments? A review. Frontiers in Environmental Science, 10, 1093920.

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Referaty na konferencjach naukowych

1. Maculewicz Jakub, Kowalska Dorota, Świacka Klaudia, Stepnowski Piotr, Dołżonek Joanna: Bioconcentration assessment of ionic and ionogenic compounds, XVI International Interdisciplinary Conference "Current Environmental Issues-2021" 2021

2. Maculewicz Jakub, Kowalska Dorota, Świacka Klaudia, Stepnowski Piotr, Dołżonek Joanna: Ocena biokoncentracji *in vitro*: powinowactwo wybranych związków do lipidów tworzących błony komórkowe, VI Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska 2021

3. Świacka Klaudia, Maculewicz Jakub, Smolarz Katarzyna, Caban Magda: Uptake, bioconcentration and mass balance of diclofenac and 4-OH diclofenac for the evaluation of their environmental fate, XVI International Interdisciplinary Conference "Current Environmental Issues-2021" 2021,

4. Kowalska Dorota, Maculewicz Jakub, Stepnowski Piotr, Dołżonek Joanna: Blood proteins as an important reservoir of charged compounds in bioconcentration phenomena, XVI International Interdisciplinary Conference "Current Environmental Issues-2021" 2021,

5. Maculewicz Jakub, Kowalska Dorota, Stepnowski Piotr, Dołżonek Joanna: Bioconcentration assessment *in vitro*: membrane partitioning of ionic liquids cations; Current Environmental Issues 2019 (CEI 2019) 24-26.09.2019, Białystok

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7. Świacka Klaudia, Caban Magda, Maculewicz Jakub, Szaniawska Anna, Smolarz Katarzyna: Bioconcentration and metabolism of diclofenac by *Mytilus trossulus*; Current Environmental Issues 2019 (CEI 2019) 24-26.09.2019, Białystok

Projekty naukowe

- NCN/2016/23/6/ST5/04245; Tytuł: Ocena biokoncentracji organicznych związków jonowych i jonogennych metodami in vitro, in vivo i in silico; 01.10.2018- 01.10.2021; Stanowisko: wykonawca

- 539-T010-B894-21; Tytuł: Nanorurki węglowe jako nośniki leków przeciwnowotworowych; 25.03.2021 - 31.12.2021; Stanowisko: kierownik projektu

- 539-T010-B473-20; Tytuł: Ocena potencjału do biokoncentracji wybranych produktów transformacji farmaceutyków; 25.03.2020 - 31.12.2020; Stanowisko: kierownik projektu

Nagrody naukowe

- Nagroda Rektora Uniwersytetu Gdańskiego pierwszego stopnia za rok akademicki 2020/2021

- Stypendium Ministra Nauki i Szkolnictwa Wyższego za wybitne osiągnięcia naukowe za lata akademickie 2016/2017 oraz 2017/2018.

Staże naukowe

- Staż w Husavik Research Centre, Islandia (1-30.09.2018). Program stażu obejmował identyfikację, obserwację i rejestrację zachowań ssaków morskich (głównie humbaków), a także tworzenie dokumentacji fotograficznej w celu identyfikacji osobników obserwowanych gatunków.

- Staż na Uniwersytecie Technicznym w Dreźnie, Niemcy (01.05-01.06.2019). Program stażu obejmował zastosowanie LC-MS/MS do analizy cieczy jonowych, a także wykonywanie testów elektrofizjologicznych z użyciem sztucznych błon lipidowych.

- Staż na Uniwersytecie Queensland w Brisbane, Australia (01.03-01.09.2023). Program stażu obejmował analizę stężenia związków przeciwdrobnoustrojowych w próbkach osadów ściekowych z Australii.