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UPTAKE, ACCUMULATION AND ELIMINATION OF ENDOCRINE DISRUPTING PHENOLIC COMPOUNDS IN SELECTED WATERBIRDS FROM THE SOUTHERN BALTIC

WNIKANIE, KUMULACJA I ELIMINACJA ENDOKRYNNIE AKTYWNYCH POCHODNYCH FENOLU U WYBRANYCH PTAKÓW WODNYCH Z REJONU POŁUDNIOWEGO BAŁTYKU

Doctoral dissertation

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UZASADNIENIE PODJĘCIA BADAŃ

2,2-bis (p-hydroksyfenylo)propan – bisfenol A (BPA) oraz alkilofenole: 4-*tert*-oktylofenol (4-*t*-OP) i 4-nonylofenol (4-NP) to pochodne fenolu, które należą do grupy związków endokrynnie aktywnych. Działanie tych ksenoestrogenów opiera się m.in. o zbliżoną budowę do endogennych hormonów, dzięki czemu łączą się one z receptorami, modulując, wywołując lub blokując odpowiedzi układu hormonalnego (Sonnenschein i Soto, 1998). Pochodne fenolu są niebezpieczne dla zachowania zdrowia i przeżycia organizmów nawet w niewielkich środowiskowo istotnych stężeniach. Ze względu na trudności wynikające z prowadzenia badań na żywych ptakach, najwięcej doniesień o negatywnym wpływie pochodnych fenolu na zwierzęta dotyczy ryb. Związki te wykazują działanie genotoksyczne, muta- oraz teratogenne, powodując m.in.: stres oksydacyjny, modulację ekspresji genów, uszkodzenia DNA, zaburzenia metaboliczne, zmiany zwyrodnieniowe narządów wewnętrznych, liczne wady rozwojowe (np. deformacje kręgosłupa, czaszki, obrzęk serca), zmiany w zachowaniu godowym, zaburzenia płodności, rozrodczości, przeżywalności zarodków i ich prawidłowego rozwoju (Chaube i in., 2012; Traversi i in., 2014; Won i in., 2014; Sharma i Chadha, 2016; Faheem i Lone, 2017; Li i in., 2017; Lee i in., 2018; Shirdel i in., 2020; Tran i in., 2020). Szczególnie niepokojące informacje dotyczą transgeneracyjnego wpływu pochodnych fenolu na sukces reprodukcyjny i rozwój zarodkowo-larwalny ryb obserwowany u potomstwa dwa, a nawet trzy pokolenia później (Gray i in., 1999; Bhandari i in., 2015). Pochodne fenolu stanowią więc zagrożenie nie tylko dla pojedynczego osobnika, ale również mogą mieć wpływ na liczebność i kondycje całych populacji. Choć niewiele wiadomo o wpływie pochodnych fenolu na ptaki, w nielicznych badaniach wskazano na możliwość podobnych zaburzeń do tych obserwowanych u ryb (Oshima i in., 2012; Cheng i in., 2017; Mentor i in., 2020).

Bisfenol A (BPA) to monomer służący głównie do syntezy żywic poliwęglanowych i epoksydowych – tworzyw syntetycznych o ważnym znaczeniu gospodarczym (US EPA, 2010). Z tego względu BPA jest obecnie jednym z najpopularniejszych związków na świecie, należących do chemikaliów o dużej wielkości produkcji (HPV, ang. high production volume chemicals) (OECD, 2004). Z kolei alkilofenole produkowane są w żywicach i oksymach fenolowych. Pochodne fenolu jako wszechobecny składnik tworzyw sztucznych znajdują się w opakowaniach i butelkach, puszkach, płytach CD, oponach, paragonach, sprzęcie sportowym, medycznym, urządzeniach elektronicznych, zabawkach, soczewkach czy materiałach dentystycznych (US EPA, 2010; Flint i in., 2012; DEPA, 2013; Rochester, 2013). BPA stosowany jest również w środkach zmniejszających palność (US EPA, 2010). Istotnym zastosowaniem alkilofenoli i ich etoksylatów są ponadto niejonowe środki powierzchniowo czynne obecne w różnego rodzaju detergentach, stabilizatorach, emulgatorach oraz środkach pianotwórczych (Ying i in., 2002; Acir i Guenther, 2018). Tak szerokie zastosowanie pochodnych fenolu pozostaje nie bez znaczenia dla ich obecności w środowisku. Związki te emitowane są głównie do mórz i oceanów wraz z wodami z przemysłowych i komunalnych oczyszczalni ścieków (Corrales i in., 2015; Acir i Guenther, 2018). BPA i alkilofenole obecne są ponadto w atmosferze nad terenami przemysłowymi i rolniczymi, jak również oddalonymi czystymi rejonami mórz (Van Ry i in., 2000; Xie i in., 2006; Graziani i in., 2019; Vasiljevic i Harner, 2021). W środowisku morskim, związki te adsorbują się na cząstkach stałych w osadach (Koniecko i in., 2014; Staniszewska i in., 2016b) i ulegają biokumulacji w organizmach ze wszystkich poziomów troficznych (Diehl i in., 2012; Staniszewska i in., 2014; Korsman i in., 2015; Nehring i in., 2018). Dodatkowo, w morzach bezpośrednim zagrożeniem dla zwierząt są również tworzywa sztuczne, które ulegając degradacji rozpadają się na mniejsze odpadki. Te mikrocząstki mogą być połykane przez ptaki, prowadząc do kumulacji pochodnych fenolu w ich organizmach (Tanaka i in., 2015; Wang i in., 2021).

W związku z doniesieniami o negatywnym wpływie pochodnych fenolu na organizmy, w 2006 r. BPA i 4-NP zostały włączone na listę substancji niedozwolonych do stosowania w kosmetykach (*Dz. U. z 2006 r. Nr 85, poz. 593*). Zakazano również wprowadzania do obrotu i stosowania niektórych produktów zawierających 4-NP oraz zawierających ten związek preparatów w stężeniach równych lub większych niż 0,1 % masowego (*Dz. U. z 2006 r. Nr 127, poz. 887 z późn. zm.*). Z kolei na terenie Unii Europejskiej zakazano stosowania BPA w butelkach do karmienia dzieci do lat 3 (EU, 2011). Ponadto, 4-*t*-OP i 4-NP włączone zostały na listę priorytetowych substancji lub grup substancji niebezpiecznych w dziedzinie polityki wodnej Wspólnoty (EC, 2001) oraz na listę priorytetowych substancji zanieczyszczających środowisko wodne (*Dz. U. z 2019 r. poz. 528*). Powyższe regulacje zminimalizowały narażenie ludzi na niektóre pochodne fenolu, jednakże nie wyeliminowały ich dopływu do środowiska morskiego. Morze Bałtyckie, ze względu na swoje położenie oraz charakter, znajduje się pod silną antropopresją, przez co narażone jest na zwiększone zanieczyszczenie chemiczne oraz koncentrację morskich odpadów. Akwen ten jest niewielki i płytki, a jego obszar jest prawie czterokrotnie mniejszy od obszaru zlewiska, który zamieszkuje ponad 85 mln ludzi. Wymiana wody z Morzem

Północnym przez wąskie i płytkie cieśniny wynosi ok. 30 lat (HELCOM, 2018). Jednym z priorytetowych działań ujętych w najnowszym strategicznym programie środków i działań HELCOM na rzecz osiągnięcia dobrego stanu środowiska morskiego, jest wprowadzenie do 2027 r. środków umożliwiających ograniczenie stosowania i zapobieganie uwalnianiu do środowiska Bałtyku, pochodnych fenolu o działaniu endokrynnie aktywnym (BSAP, 2021).

Morze Bałtyckie jest również ważnym miejscem odpoczynku, żerowania, pierzenia, lęgów i zimowania dla około 80 gatunków ptaków. Wg najnowszych danych, populacja ptaków wodnych w sezonie lęgowym i w okresie zimowania w rejonie Bałtyku w latach 2011 – 2016 zmalała odpowiednio o około 30 % i 20 % (HELCOM, 2018). Jednakże wpływ wzrostu uprzemysłowienia na ptaki morskie widoczny jest na całym świecie. Wg szacunków doprowadził on w ostatnich 60 latach do 70 % spadku światowej populacji ptaków morskich (Paleczny i in., 2015). Ptaki od dawna są dobrze znanymi bioindykatorami zanieczyszczenia środowiska, ponieważ są szczególnie wrażliwe na zmiany środowiskowe. Niemniej dotychczas skupiano się głównie na badaniach metali śladowych i trwałych zanieczyszczeń organicznych, podczas gdy niewiele uwagi poświęcono pochodnym fenolu. Tym samym, praca ta stanowi pierwszą, która szeroko dokumentuje obecność BPA, 4-*t*-OP i 4-NP w miejscach, w których zachodzi ich wnikanie, biokumulacja oraz potencjalna toksyczność, a także wybrane drogi, którymi związki te mogą być usuwane. Aby jak najlepiej odzwierciedlić los BPA i alkilofenoli w organizmach ptaków (alki *Alca torda*, lodówki *Clangula hyemalis* i nurogęsi *Mergus merganser*) postanowiono zbadać na obecność tych związków krew, tkanki (jelita, płuca, mięśnie, nerki, wątroby, mózgi, tłuszcz podskórny, gonady), a także wytwory naskórka (pazury i lotki). Analizie poddano wpływ potencjalnych czynników na wielkość stężeń pochodnych fenolu w poszczególnych tkankach, w tym cech gatunkowych (poziom troficzny, środowisko) oraz osobniczych (kondycja, wiek, płeć). Jako parametr dodatkowy do pracy włączono analizę stabilnych izotopów $\delta^{15}N$ i $\delta^{13}C$, które pozwoliły ustalić poziom troficzny wybranych gatunków ptaków i pochodzenie ich pokarmu.

W **pierwszej publikacji** skupiono się na dwóch najważniejszych drogach wnikania zanieczyszczeń do organizmów ptaków, w tym uznawanej za główną – pokarmową oraz często pomijaną – oddechową. Ptaki będąc długowiecznymi drapieżnikami, znajdującymi się u szczytu łańcucha troficznego, narażone są szczególnie na zwiększoną biokumulację zanieczyszczeń w ich organizmach (Burger i Gochfeld, 2004). Ponadto, ich jelita i płuca mogą stanowić wysoki potencjał we wskazaniu zanieczyszczenia konkretnych elementów środowiska, w tym spożywanego przez ptaki pokarmu oraz otaczającego powietrza. Należy jednak zaznaczyć, że płuca i przewód pokarmowy, podobnie jak skóra są głównymi barierami oddzielającymi organizmy wyższe od środowiska o wysokich stężeniach ksenobiotyków (Lehman-McKeeman, 2008). Pochodne fenolu jako związki endokrynnie aktywne muszą przekroczyć przynajmniej jedną z tych barier, aby móc połączyć się z receptorem i wywołać odpowiedź organizmu. Dlatego też krew okazała się w pierwszej pracy istotnym narzędziem do oceny biodostępności pochodnych fenolu.

Niezależnie od wielkości stężeń pochodnych fenolu w jelitach lub płucach, transfer ten niekoniecznie musi odzwierciedlać rzeczywistą ilość danego ksenobiotyku na jaki narażony jest ptak. Ponadto, krew dostarcza informacji o narażeniu chwilowym i jest nośnikiem ksenoestrogenów do miejsc ich oddziaływania (Espín i in., 2016). W związku z tym, aby ocenić narażenie ptaków na pochodne fenolu, w **drugiej i czwartej publikacji** skupiono się na poznaniu dystrybucji narządowej BPA i alkilofenoli z uwzględnieniem ich dróg wnikania. Mózg i gonady są tkankami szczególnie wrażliwymi na działanie endokrynnie aktywnych pochodnych fenolu (Cheng i in., 2017; Li i in., 2017; Mentor i in., 2020; Tran i in., 2020). Mięśnie i tłuszcz podskórny stanowią magazyn dla ksenobiotyków, ale mogą być również źródłem wtórnego narażenia (Lehman-McKeeman, 2008). Szczególnie w okresach silnego stresu np. podczas migracji i lęgów ptaków, zmagazynowany tłuszcz w organizmie jest metabolizowany, a wraz z nim mobilizowane są i transportowane do krwiobiegu zanieczyszczenia (Henriksen i in., 1996; Perkins i Barclay, 1997). Z kolei wątroby i nerki są narządami, dzięki którym ksenobiotyki mogą być metabolizowane i usuwane (Lehman-McKeeman, 2008). Poza tym pochodne fenole mogą wpływać również na prawidłowe funkcjonowanie tych ważnych narządów (Traversi i in., 2014; Faheem i Lone, 2017; Shirdel i in., 2020).

Ptaki będąc drapieżnikami u szczytu łańcucha troficznego, narażone są na zwiększone dawki ksenoestrogenów na skutek ich biomagnifikacji (Burger i Gochfeld, 2004). Tym samym istotnym elementem **trzeciej publikacji** było ustalenie czy zwierzęta te mają również możliwość przynajmniej częściowej eliminacji pochodnych fenolu poza organizm. Zarówno pióra jak i pazury stanowią ważne drogi usuwania ksenobiotyków, w tym również trwałych zanieczyszczeń organicznych, preferujących gromadzenie się w tkankach tłuszczowych. Ważną częścią pracy było ponadto porównanie stężeń pochodnych fenolu w lotkach pochodzących od gatunków ptaków, które wymieniają je w dwóch skrajnie różnych rejonach o odmiennych uwarunkowaniach środowiskowych oraz odległości od potencjalnych źródeł zanieczyszczeń. Ptasie pióra z powodzeniem wykorzystywane są jako wskaźniki jakości środowiska oraz nieinwazyjne narzędzie oceny obciążenia organizmów ptaków zanieczyszczeniami (Jaspers i in., 2006; Kim i Koo, 2008; Meyer i in., 2009).

CELE PRACY

Ptaki morskie doświadczają dużego stresu co odzwierciedla drastyczny spadek ich światowej populacji, a kumulacja i oddziaływanie zanieczyszczeń na ich organizmy jest jednym z bodźców zewnętrznych odpowiadających za ten spadek (Croxall i in., 2012; Paleczny i in., 2015). Badania dziko żyjących ptaków wodnych rzadko są możliwe do zrealizowania, co wynika z trudności związanych z pozyskaniem materiału. Niewiele wiadomo o ekspozycji ptaków wodnych na pochodne fenolu, a tym bardziej o dalszym losie tych ksenoestrogenów w ich organizmach oraz skutkach tego narażenia. Określone w pracy główne miejsca kumulacji pochodnych fenolu mogą wskazać kierunek badań nad potencjalnym wpływem tych związków na ptaki. Natomiast wiedza o wielkości stężeń BPA, 4-*t*-OP i 4-NP w poszczególnych tkankach stanowić może podstawę do zbadania możliwych negatywnych skutków wywoływanych przy środowiskowych stężeniach badanych związków. Przedłożona praca miała za zadanie dostarczyć również nowych informacji o potencjale jaki niosą za sobą ptaki w badaniach biomonitoringu środowiska.

Przystępując do pracy postawiono następujące hipotezy badawcze:

1. Alki *Alca torda*, lodówki *Clangula hyemalis* i nurogęsi *Mergus merganser* bytujące w rejonie południowego Bałtyku charakteryzują się zróżnicowanym narażeniem na BPA i alkilofenole.
2. Inhalacja stanowi istotną drogę narażenia alki *Alca torda*, lodówki *Clangula hyemalis* i nurogęsi *Mergus merganser* na BPA, 4-*t*-OP i 4-NP.
3. Narządy strategiczne dla prawidłowego rozwoju i funkcjonowania ptaków tj. gonady, mózgi, nerki i wątroby, stanowią główne miejsca kumulacji endokrynnie aktywnych pochodnych fenolu.
4. Pióra i pazury alki *Alca torda* oraz lodówki *Clangula hyemalis* stanowią istotną drogę eliminacji pochodnych fenolu z ich organizmów.
5. Jelita, płuca i pióra alki *Alca torda*, lodówki *Clangula hyemalis* i nurogęsi *Mergus merganser* są dobrymi wskaźnikami zanieczyszczenia środowiska pochodnymi fenolu.

Hipotezy zweryfikowano poprzez realizację następujących głównych celów badawczych:

1. Określenie głównych dróg wnikania pochodnych fenolu do organizmów ptaków oraz czynników determinujących ich ekspozycję w rejonie południowego Bałtyku (publikacja 1).
2. Rozpoznanie dystrybucji narządowej pochodnych fenolu w organizmach ptaków wodnych (publikacja 2 i 4).
3. Rozpoznanie potencjału piór i pazurów do eliminacji pochodnych fenolu z organizmów ptaków (publikacja 3).
4. Określenie potencjału wybranych tkanek i wytworów naskórka ptaków jako wskaźników zanieczyszczenia środowiska oraz obciążenia organizmów ptaków przez pochodne fenolu (publikacja 1 i 3).

ZEBRANY MATERIAŁ I ANALIZY CHEMICZNE

Badania prowadzono na martwych ptakach pozyskanych z przyłowu, wyłowionych w latach 2015 – 2016 z rejonu południowego Bałtyku. Wśród nich znajdowały się: lodówki (*Clangula hyemalis*), alki zwyczajne (*Alca torda*) oraz nurogęsi (*Mergus merganser*). Lodówki wyłowiono z dwóch akwenów – Zatoki Gdańskiej i Zatoki Pomorskiej. Alki pochodziły wyłącznie z Zatoki Gdańskiej, a nurogęsi z Zalewu Szczecińskiego. Dla alki Bałtyk jest stałym miejscem bytowania, podczas gdy nurogęsi i lodówki przylatują w rejony południowego Bałtyku jedynie w okresie pozalęgowym. Podstawę diety lodówek w okresie pozalęgowym stanowi głównie zoobentos. Natomiast alki i nurogęsi żywią się wyłącznie rybami (Cramp and Simmons, 1977; Cramp, 1985; Stempniewicz, 1995).

W czasie sekcji ptaków pobrano jelita, płuca, mięśnie piersiowe, nerki, wątroby, tłuszcz podskórny, mózg, gonady, pióra, pazury oraz krew z serca. Jelita opróżniono z zawartości pokarmowej i przepłukano wodą mili-Q. Pobrane próbki do czasu analizy zostały zamrożone (-20^{0}C). Przed analizą jelita, płuca, mięśnie piersiowe, nerki, wątroby, gonady, pióra i pazury liofilizowano, a następnie homogenizowano. Dodatkowo, przed analizą oba wytwory naskórka myto w acetonie przy użyciu ultradźwięków przez 10 min. w 20^{0}C. Tak przygotowane tkanki i wytwory naskórka przechowywano w szkle borokrzemowym w eksykatorze w stałych warunkach (temp. 20^{0}C ± 2^{0}C, wilgotność 45% ± 5%). Mózg oraz tłuszcz homogenizowano bezpośrednio przed analizą. W obu tych tkankach określono dodatkowo wilgotność w celu przeliczenia wyników w mokrej masie na suchą. Podczas sekcji ptaków, oznaczono ich wiek na podstawie cech upierzenia (Baker, 2016). Płeć określano po wyglądzie gonad. Każdy osobnik został również zważony, a stan ciała oceniano na podstawie zawartości tłuszczu jelitowego i tłuszczu podskórnego zgodnie z przyjętą skalą (Camphuysen i in., 2007). Szczegółowy opis materiału zestawiono w Tabeli 1.

Stężenia BPA, 4-*t*-OP i 4-NP w jelitach, płucach, mięśniach, nerkach, wątrobach, gonadach, piórach i pazurach oznaczano wg metody opisanej przez Xiao i in. (2006) oraz zmodyfikowanej przez Staniszewską i in. (2014; 2018). Odważony materiał biologiczny poddawano ekstrakcji w łaźni ultradźwiękowej w mieszaninie metanolu, 0,01M octanu amonu i 4M kwasu chlorowego (VII). Otrzymane ekstrakty oczyszczano na szklanych kolumienkach Oasis HLB (200 mg, 5 cm^3). Oznaczenia stężeń pochodnych fenolu we krwi przeprowadzono wykorzystując metodę opisaną przez Xiao i in. (2006). Próbki poddawano ekstrakcji w łaźni ultradźwiękowej mieszaniną n-heksanu i eteru dietylowego (70:30) z octanem amonu (0,01 M). Z kolei tłuszcz i mózg poddawano dwukrotnej ekstrakcji w acetonitrylu. Ekstrakty następnie łączono, odwirowywano i oczyszczano poprzez wytrząsanie z zastosowaniem heksanu (Geens i in., 2012). Wszystkie otrzymane ekstrakty odparowywano do sucha i uzupełniano acetonitrylem do 0,2 cm^3.

Tabela 1 Zestawienie gatunków ptaków, rodzaju oraz liczby próbek materiału biologicznego wykorzystanego do analiz w poszczególnych publikacjach

publikacja	gatunek	krew	jelita	płuca	mięśnie	nerki	wątroby	tłuszcz	mózgi	gonady	pióra	pazury
publikacja 1	*Clangula hyemalis*	30	29	30								
	Alca torda	15	15	15								
	Mergus merganser	8	8	8								
publikacja 2	*Clangula hyemalis*				29	30	30	28	28			
	Alca torda				15	15	15	15	14			
	Mergus merganser				7	8	8	8	8			
publikacja 3	*Clangula hyemalis*										29	29
	Alca torda										15	14
publikacja 4	*Clangula hyemalis*									47		

Oznaczenia końcowe stężeń bisfenolu A, 4-*tert*-oktylofenolu i 4-nonylofenolu wykonano z użyciem wysokosprawnej chromatografii cieczowej z detektorem fluorescencyjnym i kolumną chromatograficzną Thermo Scientific HYPERSIL GOLD C18 PAH (250×4,6 mm; 5 µm). Długość generowanej fali wzbudzającej wynosiła $\lambda = 275$ nm, natomiast emisję mierzono przy długości fali $\lambda = 300$ nm. Proces rozdzielenia chromatograficznego przeprowadzono w warunkach gradientowych stosując fazę ruchomą (woda:acetonitryl). Odzysk został wyznaczony w próbkach z dodatkiem znanej ilości analitu, na podstawie pięciokrotnego pomiaru stężeń BPA, 4-*t*-OP i 4-NP. Precyzja metody została wyrażona jako współczynnik zmienności. Granicę oznaczalności metody wyznaczono dla próbki z niewielką zawartością analitu jako dziesięciokrotny stosunek sygnału do szumu. Odzysk dla wszystkich próbek wynosił zawsze > 80%, a precyzja metody < 15%. Granica oznaczalności wahała się w zależności od związku i materiału biologicznego od 0,07 do 2,0 ($ng \cdot cm^{-3}$ we krwi oraz $ng \cdot g^{-1}$ s.m. w pozostałych próbkach).

Analiza stabilnych izotopów została zlecona i wykonana w laboratorium Wydziału Chemii Politechniki Łódzkiej. Analizę wykonano w uprzednio zliofilizowanych i zhomogenizowanych mięśniach ptaków, ponieważ odzwierciedlają one ostatnie 3-4 tygodnie diety ptaków (Hobson and Clark, 1992). Oznaczenia izotopów $\delta^{15}N$ i $\delta^{13}C$ przeprowadzono za pomocą Isotope Ratio Mass Spectrometer Sercon 20-22. Jako katalizatora spalania użyto V_2O_5, a lokalny standard stanowił kwas tiobarbiturowy (azot atmosferyczny i PDB odpowiednio dla $\delta^{15}N$ i $\delta^{13}C$).

WNIOSKI

Alka, lodówka i nurogęś bytujące w rejonie południowego Bałtyku narażone są na wnikanie pochodnych fenolu zarówno drogą pokarmową jak i oddechową. Najwyższe stężenia w jelitach i płucach przypadające na BPA i 4-NP są skutkiem ich wysokiej produkcji i późniejszego uwalniania do środowiska, powodującego szerokie rozpowszechnienie tych związków w jego elementach. Dominującą drogą narażenia na BPA, u wszystkich badanych gatunków ptaków była ekspozycja pokarmowa. Natomiast szlaki wnikania alkilofenoli do organizmów ptaków były bardziej zróżnicowane i uwarunkowane rejonem bytowania oraz nawykami żywieniowymi. 4-NP wnikał do organizmów ptaków rybożernych głównie drogą pokarmową, podczas gdy u bentofagów dominowała droga oddechowa. Ponadto, wraz ze wzrostem pozycji troficznej ptaków, wzrastało również ich narażenie pokarmowe na 4-NP. Z kolei 4-*t*-OP u ptaków bytujących w rejonie Zatoki Gdańskiej wnikał głównie poprzez inhalację, podczas gdy dla ptaków przebywających w rejonie Zatoki Pomorskiej przeważała droga pokarmowa (**publikacja 1**). Tym samym, zweryfikowano **1 i 2 hipotezę,** wykazując zróżnicowane narażenie alki *Alca torda*, lodówki *Clangula hyemalis* i nurogęsi *Mergus merganser* na pochodne fenolu oraz ukazując inhalację jako istotną drogę ekspozycji ptaków na te związki.

Wykazano również, że pochodne fenolu przenikają do krwi, a więc pokonują bariery biologiczne i mogą być dystrybuowane po całym organizmie, w tym również do miejsc docelowych dla ich endokrynnie aktywnego działania (**publikacja 1, 2**). W pracy ukazano szeroką dystrybucję wszystkich trzech związków do wątrób, nerek, mięśni, tłuszczu podskórnego, mózgu i gonad, co sugeruje, że pochodne fenolu podlegają w organizmach ptaków kumulacji oraz szeregowi procesów transformacji i eliminacji. Jednak szlaki dystrybucji pochodnych fenolu różniły się i były uwarunkowane najprawdopodobniej właściwościami poszczególnych ksenobiotyków, a szczególnie ich lipofilowością i potencjałem do wiązania z białkami oraz rozpuszczania w tłuszczach. BPA i 4-NP ulegały największej dystrybucji do mięśni, wątrób oraz nerek u wszystkich badanych gatunków ptaków, a 4-NP również do gonad. Natomiast 4-*t*-OP transportowany był głównie do mózgu, tłuszczu podskórnego oraz wątroby **(publikacja 2, 4)**. Uzyskane wyniki potwierdzają postawioną **3 hipotezę**, iż narządy strategiczne dla prawidłowego rozwoju i funkcjonowania ptaków tj. gonady, mózgi, nerki i wątroby, stanowią główne miejsca kumulacji endokrynnie aktywnych pochodnych fenolu.

Różne miejsca docelowej kumulacji poszczególnych pochodnych fenolu wskazują, że każdy z badanych związków może nieść za sobą odmienne skutki zdrowotne u ptaków **(publikacja 2, 4)**. W związku z największą biokumulacją BPA i 4-NP w wątrobach i nerkach, w przyszłych badaniach należy przyjrzeć się ich potencjalnemu wpływowi na upośledzenie funkcji tych ważnych narządów odpowiadających za biotransformację i eliminację zanieczyszczeń. Co więcej, wątroba była jedynym narządem, w którym wszystkie trzy związki wykazywały wysokie stężenia oraz pozytywne korelacje między sobą. Ukazuje to ważną funkcję selektywnej sekwestracji pochodnych fenolu w wątrobie, będącej pierwszym narządem, do którego transportowane są ksenobiotyki wnikające do organizmu drogą pokarmową. W pracy sugerowano, że proces ten może być determinowany dobrą kondycją ptaków, sprzyjając transferowi pochodnych fenolu z jelita do wątroby wraz ze wzrostem zawartości tłuszczu jelitowego (**publikacja 2**).

Na szczególną uwagę zasługuje odznaczająca się na tle pozostałych związków zdolność 4-*t*-OP do przenikania bariery krew-mózg, gdyż nagromadzenie tego ksenobiotyku w mózgu może prowadzić do zmian w zachowaniu np. godowym. Sprzyjać temu może również krążąca we krwi wolna frakcja 4-*t*-OP, która dostępna jest do transportowania do miejsc oddziaływania. Wykazano jednak, że mózg ptaków może być chroniony przed nagromadzeniem lipofilowych ksenoestrogenów, poprzez ich odkładanie w tłuszczu podskórnym, które zmniejsza jednocześnie transfer do mózgu (**publikacja 1, 2**).

Podkreślenia wymaga również szczególnie wysokie powinowactwo 4-NP względem gonad, wskazujące na możliwy potencjał tego związku do zaburzenia prawidłowego funkcjonowania tego ważnego gruczołu rozrodczego. Szczególnie, iż na przykładzie gonad lodówki, na podstawie otrzymanych wyników oraz analizy literatury wykazano, że stężenia pochodnych fenolu w gruczole rozrodczym ptaków były na podobnym poziomie, przy którym w dotychczas przeprowadzonych badaniach obserwowano negatywne skutki spowodowane ich endokrynnie aktywnym działaniem. Pokazuje to, że badane ksenoestrogeny mogą zaburzyć rozmnażanie i rozwój ptaków. Na przykładzie gonad lodówki, ujawniono również wpływ wieku i płci na wielkość stężeń pochodnych fenolu w gruczole rozrodczym. Wykazano, że dorosłe lodówki charakteryzują się wyższymi stężeniami pochodnych fenolu w stosunku do osobników młodych, co może wynikać z wieloletniej biokumulacji, jak również zróżnicowanego zanieczyszczenia rejonów ich bytowania. Z kolei wśród dorosłych lodówek, pochodne fenolu charakteryzowały się wyższymi stężeniami w samcach w stosunku do samic, co prawdopodobnie związane jest z posiadaniem przez samice dodatkowej drogi eliminacji zanieczyszczeń z organizmów poprzez ich transfer z matki do jaja. Tym samym, nie można wykluczyć potencjalnego wpływu pochodnych fenolu na rozwój embrionów we wrażliwym okresie wzrostu, kiedy stężenia ksenobiotyków, tak samo jak inne substancje odżywcze, mogą być przekazywane z matki do jaja (**publikacja 4**).

Dla większości tkanek wewnętrznych, poziom troficzny ptaków i ich nawyki żywieniowe nie decydowały o wielkości stężeń pochodnych fenolu. Jednak ptaki z najwyższego poziomu troficznego charakteryzowały się wyższymi stężeniami pochodnych fenolu wyłącznie w nerkach. Wskazuje to na efektywną eliminację pochodnych fenolu, zapobiegającą zwiększonej biokumulacji spowodowanej biomagnifikacją ksenobiotyków w łańcuchu troficznym. Ponadto, na przykładzie dwóch gatunków ptaków z tego samego poziomu troficznego, ale o zróżnicowanych preferencjach żywieniowych pokazano, że biomagnifikacja pochodnych fenolu może być wyższa u bentofagów w porównaniu do gatunków żerujących na rybach pelagicznych. Spośród badanych pochodnych fenolu, 4-NP odznaczał się największym potencjałem do biomagnifikacji w badanych gatunkach ptaków. Należy jednak podkreślić, że biomagnifikacja pochodnych fenolu w tkankach ptaków może być niedoszacowana i okazać się wyższa niż przedstawione w niniejszej pracy wartości. Szczególnie w przypadku 4-*t*-OP, który ulegał największej kumulacji w mózgach, a nie w mięśniach, dla których zostały obliczone współczynniki biomagnifikacji (**publikacja 2**).

Wbudowywanie pochodnych fenolu w wytwory naskórka tj. pióra oraz pazury, umożliwia ptakom ich eliminację ze swoich organizmów. Eliminacja pochodnych fenolu w zależności od związku i wytworu naskórka wynosi od 12 do 34 %. Dla większości związków i ptaków największy udział w eliminacji przypadał na pazury. Biorąc pod uwagę tylko te dwie drogi usuwania pochodnych fenolu stwierdzono, że wielkość eliminacji jest niższa od kumulacji w zbadanych dotychczas tkankach wewnętrznych ptaków. Eliminacja ta wydaje się jednak efektywna na tyle, aby zapobiec możliwej bioakumulacji z wiekiem oraz biomagnifikacji u ptaków żerujących na organizmach z wyższych poziomów troficznych. Potwierdza to postawioną **4 hipotezę**, iż pióra i pazury alki *Alca torda* oraz lodówki *Clangula hyemalis* stanowią istotne drogi eliminacji pochodnych fenolu z ich organizmów. Szczególnie, iż pochodne fenolu usuwane są prawdopodobnie ze wszystkich lub przynajmniej większości tkanek wewnętrznych, przy czym mózg może być bardziej odporny na ich eliminację. Spośród pochodnych fenolu, największym potencjałem do eliminacji charakteryzował się 4-NP, zarówno z piórami, jak i pazurami, podczas gdy najsłabiej usuwany był 4-*t*-OP (**publikacja 3**).

Ujawniono różnice w stopniu zanieczyszczenia BPA i alkilofenolami wód oraz powietrza rejonu Zatoki Gdańskiej i Zatoki Pomorskiej, wskazując na potencjał jelit i płuc ptaków jako bioindykatorów zanieczyszczenia poszczególnych elementów środowiska pochodnymi fenolu (**publikacja 1**). Również lotki ptaków okazały się być obiecującym wskaźnikiem zanieczyszczenia środowiska 4-NP. Porównanie stężeń w lotkach lodówki i alki, które pierzą je w dwóch różnych środowiskach o odmiennym stopniu zanieczyszczenia i odległości od źródeł, pozwoliło ustalić, że Morze Bałtyckie jest ok. 3-krotne bardziej zanieczyszczone 4-NP od obszarów morskich rosyjskiej Arktyki (**publikacja 3**). Tym samym, potwierdzono częściowo **5 hipotezę** postawioną w niniejszej pracy, iż jelita, płuca i pióra alki *Alca torda*, lodówki *Clangula hyemalis* i nurogęsi *Mergus merganser* są dobrymi wskaźnikami zanieczyszczenia środowiska pochodnymi fenolu. Nie udało się jednak potwierdzić użyteczności pazurów w biomonitoringu środowiska ani żadnego z wytworów naskórka jako bezinwazyjnego narzędzia badania poziomów narażenia ptaków na BPA i alkilofenole (**publikacja 3**).

Słowa kluczowe: ptaki, bioindykatory, endokrynnie aktywne pochodne fenolu, narażenie, biokumulacja, eliminacja

ABSTRACT

RATIONALE FOR THE STUDY

2,2-bis(p-hydroxyphenyl)propane – bisphenol A (BPA) and alkylphenols: 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) are phenol derivatives that fall into the category of endocrine disrupting compounds. The activity of these xenoestrogens is based, among other factors, on the similarity of their structure to endogenous hormones, thanks to which they bind to receptors, modulating, inducing or blocking the responses of the endocrine system (Sonnenschein and Soto, 1998). Phenol derivatives are dangerous for the health and survival of organisms, even at low environmentally significant concentrations. Most of the reports that are available on the negative impact of phenol derivatives on animals focus on fish. These show that the mentioned compounds have genotoxic, muta- and teratogenic effects which may cause, among other things: oxidative stress, modulation of gene expression, DNA damage, metabolic disorders, degenerative changes of internal organs, numerous developmental defects (spinal or skull deformities, heart edema), changes in mating behavior, fertility, reproduction and the survival and normal development of embryos (Chaube et al., 2012; Traversi et al., 2014; Won et al., 2014; Sharma and Chadha, 2016; Faheem and Lone, 2017; Li et al., 2017; Lee et al., 2018; Shirdel et al., 2020; Tran et al., 2020). What is particularly disturbing is the transgenerational impact of phenol derivatives on the reproductive success and embryo-larval development of fish observed in the offspring two or even three generations after exposure (Gray et al., 1999; Bhandari et al., 2015). Phenol derivatives therefore pose a threat not only to individual members of a species, but also to the size and condition of entire populations. Although little is known about the effects of phenol derivatives on birds, the few available studies have indicated the possibility of similar disorders to those observed in fish (Oshima et al., 2012; Cheng et al., 2017; Mentor et al., 2020).

Bisphenol A (BPA) is a monomer used mainly for the synthesis of polycarbonate and epoxy resins – synthetic plastics of important economic importance (US EPA, 2010). For this reason, BPA is currently one of the most popular compounds in the world and is classed as a High Production Volume (HPV) chemical (OECD, 2004). In turn, alkylphenols are produced as part of phenolic resins and oximes. Phenol derivatives as a ubiquitous component of plastics are found in packaging and bottles, cans, CDs, tyres, receipts, sports and medical equipment, electronic devices, toys, lenses and dental materials (US EPA, 2010; Flint et al., 2012; DEPA, 2013; Rochester, 2013). BPA is also used in flame retardants

(US EPA, 2010). An important application of alkylphenols and their ethoxylates is also to be found in non-ionic surfactants present in various types of detergents, stabilizers, emulsifiers and foaming agents (Ying et al., 2002; Acir and Guenther, 2018). Such a wide application of phenol derivatives is not without significance for their presence in the environment. These compounds are mainly emitted into seas and oceans along with waters from industrial and municipal wastewater treatment plants (Corrales et al., 2015; Acir and Guenther, 2018). BPA and alkylphenols are also present in the atmosphere over industrial and agricultural areas, as well as over remote areas of clean seas (Van Ry et al., 2000; Xie et al., 2006; Graziani et al., 2019; Vasiljevic and Harner, 2021). In the marine environment, these compounds adsorb onto solid particles in sediments (Koniecko et al., 2014; Staniszewska et al., 2016a) and bioaccumulate in organisms from all trophic levels (Diehl et al., 2012; Staniszewska et al., 2014; Korsman et al., 2015; Nehring et al., 2018). Additionally, a direct threat to animals is posed by synthetic materials, which break down into smaller pieces in seawater. These microparticles can be ingested by birds, resulting in the accumulation of phenol derivatives in their bodies (Tanaka et al., 2015; Wang et al., 2021).

Owing to reports on the negative impact of phenol derivatives on organisms, in 2006 BPA and 4-NP were included on the list of substances prohibited for use in cosmetics (*Dz. U. z 2006 r. Nr 85, poz. 593*). It has also been prohibited to sell, supply and use certain products containing 4-NP and pharmaceutical preparations containing this compound in concentrations equal to or greater than 0.1% by weight (*Dz. U. z 2006 r. Nr 127, poz. 887*). Moreover, the use of BPA in feeding bottles for children under the age of 3 has been banned in the European Union (EU, 2011). The compounds 4-*t*-OP and 4-NP have been included on the priority list of substances or groups of hazardous substances within the framework of the Community water policy (EC, 2001) and on the priority list of substances polluting the aquatic environment (*Dz. U. z 2019 r. poz. 528*). The abovementioned legal regulations have minimized human exposure to some phenol derivatives but have not stopped their inflow to the marine environment. The Baltic Sea, due to its location and character, remains under intense anthropopressure, which is why it is also exposed to increased chemical pollution and high concentration of marine litter. The sea is relatively small and shallow, and its area is almost four times smaller than the area of its catchment, which is inhabited by over 85 million people. Moreover, the exchange of water with the North Sea through the narrow and shallow straits takes about 30 years (HELCOM, 2018). The introduction of measures to limit the use and prevent the release of endocrine disrupting phenol derivatives into the Baltic environment by 2027 is one of the priority undertakings included in the latest strategic program of measures and actions of HELCOM for achieving good marine environment status (BSAP, 2021).

The Baltic Sea is also an important site of resting, feeding, moulting, breeding and wintering for about 80 species of birds. According to the latest data, the population of aquatic birds in the Baltic region in 2011 – 2016 decreased by approximately 30 % and 20 %, respectively in the breeding and wintering seasons (HELCOM, 2018). However, the impact of increased industrialization on seabirds is to be observed worldwide. According to estimates, it has led to a 70 % decrease in the global population of seabirds in the last 60 years (Paleczny et al., 2015). Birds have long been well-known bioindicators of environmental pollution, as they are particularly sensitive to environmental changes. However, so far the focus of research has been put mainly on trace metals and persistent organic pollutants, while little attention has been paid to phenol derivatives. Thus, the present work is the first to extensively document the presence of BPA, 4-*t*-OP and 4-NP in sites of their penetration, bioaccumulation and potential toxicity, and to discuss selected routes by which these compounds can be removed. In order to get the best picture of what happens with BPA and alkylphenols in the organisms of birds (razorbills *Alca torda*, long-tailed ducks *Clangula hyemalis* and goosanders *Mergus merganser*), it was decided to assay these compounds in their blood, tissues (intestines, lungs, muscles, kidneys, livers, brains, subcutaneous fat and gonads), as well as in epidermal products (claws and flight feathers). It was also analysed the influence of potential factors, including the characteristics of the species (trophic level and environment) and individuals (condition, age and sex) on the concentration of phenol derivatives in particular tissues. As an additional parameter, the analysis of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ was included in the work, which enabled determination of the trophic level of selected bird species and the origin of their food.

The **first paper** focuses on the two most important ways of entry for pollutants to the birds' organisms, including the alimentary route – considered to be the main one, and the frequently overlooked respiratory route. Birds, being long-lived predators at the top of the trophic chain, are particularly exposed to increased bioaccumulation of pollutants in their organisms (Burger and Gochfeld, 2004). In addition, their intestines and lungs can be high potential indicators of contamination of specific components of the environment, including the food consumed by birds and the surrounding air. It should be noted, however, that in higher organisms, the lungs and the digestive tract, as well as the skin, are the main barriers between their systems and the high concentrations of xenobiotics in the environment (Lehman-McKeeman, 2008). Phenol derivatives, as endocrine disrupting compounds, must cross at least one of these barriers in order to be able to bind to the receptor and trigger the body's response. Therefore, in the first work, it was blood that turned out to be an important tool for assessing the bioavailability of phenol derivatives.

Regardless of the degree of phenol derivative concentration in the intestines or lungs, this transfer does not necessarily reflect the actual amount of a given xenobiotic to which the bird is exposed. In addition, blood provides information about momentary exposure and is a carrier of xenoestrogens to the sites of their activity (Espín et al., 2016). Therefore, in order to assess the exposure of birds to phenol derivatives, **the second and fourth papers** focused on understanding the distribution of BPA and alkylphenols among organs, taking into account their penetration routes. The brain and gonads are tissues that are particularly sensitive to the effects of endocrine active phenol derivatives (Cheng et al., 2017; Li et al., 2017; Mentor et al., 2020; Tran et al., 2020). Muscle and subcutaneous fat are sites of xenobiotic deposition but can also be sources of secondary exposure (Lehman-McKeeman, 2008). Especially during periods of strong stress, e.g. during migration or breeding, the fat stored in the bird's body is metabolized, and along with it, pollutants are mobilized and transported to the bloodstream (Henriksen et al., 1996; Perkins and Barclay, 1997). In turn, the liver and kidney are the organs through which xenobiotics can be metabolized and removed (Lehman-McKeeman, 2008). In addition, phenol derivatives can also affect the proper functioning of these important organs (Traversi et al., 2014; Faheem and Lone, 2017; Shirdel et al., 2020).

Birds, being predators at the top of the trophic chain, are exposed to increased doses of xenoestrogens as a result of their biomagnification (Burger and Gochfeld, 2004). Thus, an important element of **the third paper** was to determine whether these animals also have the ability to at least partially remove phenol derivatives from their bodies. Both feathers and claws are important ways of removing many xenobiotics, including persistent organic pollutants, which prefer to accumulate in fatty tissues. An important part of the work was also to compare the concentrations of phenol derivatives in the remiges of bird species that exchange these feathers in two extremely different regions with different environmental conditions and distances from potential pollution sources. Bird feathers are successfully used as indicators of environmental quality, but also as a non-invasive tool for assessing the pollution load in birds (Jaspers et al., 2006; Kim and Koo, 2008; Meyer et al., 2009).

AIMS OF THE STUDY

Seabirds experience a lot of stress, which is reflected by the drastic decrease in their global population, and one of the external factors responsible for this decline is the accumulation and activity of pollutants in their organisms (Croxall et al., 2012; Paleczny et al., 2015). Carrying out research on wild aquatic birds is rarely possible owing to the difficulties associated with obtaining study material. Little is known about the exposure of water birds to phenol derivatives, and even less about what happens after these xenoestrogens enter their organisms and what effect they have. The main sites of accumulation of phenol derivatives identified in this paper may indicate the future direction for research on the potential impact of these compounds on birds. On the other hand, the knowledge pertaining to the concentrations of BPA, 4-*t*-OP and 4-NP in individual tissues may serve as a basis for examining the possible negative effects caused by environmental concentrations of the tested compounds. The submitted work was also intended to provide new information about the potential of birds in environmental biomonitoring research.

When commencing the study, the following research hypotheses were put forward:

1. Razorbills *Alca torda*, long-tailed ducks *Clangula hyemalis* and goosanders *Mergus merganser* inhabiting the southern Baltic region are exposed to BPA and alkylphenols to varying degrees.
2. Inhalation is a significant route of exposure to BPA, 4-*t*-OP and 4-NP for razorbills *Alca torda*, long-tailed ducks *Clangula hyemalis* and goosanders *Mergus merganser*.
3. Strategic organs for the proper development and functioning of birds, i.e. gonads, brains, kidneys and livers, are the main sites of accumulation for endocrine disrupting phenol derivatives.
4. In razorbills *Alca torda* and long-tailed ducks *Clangula hyemalis* feathers and claws provide important means of eliminating phenol derivatives from their organisms.
5. The intestines, lungs, and feathers of razorbills *Alca torda*, long-tailed ducks *Clangula hyemalis* and goosanders *Mergus merganser* are good indicators of environmental pollution with phenol derivatives.

The hypotheses were verified by implementing the following main study objectives:

1. Determination of the main penetration routes of phenol derivatives into the organisms of birds and the factors determining birds' exposure in the southern Baltic region (paper 1).
2. Recognition of the organ distribution of phenol derivatives in the organisms of aquatic birds (papers 2 and 4).
3. Recognition of the potential of feathers and claws to remove phenol derivatives from bird organisms (paper 3).
4. Determination of the potential of selected tissues and epidermal formations of birds as indicators of environmental pollution and the phenol derivative load on birds' organisms (papers 1 and 3).

COLLECTED MATERIAL AND CHEMICAL ANALYSIS

The research was carried out on dead birds obtained in 2015 – 2016 from bycatches in the region of the southern Baltic Sea. Among them were: long-tailed ducks *Clangula hyemalis*, razorbills *Alca torda* and goosanders *Mergus merganser*. The long-tailed ducks originated from two bodies of water – the Gulf of Gdansk and the Pomeranian Bay. The razorbills came only from the Gulf of Gdansk, and goosanders from the Szczecin Lagoon. For the razorbills, the Baltic Sea is a permanent habitat, while goosanders and long-tailed ducks come to the southern Baltic only during the non breeding period. The diet of long-tailed ducks in the non-breeding period is mainly based on zoobenthos. In contrast, razorbills and goosanders feed exclusively on fish (Cramp and Simmons, 1977; Cramp, 1985; Stempniewicz, 1995).

A number of tissues were collected from the birds during dissection, including: intestines, lungs, pectoral muscles, kidneys, livers, subcutaneous fat, brain, gonads, feathers, claws and cardiac blood. The intestines were emptied of content and rinsed with milli-Q water. The collected samples were frozen (-20^{0}C) until analysis. Prior to analysis, the samples of intestines, lungs, pectoral muscles, kidneys, livers, gonads, feathers and claws were

freeze-dried and then homogenized. In addition, before analysis, both epidermal products were washed in acetone using ultrasound for 10 min. at 20^{0}C. Tissues and epidermal products prepared in this way were stored in borosilicate glass in a desiccator under constant conditions (temp. 20^{0}C ± 2^{0}C, humidity 45% ± 5%). Brain and fat samples were homogenized immediately before analysis. Wetness was additionally determined in both of these tissues in order to convert the results from wet weight to dry weight. During dissection of the birds, their age was determined based on plumage characteristics (Baker, 2016). Sex was determined by the appearance of the gonads. Each individual was also weighed and body condition was assessed based on the content of intestinal fat and subcutaneous fat according to an adopted scale (Camphuysen et al., 2007). A detailed description of the material is presented in Table 1.

BPA, 4-*t*-OP and 4-NP in the intestines, lungs, muscles, kidneys, livers, gonads, feathers and claws were determined according to the method described by Xiao et al. (2006) and modified by Staniszewska et al. (2014; 2018). The weighed biological material was extracted in an ultrasonic bath in a mixture of methanol, 0.01M ammonium acetate and 4M chloric acid (VII). The obtained extracts were purified on Oasis HLB glass columns (200 mg, 5 cm^3). The concentrations of phenol derivatives in blood were determined using the method described by Xiao et al. (2006). The samples were extracted in an ultrasonic bath with a mixture of n-hexane and diethyl ether (70:30) with ammonium acetate (0.01 M). Fat and brain were extracted twice in acetonitrile. The extracts were then combined, centrifuged and purified by shaking with hexane (Geens et al., 2012). All obtained extracts were evaporated to dryness and topped up with acetonitrile to 0.2 cm^3.

Table 1 List of bird species, type and number of biological material samples for analysis in particular papers

paper	species	blood	intestines	lungs	muscles	kidneys	livers	fat	brains	gonads	feathers	claws
paper 1	*Clangula hyemalis*	30	29	30								
	Alca torda	15	15	15								
	Mergus merganser	8	8	8								
paper 2	*Clangula hyemalis*				29	30	30	28	28			
	Alca torda				15	15	15	15	14			
	Mergus merganser				7	8	8	8	8			
paper 3	*Clangula hyemalis*										29	29
	Alca torda										15	14
paper 4	*Clangula hyemalis*									47		

The final assays of the concentrations of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol were performed using high-performance liquid chromatography with a fluorescence detector and a Thermo Scientific HYPERSIL GOLD C18 PAH chromatographic column (250×4.6 mm; 5 μm). The length of the generated excitation wave was λ = 275 nm, while emission was measured at the wavelength of λ = 300 nm. The chromatographic separation process was performed under gradient conditions using a mobile phase (water:acetonitrile). The recovery was determined in samples with the addition of a known amount of analyte, based on five measurements of the concentrations of BPA, 4-*t*-OP and 4-NP. The precision of the method was expressed as a coefficient of variation. The limit of determination of the method was established for a sample with a small content of analyte as a tenfold signal to noise ratio. Recovery for all samples was always > 80% and method precision < 15%. The limit of quantification ranged, depending on the compound and biological material, from 0.07 to 2.0 ($ng \cdot cm^{-3}$ in blood and $ng \cdot g^{-1}$ dw in other samples).

The analysis of stable isotopes was commissioned and performed in the laboratory of the Faculty of Chemistry of the Lodz University of Technology. The analysis was performed in previously freeze-dried and homogenized bird muscles as they reflect the last 3-4 weeks of the birds' diet (Hobson and Clark, 1992). Determination of isotopes $\delta^{15}N$ and $\delta^{13}C$ was performed using the Isotope Ratio Mass Spectrometer Sercon 20-22. V_2O_5 was used as the combustion catalyst and the local standard was thiobarbituric acid (atmospheric nitrogen and PDB for $\delta^{15}N$ and $\delta^{13}C$, respectively).

CONCLUSIONS

The razorbill, the long-tailed duck and the goosander living in the southern Baltic region are exposed to phenol derivative penetration both via ingestion and inhalation. The fact that BPA and 4-NP account for the highest concentrations in the intestines and lungs is the result of high production and subsequent release of these compounds into the environment, causing their widespread distribution in its various components. Ingestion was the dominant route of BPA exposure in all of the studied bird species. In the case of alkylphenols, however, the routes of penetration into bird organisms were more diverse and conditioned by the region of residence and eating habits. 4-NP entered the organisms of piscivorous birds mainly through the alimentary route, while the respiratory route dominated in bentophages. In addition, the higher the trophic position of the birds, the greater was their alimentary exposure to 4-NP. As for 4-*t*-OP, it was absorbed mainly by inhalation in birds living in the area of the Gulf of Gdansk, while for birds living in the area of the Pomeranian Bay, the alimentary route prevailed (**paper 1**). Thus, **hypotheses 1 and 2** were verified, showing the varied exposure of the species *Alca torda*, *Clangula hyemalis* and *Mergus merganser* to phenol derivatives, and indicating inhalation as an important route of exposure of birds to these compounds.

It has also been shown that phenol derivatives penetrate into the blood, which means that they penetrate through biological barriers and can be distributed throughout the body, including target sites for their endocrine activity (**paper 1, 2**). The present work shows wide distribution of all three compounds to the liver, kidneys, muscles, subcutaneous fat, brain and gonads, which suggests that phenol derivatives are subject to accumulation in the organisms of birds and undergo a series of transformation and elimination processes. However, in the study, the distribution pathways of phenol derivatives differed and were most likely determined by the properties of particular xenobiotics, especially their lipophilicity and the potential to bind to proteins and dissolve in fats. In all of the studied bird species, BPA and 4-NP were distributed in the largest amounts to the muscles, livers and kidneys, and in the case of 4-NP also to the gonads. On the other hand, 4-*t*-OP was transported mainly to the brain, subcutaneous fat and liver (**papers 2, 4**). The obtained results confirm **hypothesis 3**, that strategic organs for the proper development and functioning of birds, i.e. gonads, brains, kidneys and livers, are the main accumulation sites for endocrine active phenol derivatives.

The different target sites for accumulation of individual phenol derivatives indicate that each of the tested compounds may have different health effects on birds (**papers 2, 4**).

Due to the highest bioaccumulation of BPA and 4-NP being found in the liver and kidneys, future research should look at their potential detrimental effect on the functions of these important organs responsible for biotransformation and elimination of pollutants. Moreover, the liver was the only organ in which all three compounds showed high concentrations and positive mutual correlations. This shows the important function of selective sequestration of phenol derivatives in the liver, which is the first organ to which xenobiotics are transported after entering the body through the digestive tract. The study suggested that this process may be determined by the condition of birds, favoring the transfer of phenol derivatives to the liver from the intestine when the content of intestinal fat was higher (**paper 2**).

It is worth noting that 4-*t*-OP differs from the other compounds in its ability to penetrate the blood-brain barrier, as the accumulation of this xenobiotic in the brain can lead to changes in, for example, mating behavior. That may also be facilitated by the free 4-*t*-OP fraction circulating in the blood, which is available for transport to the sites of impact. However, it has been shown that a bird's brain can be protected against the accumulation of lipophilic xenoestrogens by means of their deposition in subcutaneous fat, which reduces transfer to the brain (**papers 1, 2**).

It also needs to be emphasized that 4-NP shows particularly high affinity for the gonads, indicating the possible potential of this compound to disrupt the proper functioning of this important reproductive gland. Even more so, based on the results obtained for the gonads of long-tailed ducks and the analysis of literature, it was shown that the concentration levels of phenol derivatives in the reproductive gland were similar to those at which negative effects caused by their endocrine activity had been observed in studies carried out so far. This shows that the tested xenoestrogens can interfere with the reproduction and development of birds. On the example of the long-tailed duck gonads, the influence of age and sex on the concentration of phenol derivatives in the reproductive gland was also revealed. It has been shown that mature long-tailed ducks are characterized by higher concentrations of phenol derivatives compared to immature specimens, which may be the result of long-term bioaccumulation, as well as diversified contamination of the areas of their residence. In turn, among mature long-tailed ducks, phenol derivatives were characterized by higher concentrations in males than in females, probably related to the fact that females have an additional means of eliminating pollutants via transfer from mother to egg. Thus, one cannot rule out the potential impact of phenol derivatives on the development of embryos during the sensitive period of growth, when concentrations of xenobiotics, as well as other nutrients, can be transferred from the mother to the egg (**paper 4**).

For most of the internal tissues, the trophic level of birds and their eating habits did not determine the concentrations of phenol derivatives. Nonetheless, birds from the highest trophic level were only found to have higher concentrations of phenol derivatives in the kidneys. This indicates the effective elimination of phenol derivatives, preventing increased bioaccumulation caused by biomagnification of xenobiotics along the trophic chain. In addition, on the example of two species of birds from the same trophic level, but with different feeding preferences, it was shown that the biomagnification of phenol derivatives may be higher in bentophages compared to species feeding on pelagic fish. Among the tested phenol derivatives, 4-NP was characterized by the greatest potential for biomagnification in the studied species of birds. It should be emphasized, however, that the biomagnification of phenol derivatives in bird tissues may be underestimated and may turn out to be higher than the values presented in this paper. That is particularly true for 4-*t*-OP, which mostly accumulated in brains, as opposed to muscles, for which biomagnification coefficients were calculated (**paper 2**).

The incorporation of phenol derivatives into the products of the epidermis, i.e. feathers and claws, enables birds to remove these xenobiotics from their bodies. The elimination of phenol derivatives, depending on the compound and epidermal product, ranges from 12 % to 34 %. For most compounds and birds, claws account for the largest proportion of removal. Considering only these two ways of removing phenol derivatives, it was found that the level of elimination is lower than the accumulation in the internal tissues of birds examined so far. However, this elimination seems to be effective enough to prevent possible bioaccumulation with age and biomagnification in birds feeding on organisms from higher trophic levels. This confirms **hypothesis 4** that the feathers and claws of *Alca torda* and *Clangula hyemalis* are important ways of removing phenol derivatives from their organisms, especially given that phenol derivatives are probably removed from most (if not all) internal tissues, while the brain may be more resistant to their elimination. Among the phenol derivatives, 4-NP had the greatest potential for being removed, both with feathers and claws, while 4-*t*-OP was the least removed of the compounds (**paper 3**).

This study has revealed differences in the levels of contamination with BPA and alkylphenols between the water and air of the Gulf of Gdansk and the Pomeranian Bay, and indicates the intestines and lungs of birds as potential bioindicators of phenol derivative contamination in the particular components of the environment (**paper 1**). Bird remiges have also turned out to be a promising indicator of environmental pollution with 4-NP. A comparison of concentrations in the remiges of the long-tailed duck and the razorbill, moulted in two different environments with different degrees of pollution and distance from sources, allowed us to establish that the Baltic Sea is about 3 times more polluted with 4-NP than the sea regions of the Russian Arctic (**paper 3**). Thus, **hypothesis 5** posed in this study was partially confirmed that the intestines, lungs and feathers of *Alca torda*, *Clangula hyemalis* and *Mergus merganser* are good indicators of environmental pollution with phenol derivatives. It was not, however, possible to confirm the usefulness of claws or any of the epidermal products in environmental biomonitoring as a non-invasive tool for examining the levels of exposure of birds to BPA and alkylphenols (**paper 3**).

Keywords: birds, bioindicators, endocrine disrupting phenol derivatives, exposure, bioaccumulation, elimination

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Gastrointestinal and respiratory exposure of water birds to endocrine disrupting phenolic compounds

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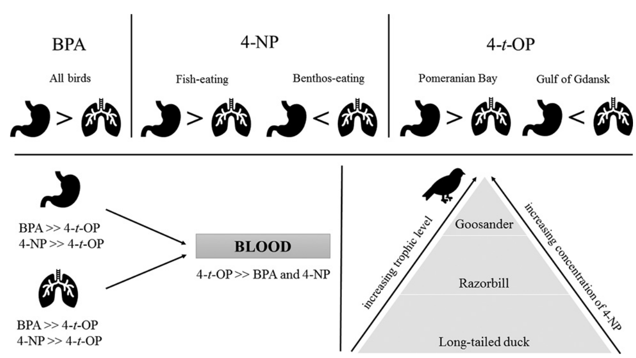
HIGHLIGHTS

- Birds eating fish and benthos are exposed to BPA mainly via the oral route.
- Exposure of birds to alkylphenols occurs both by ingestion and inhalation.
- Exposure is conditioned by the bird's location (4-*t*-OP) and trophic level (4-NP).
- 4-*t*-OP may have the greatest potential for endocrine disruption in birds.
- Birds intestines and lungs may indicate pollution of sea and air by phenols.

GRAPHICAL ABSTRACT



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ABSTRACT

Aquatic birds found at the top of the trophic chain are exposed to xenobiotics present both in food and inhaled air. The aim of this study was to indicate and assess the routes and levels of exposure of aquatic birds to bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP). The birds constituting the study material (*Clangula hyemalis*, *Alca torda*, *Mergus merganser*) originated from by-catches (winter 2014–2016) in the Southern Baltic. The studies show that the exposure of birds to phenol derivatives is determined by the specifics of a compound, the habitat area, trophic level and food consumed. BPA was characterized by the highest intestinal concentrations in all birds (6.6–1176.2 $ng \cdot g^{-1}$ dw). Higher concentrations of 4-*t*-OP were determined in the lungs of birds from the eastern part of the Southern Baltic (9.1–135.7 $ng \cdot g^{-1}$ dw) and in the intestines of birds from the western part (<0.5–191.4 $ng \cdot g^{-1}$ dw). In the case of 4-NP, higher concentrations were found in the intestines of carnivorous species (64.9–524.5 $ng \cdot g^{-1}$ dw), and the lungs of benthos-eating species (39.4–399.7 $ng \cdot g^{-1}$ dw). The intestines that were most burdened with 4-NP were those of birds from the highest trophic level. Correlations between the concentrations of phenol derivatives in the blood and the intestines and lungs indicated that birds are exposed to the penetration of phenol derivatives through the digestive and respiratory tracts. BPA and 4-NP were characterized by the highest concentrations in the intestines and lungs, whereas 4-*t*-OP in blood (3.2–39.2 $ng \cdot cm^{-3}$), which may indicate the largest endocrine potential of this compound in birds. Significant differences in phenol derivatives concentrations in the intestines and lungs of birds from the western and eastern part of the Southern Baltic, shows that these tissues can be useful for assessing the contamination of the environment with EDCs.

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1. Introduction

Wildlife is constantly exposed to a wide range of substances that humans introduce into the environment. It has been shown that even low concentrations of such peculiar pollutants as endocrine disrupting compounds (EDCs) pose a threat to the health and survival of animals at all trophic levels as well as humans. These xenobiotics include phenol derivatives i.e.: bisphenol A (BPA) and alkylphenols: 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP). These compounds, when transported throughout the food chain, can disrupt homeostasis of the organism and as a result, lead to changes in behavior and abnormalities in development and reproduction, including genital malformation and cancers (Oehlmann et al., 2000; Sohoni et al., 2001; Honkanen et al., 2004; Levy et al., 2004; Matsumoto et al., 2005; Lee et al., 2006; Oehlmann et al., 2006; Xia et al., 2010; Senthil Kumaran et al., 2011; Chaube et al., 2012; Traversi et al., 2014; Bhandari et al., 2015; Kinch et al., 2015; Li et al., 2017; Sharma and Chadha, 2017; Abdel-Tawwab and Hamed, 2018; Huang et al., 2018; Lee et al., 2018; Wang et al., 2019). Therefore, phenol derivatives pose a threat not only to individual organisms, but can also affect entire populations.

The use of phenol derivatives is mainly associated with the large-scale production of plastics. Bisphenol A is added as raw material to polycarbonate and epoxy resins, and alkylphenols are used in phenolic resins and oximes. 4-NP, 4-*t*-OP and their ethoxylates are also components of non-ionic surfactants and detergents. As a result, phenol derivatives are emitted into the environment mainly in the form of solid waste and together with discharged waters from industrial and municipal wastewater treatment plants (WWTP) (Giger et al., 1984; Ahel et al., 1994; Staples et al., 1998; Ying et al., 2002; Flint et al., 2012; Corrales et al., 2015; Acir and Guenther, 2018). The main recipients of these compounds are rivers, seas and oceans, where they accumulate in sediments and in the trophic chain (Koniecko et al., 2014; Staniszewska et al., 2014, 2015a, 2016a; Nehring et al., 2017; Staniszewska et al., 2017; Nehring et al., 2018).

Birds may be one of the groups of marine animals that are the most exposed to phenol derivatives. They are long-living predators at the top of the trophic chain, a fact which enables effective bioaccumulation and biomagnification of xenobiotics in their tissues (Burger and Gochfeld, 2004). In addition, due to the high respiration rate, unidirectional air flow and cross-current gas exchange in the respiratory system, these animals are also exposed to air pollutants (Brackenbury et al., 1981; Brown et al., 1997). It has been shown that the marked decrease in the global population of seabirds by 69.7% over the last 60 years has been associated with increased industrialization (Paleczny et al., 2015). Thus, the stress experienced by seabirds around the world today may be additionally aggravated by the pollution of air and water, and consequently food, interfering with reproduction and development.

Birds have long been well-known bioindicators of environmental pollution (Furness and Camphuysen, 1997; Burger and Gochfeld, 2004; Szumiło-Pilarska et al., 2017). However, until now xenobiotics, i.e. trace metals and persistent organic pollutants, have been assayed mainly in muscles, liver, kidneys, feathers, eggs and blood. All of those provided basic information about distribution, accumulation or elimination of such pollutants (Furness et al., 1986; Evers et al., 1998; Burger and Gochfeld, 2001; Franson et al., 2004; Rocque and Winker, 2004; Dauwe et al., 2005; Hela et al., 2006; Ribeiro et al., 2009; Espín et al., 2010, 2012a, 2012b; Pilarczyk et al., 2012; Binkowski et al., 2016; Espín et al., 2016; Dahlberg et al., 2016; Fromant et al., 2016; Falkowska et al., 2016; Nehring et al., 2017; Szumiło-Pilarska et al., 2017). However, little attention was paid to phenol derivatives and the penetration routes through the intestines and lungs. In particular, the respiratory exposure of birds to these compounds is still poorly researched. These tissues seem to have great potential for indicating the pollution of specific components of the environment, including the food consumed by the birds and the surrounding air. In addition, birds and people exposed to both gastrointestinal exposure and inhalation appear to share a similar pattern of penetration for foreign substances entering the body (Carere et al., 2010). All of these characteristics make birds suitable as early warning biomonitors, which makes it possible to use their tissues to assess xenobiotic exposure of the human population in a given region. In addition, tracking the concentrations of impurities in the intestines and lungs may indicate the effectiveness of the protective functions of these organs, which, being barriers to the absorption of pollutants into the bloodstream, are also interesting tissues from the toxicological point of view (Lehman-McKeeman, 2008).

The authors aimed to investigate the main penetration routes of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol into the bodies of birds and the factors determining bird exposure in the Southern Baltic region. The study hypothesis is to show that birds are exposed to EDCs not only via ingestion but also through inhalation, where penetration of phenol derivatives through inhalation can be significant. The paper also attempts to the risk assessment associated with the presence of those endocrine disrupting compounds in birds' organisms. In addition, the intestines and lungs of birds were used as indicator tissues for the assessment of phenol derivatives pollution of individual components and regions of the Southern Baltic and as indicators of potentially different sources of origin for EDCs. Three species of water birds were selected for the study: the long-tailed duck (*Clangula hyemalis*), the razorbill (*Alca torda*) and the goosander (*Mergus merganser*), which in the non-breeding period inhabit the Southern Baltic and obtain food from various trophic levels. The composition of the diet of these species was determined based on literature data, as well as the analysis of stable isotopes $\delta^{15}N$ and $\delta^{13}C$. The present publication does not take into account the age and sex of birds because the tissues studied are primarily the sites through which xenobiotics flow, rather than their bioaccumulation targets.

2. Sampling area and studied species

The Baltic Sea is a brackish, semi-enclosed, shallow reservoir with an average depth of 52 m. There is free water exchange between the Baltic Sea and the North Sea, which takes about 30 years. These features, combined with the large population (85 million people) and high industrialization of the Baltic basin have led to many problems, i.e. increased concentration of pollution, marine hypoxia, eutrophication and cyanobacteria blooms. The Southern Baltic region, which includes the Gulf of Gdansk, Pomeranian Bay and Szczecin Lagoon, is characterized by the greatest anthropopressure (Fig. 1). Those parts of the Baltic are also considered to be the most polluted within Polish coastal waters (Kot-Wasik et al., 2003; HELCOM, 2010). On the other hand, the Baltic Sea is also an important place of rest, foraging, moulting, breeding and wintering for about 80 species of birds. According to the latest report by Helcom (2018), the populations of water birds during the breeding season and during the wintering period in 2011–2016 decreased by about 30% and 20%, respectively. There are many causes for that, but all of them are related to anthropogenic human activity (Helcom, 2018) e.g. habitat destruction, pollution emissions, getting caught in fishing nets, overfishing of food sources and energy production (Žydelis et al., 2009; Croxall et al., 2012).

2.1. Gulf of Gdansk

The Gulf of Gdansk is located in the southeastern part of the Baltic Sea between Poland and Russia (Fig. 1). The main inflow to the bay is the Vistula River, which is the largest river in Poland. Its drainage introduces water from an area of 194,424km^2, encompassing 54% of Poland (Kot-Wasik et al., 2003; Pastuszak et al., 2018). In the Gulf of Gdansk, most anthropogenic pollution sources are located in the Tri-City agglomeration, situated directly along the shoreline, with a total population of over 1.2 million (GUS, 2020. Shipbuilding, oil refining, chemical,

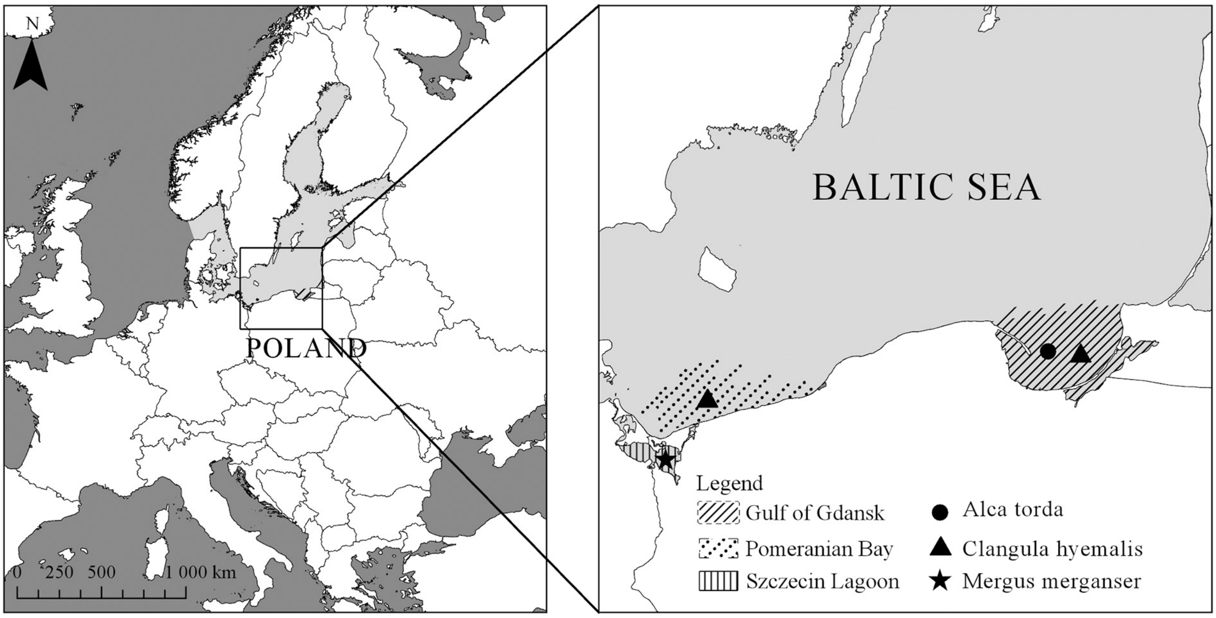

Fig. 1. Areas of bird by-catches in the Southern Baltic region (2014–2016).

pharmaceutical, paper and textile industries are rapidly developing here. The western part of the Gulf of Gdansk has the greatest biodiversity in the Polish coastal zone of the Baltic Sea. In 1992, it was incorporated into the Baltic Sea Protected Areas (BSPA), and a significant part of it (Puck Bay) is a special bird protection area belonging to the Natura 2000 network. In the non-breeding period, it is one of the most important places for waterbirds in the Polish zone of the Baltic (Durinck et al., 1994; Skov et al., 2011).

2.2. Pomeranian Bay and Szczecin Lagoon

The Pomeranian Bay and Szczecin Lagoon are located in the south-western part of the Baltic Sea, between Poland and Germany (Fig. 1). The main inflow to this area is the Odra - the second largest river in Poland behind the Vistula and one of the most significant in the Baltic basin. Its drainage introduces water from many different industrial hotspots and agricultural areas, incorporating a total area of approx. 118,861 km^2 (34% of the territory of Poland) (Kot-Wasik et al., 2003; Daniszewski, 2014; Pastuszak et al., 2018). In contrast to the Vistula, which flows directly into the Gulf of Gdansk, the waters of the Odra flow first into the Szczecin Lagoon, which acts as a buffer and protects the Baltic Sea against its pollution (Glasby et al., 2004). For that reason, the waters of the Szczecin Lagoon are characterized by high eutrophication and sedimentation rates, as well as high levels of nutrients and impurities, which ultimately also affects environmental quality in the Pomeranian Bay (Neumann et al., 1996, 1998; Lampe, 1999). The largest urban center located in this area is the Szczecin agglomeration with nearly 700,000 inhabitants (GUS, 2020). There are numerous industrial plants and shipyards, as well as an important shipping route connecting the port of Szczecin with the port of Świnoujście. The entire Pomeranian Bay and Szczecin Lagoon region is a special bird protection area belonging to the Natura 2000 network. These two reservoirs are one of the most important wintering sites for aquatic birds in the Baltic basin (Durinck et al., 1994; Skov et al., 2011).

2.3. Characteristics of the studied species

The research was carried out on dead birds caught in fishing nets (by-catch birds) during winter in the years 2014–2016 in three regions of the Southern Baltic: the Gulf of Gdansk, Pomeranian Bay and Szczecin Lagoon. Among them were: 30 long-tailed ducks (15 from the Gulf of Gdansk and 15 from Pomeranian Bay), 15 razorbills from the Gulf of Gdansk and 8 goosanders from the Szczecin Lagoon (Fig. 1). The most important information about each species is summarized in Table 1S (Supplementary material).

3. Materials and methods

3.1. Biological material for analyses

During autopsy of the birds, intestines, lungs, muscles and blood from the heart were collected. The intestines were emptied of alimentary content and rinsed with milli-Q water. The collected samples were frozen (−20 °C) until analysis. Then the soft tissues were lyophilized, homogenized and stored in a borosilicate glass container in a desiccator under constant conditions (temperature 20 °C ± 2 °C, humidity 45% ± 5%).

3.2. Chemical analyses of bisphenol A, 4-tert-octylphenol and 4-nonylphenol

All solvents, ie, water, acetonitrile, and methanol, were manufactured by Merck and were HPLC grade. Chloric acid (VII) (70% concentration) and ammonium acetate (p.a.) were produced by POCh. High purity (>97%) bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol standards were produced by SIGMA-ALDRICH. The calibration curves were prepared on the basis of working solutions prepared in methanol of the following concentrations: 10, 25, 50, 75 and 100 ng·cm^{-3}. The standards were stored in a refrigerator, and before use, they were exposed in order for them to reach room temperature and appropriate volume. Prior to analysis, the laboratory glass was first etched in nitric acid (3.5 mol · dm^{-3}) for 24 h and then dried at 120 °C.

In order to determine the concentrations of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol in the intestines and lungs of birds, weighed biological material (0.1 g ± 10^{-3} g) was extracted in an ultrasonic bath (10 min, 20 °C) in the mixture of the following solvents: 8 cm^3 methanol, 2 8 cm^3 0.01 M ammonium acetate and 100 µcm^3 4 M chloric acid (VII). The obtained extracts were purified on Oasis HLB glass columns (200 mg, 5 ml) (produced by Waters), according to the method described by Staniszewska et al. (2014). On the other hand, the concentrations of phenol derivatives in the blood were determined using the method described by Xiao et al. (2006). For that purpose, 0.5 cm^3 samples were taken and extracted in an ultrasonic bath with a mixture of n-hexane and diethyl ether (70:30) with ammonium acetate (0.01 M). All obtained extracts were evaporated to dryness and topped up with acetonitrile to 0.2 cm^3.

Final assays of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol concentrations were performed using high performance liquid chromatography with a fluorescence detector and a Thermo Scientific HYPERSIL GOLD C18 PAH chromatographic column (250 cm / 4.6 μm). The generated excitation wavelength was $\lambda = 275$ nm, while emission was measured at $\lambda = 300$ nm. The chromatographic separation process was performed using a mobile phase (water: acetonitrile) under gradient conditions. Mean recovery, based on quintuple measurements of BPA, 4-*t*-OP and 4-NP concentrations in the samples with the addition of a known amount of analyte, was above 80% for each compound. However, the precision of the method, expressed as the coefficient of variation, was <15%. The limit of quantification of the method was determined as the 10-fold signal-to-noise ratio of a sample with a low analyte content. For blood it was 0.07 $ng \cdot cm^{-3}$ (BPA, 4-*t*-OP and 4-NP), and in soft tissues 2 $ng \cdot g^{-1}$ (BPA) and 0.5 $ng \cdot g^{-1}$ (4-*t*-OP and 4-NP).

3.3. Stable isotope analysis and trophic level of birds

Assay of the stable $\delta^{15}N$ and $\delta^{13}C$ isotopes was performed in the previously freeze-dried and homogenized muscles of birds, as these tissues reflect the final 3–4 weeks of the birds' diets (Hobson and Clark, 1992). For this purpose, homogenized material in the amount of approx. 4 mg $\pm$ 10^{-5} mg was weighed out into tin capsules (cylinder, 4 × 6 mm). V_2O_5 was used as the combustion catalyst, and the local standard was thiobarbituric acid (atmospheric nitrogen and PDB for $\delta^{15}N$ and $\delta^{13}C$, respectively). Analysis of the stable isotopes $\delta^{15}N$ and $\delta^{13}C$ was performed by means of an Isotope Ratio Mass Spectrometer Sercon 20–22. The results were expressed in (‰) according to the equation:

$$\delta X = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \cdot 1000 \quad (1)$$

where X represents $\delta^{15}N$ and $\delta^{13}C$ and is the corresponding $^{15}N/^{14}N$ and $^{13}C/^{12}C$ respectively.

The values of the stable nitrogen isotope ($\delta^{15}N$) were used to calculate the trophic level (TL) of birds in the food web according to the formula by Hobson and Welch (1992):

$$TL = \frac{\delta^{15}N_{consumer} - \delta^{15}N_{baseline}}{\Delta^{15}N} + 2 \quad (2)$$

where:

- TL is the trophic level of birds
- $\delta^{15}N_{consumer}$ is the nitrogen isotope ratio of a consumer,
- $\delta^{15}N_{baseline}$ is the nitrogen isotope baseline,
- $\Delta^{15}N$ is the ^{15}N trophic enrichment factor.

As the $\delta^{15}N$ baseline for the Gulf of Gdansk region we adopted the mean value of $\delta^{15}N$ (8.4) calculated by Sokołowski (2009) for all primary consumers, as they show less time variability than the primary sources of organic matter. On the other hand, $\Delta^{15}N$ was substituted by 3.4 - the value by which $\delta^{15}N$ increases in subsequent links of the trophic chain (Post, 2002; Olive et al., 2003; Sokołowski et al., 2012).

3.4. Statistical analyses

Using the Shapiro-Wilk test, the data was found to be characterized by a non-parametric distribution. Therefore, in order to check the relationship between the selected parameters and the concentrations of BPA, 4-*t*-OP and 4-NP in the tissues and blood of birds, two tests were used: the Mann-Whitney *U* test (two variables) and the Kruskal-Wallis test (many variables). The relationships between the concentrations of phenol derivatives in the tested samples were determined using the Spearman's rank correlation (ρ) in order to take into account the non-linear relationships. The level of significance was $p = 0.05$. The above analyses were performed using StatSoft STATISTICA12.

4. Results

4.1. Intestines

Bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol were identified in 98% of the intestines of the studied bird species: the long-tailed duck, the razorbill and the goosander (Table 1). The long-tailed duck intestines from both study areas were characterized by the highest BPA concentrations (6.6–1176.2 $ng \cdot g^{-1}$ dw), while goosander intestines had the highest 4-NP concentrations (122.2–524.5 $ng \cdot g^{-1}$ dw). The razorbill was characterized by the highest concentration range observed for BPA (25.6–315.0 $ng \cdot g^{-1}$ dw), while the highest median was for 4-NP (165.8 $ng \cdot g^{-1}$ dw). On the other hand, the lowest concentrations in the intestines of all bird species were determined for 4-*t*-OP (<0.5–233.6 $ng \cdot g^{-1}$ dw). The median concentration of this compound was 3 to 20 times lower than that of BPA and 4-NP, with the greatest differences occurring in the case of goosander.

4.2. Lungs

The only tissues where phenol derivatives were found were in the lungs of the razorbills (all) and of the long-tailed ducks (97%) (Table 1). In contrast, among the goosanders, the only compound that was identifiable in all lungs was 4-NP, while BPA was not determined in 43% of the samples and 4-*t*-OP in more than half of them. The latter bird species was also characterized by the lowest concentrations of all three compounds. The highest concentrations found in lungs were those of 4-NP in long-tailed duck (39.4–399.7 $ng \cdot g^{-1}$ dw) and of BPA in long-tailed duck (<2.0–331.7 $ng \cdot g^{-1}$ dw) and in razorbill (13.6–329.1 $ng \cdot g^{-1}$ dw). On the other hand, the lowest concentrations found in all of the bird species were those of 4-*t*-OP (<0.5–120.0 $ng \cdot g^{-1}$ dw), the medians of which, depending on the bird species studied, were 2 to 11 times lower than of other compounds.

4.3. Blood

Phenol derivatives were assayed in all of the razorbill and goosander blood samples (Table 1). On the other hand, 37% of long-tailed duck blood samples were found to contain no bisphenol A. The highest concentrations determined in bird blood were those of 4-*tert*-octylphenol (3.2–39.2 $ng \cdot cm^{-3}$). In turn, the lowest concentrations in blood were found for BPA (<0.07–8.7 $ng \cdot cm^{-3}$) in the long-tailed duck and for 4-NP (1.0–9.7 $ng \cdot cm^{-3}$) in the razorbill and the goosander. The medians of these compounds were 2 to 10 times lower than those of 4-*t*-OP depending on the species of bird.

4.4. Stable isotopes of $\delta^{15}N$, $\delta^{13}C$ in the birds' muscles and trophic level

Statistical analysis showed significant differences ($p < 0.05$; Kruskal-Wallis test) in the values of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ between the particular species and populations of birds. The smallest range of $\delta^{15}N$ and $\delta^{13}C$ values was found in the razorbill (12.1–13.5‰ and − 21.7 - (−21.3‰) respectively), and the widest range in the goosander

Table 1
Concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the intestines and lungs [$ng \cdot g^{-1}$ dw] and blood [$ng \cdot cm^{-3}$] of the long-tailed ducks, razorbills and goosanders by-caught in the Southern Baltic region in winter in the years 2014–2016.

		Long-tailed duck GG			Razorbill GG			Long-tailed duck PB			Goosander SL		
		Inte-stines	Lungs	Blood	Inte-stines	Lungs	Blood	Inte-stines	Lungs	Blood	Inte-stines	Lungs	Blood
BPA	Min	6.6	<2.0	<0.07	25.5	13.6	7.4	25.5	27.6	<0.07	53.3	<2.0	2.7
	Max[a]	686.5	331.7	8.7	315.0	329.1	30.6	1176.2	168.7	6.8	260.3	27.3	23.6
	x	126.7	90.0	5.6	113.4	83.1	13.0	209.7	67.0	4.2	114.3	23.1	9.4
	Md	76.0	67.8	5.6	98.0	46.4	11.6	117.6	52.7	4.2	93.7	22.7	3.8
	SD	165.8	86.1	1.4	89.9	99.5	5.4	290.8	39.5	1.6	62.4	3.5	8.7
4-NP	Min	6.7	39.4	1.5	64.9	33.9	1.0	25.0	49.2	1.8	122.2	56.7	1.6
	Max[a]	100.6	399.7	27.4	268.5	136.4	4.0	227.6	261.8	17.8	524.5	122.1	9.7
	x	54.6	129.4	13.1	171.4	80.8	1.9	114.3	152.3	6.9	314.1	90.9	4.0
	Md	54.9	100.0	15.3	165.8	93.0	1.8	107.8	150.9	6.5	301.8	90.9	2.3
	SD	26.6	95.3	7.5	69.4	34.2	0.7	60.8	67.0	4.7	152.9	24.1	3.4
4-*t*-OP	Min	3.1	9.1	3.2	12.0	20.6	15.4	5.3	7.0	6.6	<0.5	<0.5	10.8
	Max[a]	43.5	99.4	20.6	233.6	135.7	35.8	191.4	120.0	18.5	49.6	9.9	39.2
	x	18.6	33.0	9.9	55.7	62.5	23.5	63.1	23.9	11.9	19.2	6.9	21.5
	Md	15.7	26.1	8.5	33.1	58.8	21.1	29.1	18.4	11.2	15.1	7.7	17.2
	SD	12.6	22.5	4.9	56.6	32.6	6.9	64.4	27.3	3.3	15.4	3.5	10.9
	n	15	15	15	15	15	15	14	15	15	8	8	8

n – number of samples; Min – minimum value; Max – maximum value; Md – median value; x – mean value; SD – standard deviation, dw – dry weight; GG – Gulf of Gdansk, PB – Pomeranian Bay, SL – Szczecin Lagoon.
[a] Maximum values also include outliers and extreme values.

(12.0–20.1‰ and − 26.5 - (−21.3‰) respectively) (Table 2). The muscles of the long-tailed duck from Gulf of Gdansk had a narrow range of $\delta^{15}N$ (10.5–14.2‰) and a wide range of $\delta^{13}C$ values (−22.8 – (−18.4‰)). On the contrary, in the long-tailed ducks from the Pomeranian Bay the correlation was reversed, with $\delta^{15}N$ ranging from 10.7 to 16.9‰, and $\delta^{13}C$ from −24.6 to −22.5‰. The calculated trophic level occupied by the birds ranged from 2.6 to 5.5, with the highest value in the goosander and the lowest in the long-tailed duck from the Gulf of Gdansk.

5. Discussion

5.1. The intestines and lungs of birds as indicators of environmental pollution with phenol derivatives

From the toxicological point of view, both the intestines and the lungs are places via which xenobiotics enter the body, as opposed to being the target tissues for storage (Lehman-McKeeman, 2008; Falkowska et al., 2017). Therefore, these organs represent a short-term exposure to pollution, which makes it possible to associate the xenobiotics assayed in them with the area where the exposure occurred. All three bird species were caught in January. Thus, both the birds which inhabit the Baltic Sea permanently (razorbills) and those staying here in the non-breeding season (goosanders and long-tailed ducks) were exposed to pollution from the Southern Baltic region for a sufficiently long time. In all of these species, bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol were measured in the intestines as well as in the lungs (Table 1). This indicates an effective flow of phenol derivatives in the Baltic trophic chain as well as the uptake of these compounds by birds via inhalation.

Table 2
Stable isotopes of $\delta^{15}N$, $\delta^{13}C$ [‰] in the muscle tissues of the studied species and trophic levels (TL) of long-tailed duck, razorbill and goosander.

	n	$\delta^{15}N$			$\delta^{13}C$			TL		
		Min	Max	x	Min	Max	x	Min	Max	x
LO GG	15	10.5	14.2	12.5	−22.8	−18.4	−21.6	2.6	3.7	3.2
RA GG	15	12.1	13.5	12.9	−21.7	−21.3	−21.5	3.1	3.5	3.3
LO PB	14	10.7	16.9	14.4	−24.6	−22.5	−23.9	2.7	4.5	3.8
GO SL	7	12.0	20.1	16.6	−26.5	−21.3	−24.3	3.1	5.5	4.4

n – number of samples; LO – long-tailed duck; RA – razorbill; GO – goosander; GG – Gulf of Gdansk; PB – Pomeranian Bay; SL – Szczecin Lagoon.

In the case of the long-tailed duck, it was possible to obtain information on the geographically diverse exposure of birds to EDCs. Concentrations of phenol derivatives in the intestines of long-tailed ducks from the Pomeranian Bay were higher than in the intestines of long-tailed ducks from the Gulf of Gdańsk. Moreover, for both alkylphenols, the differences were statistically significant (Mann-Whitney U test; $p < 0.05$; Fig. 2a). These results are consistent with the reports of the General Inspectorate of Environmental Protection in Poland, which describes the chemical status of the transitional and coastal waters of the Pomeranian Bay as bad, and that of Gdańsk Bay as good (GIOŚ, 2016a, 2016b). This assessment was made on the basis of the classification recommended by the EU Water Framework Directive, and concerned the period during which the birds were caught - 2015. Moreover, the obtained values of $\delta^{13}C$ indicated that both the piscivorous and benthos-eating birds from the eastern part of the Southern Baltic (Gulf of Gdańsk) seem to feed on a trophic chain based on autochthonous organic matter (Maksymowska et al., 2000) (Fig. 3). On the other hand, birds from its western part (Pomeranian Bay and Szczecin Lagoon) consume organisms based rather on allochthonous organic matter (Fig. 3), thus indicating its anthropogenic origin, probably associated with discharges from sewage treatment plants (Maksymowska et al., 2000). Alkylphenols are also transported from WWTP to the marine environment mainly via rivers and show high affinity for binding with organic matter (Ahel et al., 1994; Ying et al., 2003). Earlier studies have shown several times higher concentrations of these compounds in rivers compared to marine subsurface or near-bottom waters, which in many cases exceeded the PNEC (predicted no effect concentration) values Staniszewska et al., 2015b). Thus, the high contribution of anthropogenic organic matter introduced by rivers into Pomeranian Bay and Szczecin Lagoon may also be a source of greater accumulation of alkylphenols in the trophic chain of these parts of the Southern Baltic.

In turn, the lungs of the long-tailed duck from the Gulf of Gdansk were characterized by higher concentrations of 4-*t*-OP and BPA compared to the long-tailed duck from Pomeranian Bay (Fig. 2b), although only for 4-*t*-OP these differences were statistically significant (Mann-Whitney U test; $p < 0.05$). The coastal zone of the Gulf of Gdansk, due to the proximity of the urbanized and industrialized Tri-City agglomeration, seems to be particularly susceptible to the emission of phenol derivatives into the atmosphere. In its vicinity there are numerous industrial plants associated with the production and processing of plastics, as well as the processing of crude oil (e.g. LOTOS SA). As demonstrated earlier, in the Gulf of Gdansk area the main source of phenol derivatives in atmospheric aerosols is the uncontrolled burning of

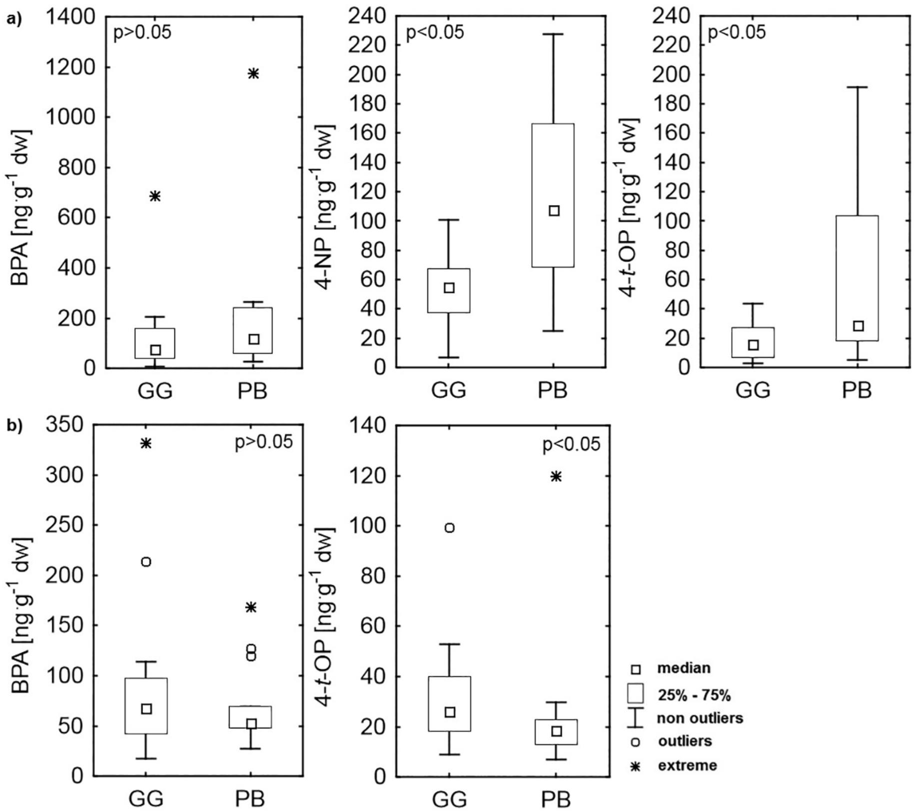

Fig. 2. Concentrations of phenol derivatives in the a) intestines and b) lungs of long-tailed ducks from the Gulf of Gdansk (GG) and Pomeranian Bay (PB).

mixed waste (especially plastics), and emission into the atmosphere increases in winter (Lewandowska et al., 2013). That is also the period when long-tailed ducks stay in this area. Moreover, similarities were observed between the mean BPA concentrations in the lungs of razorbills from the Gulf of Gdansk, for which the Baltic Sea is a permanent habitat, and the lungs of long-tailed ducks, for which this area is only a winter residence (Table 1). This suggests that both species breathed the same air, at the same time confirming that the pollutants accumulated in their lungs originated in the Gulf of Gdansk area. Unfortunately, to our knowledge, no research has been carried out to date on the presence of phenol derivatives in the atmosphere of the Pomeranian Bay and defining potential sources of these compounds in this region. On the other hand, based on the results obtained for the lungs of birds, it can be assumed that exposure to BPA and 4-*t*-OP through inhalation of both birds and humans is greater in the Gulf of Gdansk than in Pomeranian Bay.

5.2. Factors determining the routes of exposure to phenol derivatives for birds

5.2.1. Bisphenol A

Bisphenol A was characterized by higher concentrations in the intestines of birds in relation to their lungs. This tendency was maintained for all species and populations, and for long tailed-duck from Pomeranian Bay and goosander these differences were statistically significant (Mann-Whitney *U* test; $p < 0.05$, Fig. 4a). This proves that the alimentary route is the key route of introduction for BPA into birds' bodies. Similar observations were made in previous studies, which showed

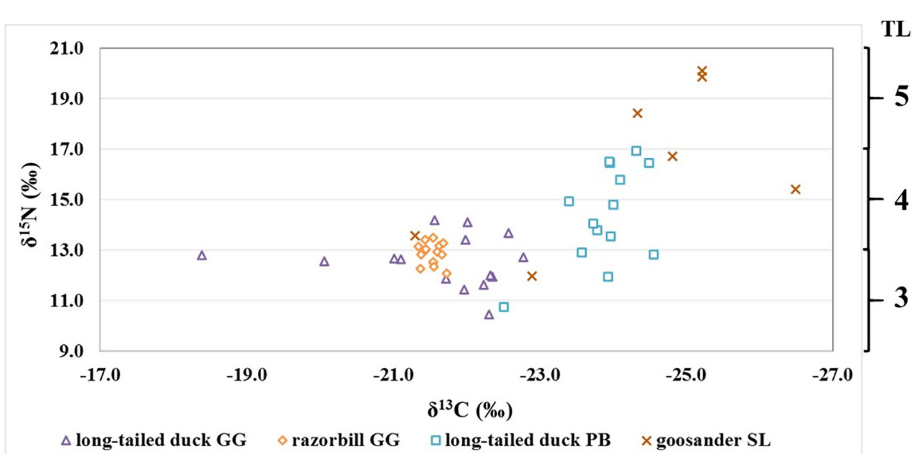

Fig. 3. Stable isotopes of $\delta^{15}N$ and $\delta^{14}C$ in the muscles of the studied birds living in the Southern Baltic region and the trophic level they occupy; footnotes as in Table 2.

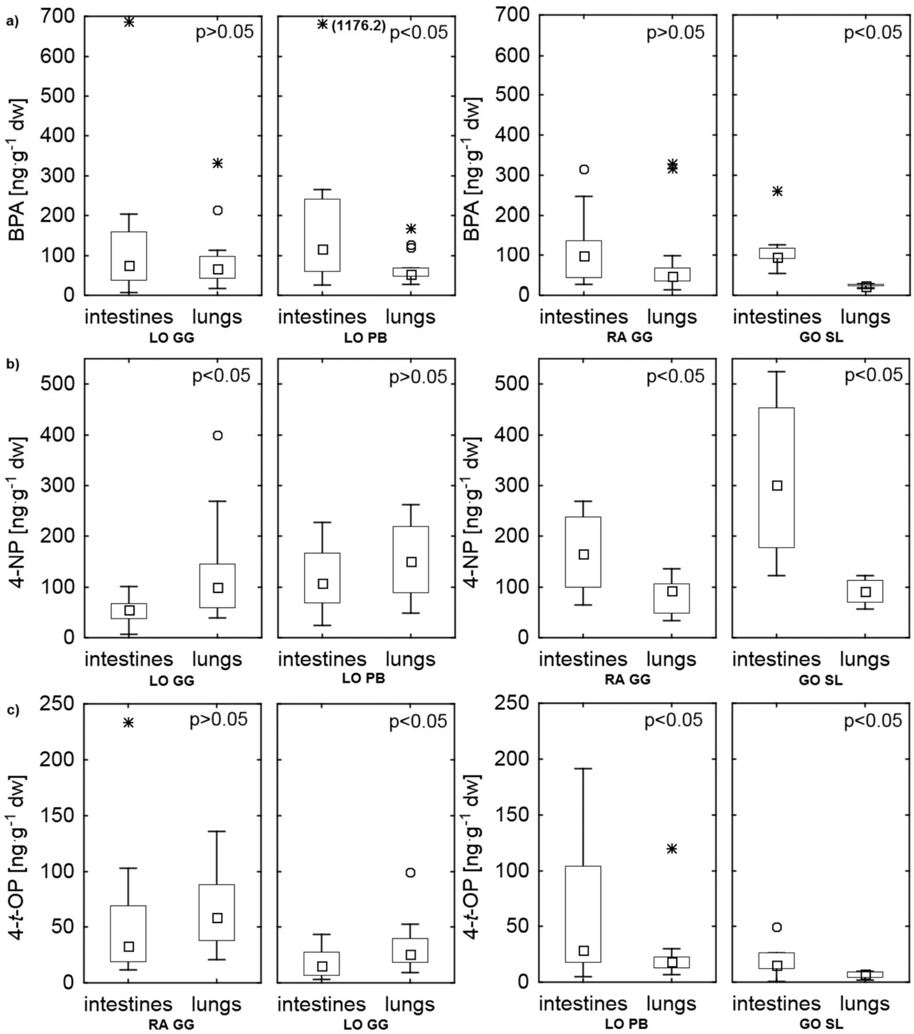

Fig. 4. Concentrations of a) bisphenol A (BPA), b) 4-nonylphenol (4-NP) and c) 4-*tert*-octylphenol (4-*t*-OP) in the intestines and lungs of birds staying in the Southern Baltic region; footnotes as in Table 2.

that out of the three phenol derivatives, the highest concentrations in organisms from different trophic levels in the Southern Baltic region are reached by BPA, while being relatively low in the air (Lewandowska et al., 2013; Staniszewska et al., 2014). Bisphenol A is a high production volume chemical released into the environment mainly with water discharged from industrial and municipal wastewater treatment plants. Although the effectiveness of BPA removal by WWTP reaches 85% (Sun et al., 2017), due to its high production which reaches almost 4 million tons per year (Rubin, 2011), its post-treatment leftovers still constitute high emissions into the environment. In the Gulf of Gdansk region, BPA is widely distributed among all organisms from different trophic levels, reaching high concentrations in practically every link of the chain (Staniszewska et al., 2014). This is confirmed by the lack of statistically significant differences in BPA concentrations between the intestines of individual bird species, despite the fact that their diet is based on organisms from different trophic levels (Fig. 3). However, the largest range of BPA concentrations was observed in the long-tailed ducks from the Gulf of Gdansk and from Pomeranian Bay (Table 1). Long-tailed ducks have the most varied diet of the three species studied. According to Cramp and Simmons (1977), in winter these birds mainly eat mussels but supplement their diet with smaller fish and crustaceans. By means of comparison, goosanders and razorbills are ichthyophages and feed solely on fish in winter (Cramp and Simmons, 1977, 1985). Both of these species were characterized by much smaller BPA concentration ranges in the intestines (Table 1). This shows that food is an important source of BPA for birds, including those that feed at different trophic levels, a fact which is probably related to its ubiquitous presence in the marine environment.

5.2.2. 4-nonylphenol

In the Southern Baltic region, the exposure of birds to 4-nonylphenol was characterized by greater variability compared to bisphenol A. Among the piscivorous species (razorbill and goosander), the predominant route of exposure was alimentary, whereas in the benthivorous long-tailed duck exposure occurred mainly via inhalation (Fig. 4b). Moreover, differences in 4-NP concentrations between the intestines and lungs were statistically significant for all of the bird species except for the long-tailed duck from Pomeranian Bay (Mann-Whitney *U* test; $p < 0.05$). However, the predominant role of the respiratory route in the long-tailed duck does not result from higher exposure to 4-NP inhalation compared to the razorbill and the goosander. That is confirmed by the lack of statistically significant differences in 4-NP concentrations between the lungs of the individual bird species (Kruskal Wallis test; $p > 0.05$). Thus, the observed correlation on the different routes of exposure of fish-eating and benthos-eating birds is most likely due to their different diets, as well as the lipophilic properties of 4-NP. Out of the three phenol derivatives, 4-NP has the highest affinity with animal adipose tissue (the log Kow partition coefficient for phenol derivatives is: 3.3 for BPA; 5.3 for 4-t-OP and 5.9 for 4-NP) (Grover, 2008), which may favor its bioaccumulation in the trophic chain (Diehl et al., 2012;

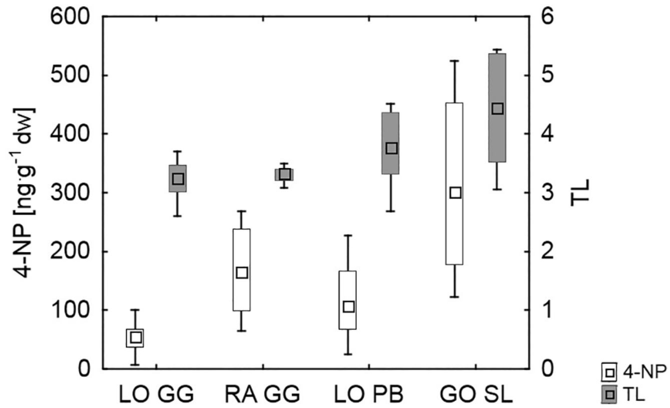

Fig. 5. The trophic level (TL) and concentration of 4-nonylphenol (4-NP) in the intestines of individual species and populations of birds living in the Southern Baltic region; footnotes as in Table 2.

Gautam et al., 2015; Korsman et al., 2015; Lee et al., 2015). In the intestines of the studied birds, the concentrations of 4-NP increased with the each trophic level occupied by the birds (Fig. 5), and the differences between the individual species were statistically significant (Kruskal Wallis test; $p < 0.05$). The goosanders, birds from the highest trophic level, had the greatest 4-NP load in their intestines (Fig. 5). On the other hand, the lowest concentrations of 4-NP were determined in the benthivorous long-tailed duck from the Gulf of Gdansk, which occupies the lowest trophic level (Fig. 5). Moreover, the fish-eating goosanders from the Szczecin Lagoon had higher concentrations of 4-NP in their intestines than the fish-eating razorbills from Gdansk Bay (Fig. 5). Therefore, it seems that the trophic level of birds and their place of origin played a major role in introducing the lipophilic 4-NP into their bodies. It is therefore likely that the respiratory route was the dominant route of 4-NP exposure in long-tailed ducks, as these birds feed on the lower links of the trophic chain and are therefore less exposed to this xenobiotic via the alimentary route. Nevertheless, the example of the long-tailed duck from the Southern Baltic shows that in the case of lower exposure of birds to 4-NP via the alimentary route, inhalation turns out to be an important factor in the penetration of pollutants and thus cannot be ignored.

5.2.3. 4-tert-octylphenol

The exposure of birds to 4-*tert*-octylphenol was conditioned by their place of habitation in the Southern Baltic region and the discussed differences in the degree of contamination of the particular components of its eastern and western parts. Both bird populations from the Gulf of Gdansk were exposed to 4-*t*-OP penetration mainly by inhalation (Fig. 4c), and the differences in its concentrations between the intestines and lungs of the long-tailed duck were statistically significant (Mann-Whitney U test; $p < 0.05$). On the other hand, a different correlation was observed in the birds from Pomeranian Bay and Szczecin Lagoon, namely higher concentrations of this compound in their intestines compared to the lungs (Fig. 4c). Birds are exposed to a wide range of contaminants, especially lipophilic ones, mainly with food (Burger and Gochfeld, 2004). The compound 4-*t*-OP also has affinity with animal adipose tissue, which results in its bioaccumulation in the marine trophic chain (Staniszewska et al., 2014; Staniszewska et al., 2017; Nehring et al., 2018). Whereas, the identification of the respiratory tract as the dominant route of exposure of birds living in the Gulf of Gdańsk region to 4-*t*-OP, including the fish-eating razorbill, comes as a surprise and seems to shed a new light on the fate of 4-*t*-OP in the environment. On the other hand, it should be noted that the production of 4-*t*-OP is much lower in comparison with BPA and 4-NP (White et al., 1994; Ying et al., 2002; Rubin, 2011), and that this may be the reason for its lower supply to the marine environment. This is confirmed by previous studies of the water and organisms of the Gulf of Gdansk, where 4-*t*-OP was characterized by several to 20 times lower concentrations compared to BPA and 4-NP (Staniszewska et al., 2014; Staniszewska et al., 2015b). The same correlation is also to be observed for the intestines of all the studied bird populations and species (Table 1). However, in atmospheric aerosols measuring <2.5 μm and 1 μm in size, 4-*t*-OP was characterized by the highest concentrations among the studied phenol derivatives, especially in the winter season (Lewandowska et al., 2013). The presence in aerosol particles makes it possible for this compound to enter the lungs and even the alveoli, then penetrating into the bloodstream. Another important source of 4-*t*-OP in the atmosphere may be the water surface microlayer (SML), which influences the transport mechanisms of aerosols containing EDCs from water to atmosphere and the other way round. As shown by Staniszewska et al. (2015b), the SML is characterized by much higher concentrations of 4-*t*-OP than the subsurface or the near-bottom water, and the concentrations reached in the SML often exceed the PNEC values. Thus, greater exposure of birds from the Gulf of Gdansk to 4-*t*-OP by inhalation than via ingestion may result from both higher concentrations of this compound in the atmosphere of this region, and lower concentrations in the trophic chain. Nevertheless, that does not change the fact that the often overlooked respiratory exposure to 4-*t*-OP is an important route of exposure to this xenobiotic for birds. Therefore, in view of the poor literature on the presence of phenol derivatives in the atmosphere, the results obtained in the lungs of birds indicate that further research on this subject should be undertaken.

What is more, surprising results of 4-*t*-OP were obtained for birds from the west part of the Southern Baltic, where the benthivorous long-tailed ducks were characterized by higher concentrations of this compound in the intestines compared to the fish-eating goosander (Table 1, Fig. 4c). The long-tailed duck's diet is mainly related to the benthic environment. Thus, the quality of the sediment itself has a large impact on the bioaccumulation of pollutants in benthic organisms and in the birds that eat them. 4-*t*-OP shows strong properties for binding with organic matter (Ying et al., 2003), which is evident in the sediments of the Gulf of Gdansk, where this compound reached the highest concentrations among the studied phenol derivatives (Koniecko et al., 2014; Staniszewska et al., 2016b). In addition, benthic organisms may contain up to 18 times higher concentrations of 4-*t*-OP than fish muscles and livers (Staniszewska et al., 2014; Staniszewska et al., 2017). This leads to the conclusion that in more contaminated basins sediment may turn out to be an important source of 4-*t*-OP for benthic organisms, and consequently also for birds feeding on them, such as long-tailed ducks.

5.3. Risk assessment of the presence of endocrine disrupting phenols in birds' organisms

5.3.1. Bioavailability of phenol derivatives

The skin, lungs and digestive tract are the main barriers separating higher organisms from an environment contaminated with xenobiotics. Phenol derivatives are endocrine disrupting compounds, and therefore must transgress at least one or more of these barriers in order to have a harmful effect on the organisms. Therefore, irrespective of the varied emissions of phenol derivatives into the environment and their subsequent introduction into the intestines or lungs, that transfer does not necessarily reflect the actual amount of a particular xenobiotic to which the body is exposed. The actual harmful exposure of the organism is what happens after the pollutant passes through the biological membrane, as a result of which the xenobiotic is transported with blood to the target tissue (Lehman-McKeeman, 2008). The obtained correlations between the concentrations of phenol derivatives in the intestines and lungs, and the concentrations of phenol derivatives in the blood of the long-tailed duck (Table 3) prove that these compounds penetrate through the biological membranes of the lungs and intestines into the bloodstream. Moreover, these correlations seem to confirm the previously formulated theses that 4-*t*-OP enters a bird organism mainly

Table 3
Spearman's correlations of phenol derivatives in long-tailed duck tissues and blood.

	lungs-blood	intestines-blood	lungs-intestines
BPA	GG: $r = -0.57$ p = 0.05 $n = 12$	GG + PB: $r = -0.49$ p < 0.05 $n = 17$	GG: $r = 0.58$ p < 0.05 n = 13
4-*t*-OP	PB: $r = 0.51$ p = 0.06 n = 14	–	GG + PB: r = 0.51 $p < 0.05$ $n = 26$ GG: $r = 0.73$ p < 0.05 $n = 14$ PB: $r = 0.49$ $p = 0.06$ $n = 13$
4-NP	–	GG + PB: $r = -0.46$ p < 0.05 $n = 28$ PB: $r = -0.70$ p < 0.05 n = 13	GG: $r = -0.58$ p < 0.05 $n = 15$ PB: $r = -0.55$ p < 0.05 n = 14

r – Spearman's correlation; p - level of significance; n – number of samples; GG – Gulf of Gdansk; PB – Pomeranian Bay.

through the respiratory tract, while 4-NP does so through the gastrointestinal tract.

Moreover, as shown in Table 1, in the intestines and lungs of birds the concentrations of 4-*t*-OP are up to 20 times lower than those of BPA and 4-NP. In bird blood, however, the situation was reversed and 4-*t*-OP was found in statistically significantly higher concentrations (Kruskal - Wallis test; $p < 0.05$). Specialized blood proteins such as albumin, alpha and beta lipoproteins have the ability to bind the xenobiotics that are present in the bloodstream. This phenomenon is important from a toxicological point of view, as toxicity usually manifests itself in the amount of unbound xenobiotics. That is because the toxic substances associated with plasma proteins are too high in molecular weight and therefore cannot penetrate the capillary walls and are therefore not available for distribution in target tissues. Therefore, a compound highly bound with plasma proteins may not be toxic as opposed to a compound that is less bound (Lehman-McKeeman, 2008). In this study, the assayed concentrations of phenol derivatives in the blood of birds constitute their free fraction, i.e. not bound to plasma proteins. Therefore, it can be assumed that 4-*t*-OP, characterized by the highest concentration in blood, has the lowest ability to bind to its proteins. However, in the case of the compounds that demonstrated lower concentrations in blood (BPA and 4-NP), a higher binding potential can be presumed. Thus, despite the lower doses of 4-*t*-OP introduced into the organisms of birds via the gastrointestinal and respiratory routes compared to BPA and 4-NP, this xenobiotic may show a high endocrine disrupting potential because only the free fraction can bind to the estrogen receptor. The current findings are in line with previous studies which showed that only a small percentage of BPA (approx. 5%) in human plasma is found as the free fraction in a non-protein-bound form (Csanady et al., 2002). Xie et al. (2013) proved that 4-*t*-OP does not bind with plasma proteins as well as 4-NP. In addition, there have been experiments which showed a greater toxic and estrogenic potential of 4-*t*-OP compared to 4-NP (White et al., 1994; Nagel et al., 1997; Senthil Kumaran et al., 2011; Traversi et al., 2014). Being an endocrine disrupting compound, 4-*t*-OP even through a low, but environmentally significant and chronic exposure, may lead to e.g. malfunctioning of the thyroid gland or abnormal development, including feminization and congenital defects (Gray et al., 1999; Seki et al., 2003; Croteau et al., 2008; Croteau et al., 2010).

5.3.2. Presystemic elimination

There are several mechanisms for removing chemicals before they enter systemic circulation, known as presystemic or first pass elimination. The process serves to limit the exposure of the organism and minimizes the harmful potential of a xenobiotic (Lehman-McKeeman, 2008). In our study, the correlations obtained (Table 3) between the concentrations of phenol derivatives in the lungs and those in the intestines of the long-tailed duck may indicate that birds possess at least one such mechanism. As has been shown, birds living in the Southern Baltic region are exposed to phenol derivatives through the inhalation of small aerosol particles. However, solid particles with a diameter of about 2.5 μm are deposited mainly in the tracheobronchiolar regions of the lungs, from which they can be removed by the retrograde movement of the mucus layer in the ciliated portions of the respiratory tract. This transport is considered fast (0.1 to 1 mm per minute) and effective (removal half-lives between 30 and 300 min) (Lehman-McKeeman, 2008). Thus, some of the phenol derivatives adsorbed on the surface of aerosols, having entered the bird lungs in this way, are probably also removed with them by the mucociliary reverse movement. Some of these particles can be expectorated, and this mechanism seems to protect the lungs and the organism of birds, preventing the contaminants deposited on larger aerosol particles from penetrating into the bloodstream. On the other hand, a certain proportion of the particles may be ingested, thus increasing the load in the intestines. Nevertheless the presence of phenol derivatives in the gastrointestinal tract of birds increases their chances of removing them by excretion or via direct transfer through the hepatic vein to the liver, where they can be biotransformed into more easily excreted forms (Lehman-McKeeman, 2008).

6. Conclusions

This paper presents a new approach to the use of bird lungs and intestines as indicators of air and marine environment pollution with phenol derivatives. It has been shown that birds inhabiting the Southern Baltic region permanently or only in the winter season, are exposed to endocrine disrupting compounds both through food and air. Bearing in mind literature reports spanning a long period of time, the gastrointestinal exposure to phenol derivatives is not surprising, as it is the main route for many contaminants entering animal organisms. On the other hand, the exposure to these xenobiotics through the respiratory tract, which turns out to be a significant, if not equivalent, factor in the penetration of phenol derivatives into bird organisms, is quite new information that is worth investigating further. The authors emphasize the need to undertake such research in the Southern Baltic region, taking into account the seasonality and the level of occurrence of phenol derivatives in aerosols. The exposure of birds to individual xenobiotics was determined by the specification of the compound, their diet and their area of habitation. In the case of BPA, the main route of entry was the alimentary route. However, exposure to alkylphenols (4-NP and 4-*t*-OP) was more varied and both the gastrointestinal and respiratory tracts played a significant role in introducing these compounds into the body. Moreover, it has been shown that phenol derivatives from both sites of exposure penetrate the bloodstream of birds from where they can be distributed to the site of action. The fact that the highest concentrations in the blood of birds were found for 4-*t*-OP indicates that, out of the tested EDCs, this compound has the greatest potential to disturb the functioning of the bird organism. The paper also indicates a potential mechanism of presystemic elimination of phenol derivatives from the lungs of birds.

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CRediT authorship contribution statement

Karina Bodziach: Resources, Conceptualization, Investigation, Validation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Marta Staniszewska:** Resources, Validation, Writing - original draft, Writing - review & editing, Supervision. **Lucyna Falkowska:** Writing - review & editing. **Iga Nehring**: Investigation, Writing - review & editing. **Agnieszka Ożarowska:** Investigation, Writing - review & editing. **Grzegorz Zaniewicz:** Investigation, Writing - review & editing. **Włodzimierz Meissner:** Investigation, 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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SUPPLEMENTARY MATERIAL

Bodziach K., Staniszewska M., Falkowska L., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2021. Gastrointestinal and respiratory exposure of water birds to endocrine disrupting phenolic compounds. Science of The Total Environment, 754, 142435, doi: 10.1016/j.scitotenv.2020.142435.

Table 1S Characteristics of the studied species living in the Southern Baltic region (data from BirdLife International, 2020; Wetlands International, 2020)

Species	Razorbill (*Alca torda*)	Long tailed duck (*Clangula hyemalis*)	Goosander (*Mergus merganser*)
	 Photo: Ken Canning	 Photo: Wolfgang Wander	 Photo: Hal and Kirsten Snyder
Family	*Alcidae*	*Anatidae*	*Anatidae*
Capture sites	Gulf of Gdansk	Gulf of Gdansk Pomeranian Bay	Szczecin Lagoon
Diet	piscivorous	mainly benthos i.e. crustaceans, molluscs; occasionally small fish, insects, plants	piscivorous
Local population trend	decreasing	decreasing	decreasing
Red list category	near threatened	vulnerable	least concern
Conservation status	protected species	protected species	protected species

STATEMENTS OF CO – AUTHORS

Bodziach K., Staniszewska M., Falkowska L., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2021. Gastrointestinal and respiratory exposure of water birds to endocrine disrupting phenolic compounds. Science of The Total Environment, 754, 142435, doi: 10.1016/j.scitotenv.2020.142435.

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Gdynia, 22.05.2023 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Bodziach K., Staniszewska M., Falkowska L., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2021. Gastrointestinal and respiratory exposure of water birds to endocrine disrupting phenolic compounds. Science of The Total Environment, 754, 142435, doi: 10.1016/j.scitotenv.2020.142435,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the southern Baltic", stanowił około **50%** całości i obejmował:

- sformułowanie problemu badawczego,
- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- analizę statystyczną wyników,
- graficzne i statystyczne przedstawienie wyników pochodnych fenolu,
- interpretację pozyskanych wyników pochodnych fenolu w świetle posiadanej wiedzy oraz zgromadzonego przeglądu literatury przedmiotowej,
- tworzenie manuskryptu.

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wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **20%** całości i obejmował:

- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną na każdym etapie tworzenia manuskryptu, w szczególności: w interpretacji wyników i redagowaniu manuskryptu,
- pełnienie funkcji autora korespondencyjnego.

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Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Bodziach K., Staniszewska M., Falkowska L., **Nehring I.**, Ożarowska A., Zaniewicz G., Meissner W., 2021. Gastrointestinal and respiratory exposure of water birds to endocrine disrupting phenolic compounds. Science of The Total Environment, 754, 142435, doi: 10.1016/j.scitotenv.2020.142435,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the southern Baltic", stanowił około **10%** całości i obejmował:

- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną.

Iga Nehring
....

IN MARI VIA TUA

dr Agnieszka Ożarowska

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Katedra Ekologii i Zoologii Kręgowców

Gdańsk, 18.05.2023 r.

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wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **5%** całości i obejmował:

- przygotowanie materiału biologicznego do analiz chemicznych.

....

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- pomoc w interpretacji wyników i redagowaniu manuskryptu.

....

PUBLICATION 2

Bodziach K., Staniszewska M., Falkowska L., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2021. Distribution path of endocrine disrupting phenolic compounds in waterbirds (*Mergus merganser*, *Clangula hyemalis*, *Alca torda*) from southern Baltic. Science of The Total Environment 793, 148556, doi: 10.1016/j.scitotenv.2021.148556.

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Distribution paths of endocrine disrupting phenolic compounds in waterbirds (*Mergus merganser, Alca torda, Clangula hyemalis*) from the Southern Baltic

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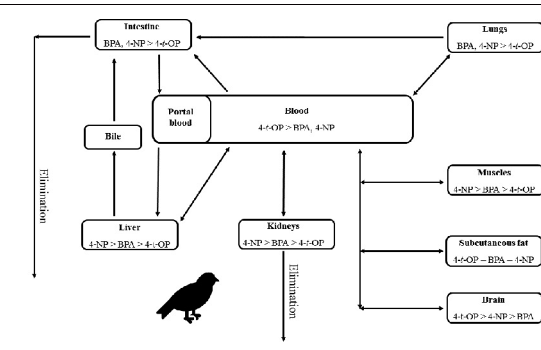
HIGHLIGHTS

- BPA, 4-NP and 4-*t*-OP have different distribution pathways in waterbirds.
- 4-NP has the greatest potential for biomagnification in waterbirds.
- Benthivorous birds are exposed to higher accumulation of phenol derivatives.
- Higher intestinal fat content leads to greater exposure of birds to EDCs.
- The accumulation of phenol derivatives in fat reduces their transfer to the brain.

GRAPHICAL ABSTRACT



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ABSTRACT

This study determined the distribution of phenol derivatives in the organisms of waterbirds and the factors influencing their bioaccumulation and affinity to specific tissues. Concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) were determined in the brains, subcutaneous fat, kidneys, livers and pectoral muscles of goosanders *Mergus merganser* (GO), long-tailed ducks *Clangula hyemalis* (LO) and razorbills *Alca torda* (RA). The birds came from the winter by-catch (2014–2016) in the Southern Baltic. Different distribution routes of individual phenol derivatives in the birds were established, most likely due to their ability to bind to proteins and/or dissolve in lipids. BPA and 4-NP accumulated most in the muscles (BPA <2.0–223.0 $ng{\cdot}g^{-1}$ dw, 4-NP 26.0–476.4 $ng{\cdot}g^{-1}$ dw), livers (BPA <2.0–318.2 $ng{\cdot}g^{-1}$ dw, 4-NP 60.7–525.8 $ng{\cdot}g^{-1}$ dw), and kidneys (BPA <2.0–836.1 $ng{\cdot}g^{-1}$ dw, 4-NP 29.3–469.2 $ng{\cdot}g^{-1}$ dw), while 4-*t*-OP was stored mainly in the brains (2.6–341.1 $ng{\cdot}g^{-1}$ dw), subcutaneous fat (0.7–173.7 $ng{\cdot}g^{-1}$ dw) and livers (<0.5–698.8 $ng{\cdot}g^{-1}$ dw). The liver was the only organ where all compounds showed a positive correlation with each other and alkylphenols were also positively correlated with each other in tissues with high fat content (brains and subcutaneous fat), and negatively in muscles. Despite the different trophic levels of birds, the concentrations of phenol derivatives in the tissues between individual species in most cases did not differ significantly. However, between the species on a similar trophic level, the higher biomagnification coefficient was calculated for LO feeding on benthos, and the lower for RA feeding on pelagic fish ($p < 0.05$). The good condition of birds, resulting in large intestinal fat stores, promoted on the one hand the penetration of phenol derivatives from the intestine to the liver, and on the other hand their accumulation in subcutaneous fat, thereby protecting the brain.

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1. Introduction

In the last 6 decades, a very disturbing and drastic decrease, as much as 70%, has been observed in the global population of seabirds (Paleczny et al., 2015). This can undoubtedly be associated with the increase in industrialisation and, consequently, pollution of the seas, by-catch of birds in fishing nets, overfishing of the food base of birds, introduction of predatory alien species to their colonies, and disturbance of peace and habitat degradation by humans (Žydelis et al., 2009; Croxall et al., 2012). The exposure of waterbirds to pollution is compounded by the fact that they are long-lived predators at the top of the trophic chain (Burger and Gochfeld, 2004). The higher oxygen demand and therefore greater respiratory efficiency of these animals when compared to other vertebrates, which is related to their ability to fly, is also important (Brown et al., 1997; Sanderfoot and Holloway, 2017). These conditions make waterbirds particularly susceptible to the penetration of pollutants both by food and respiration, and consequently to bioaccumulation and/or the negative impact of xenobiotics on their organisms. Birds are exposed to xenobiotics mainly through contaminated food and air. However, the penetration of xenobiotics into the body through the alimentary and respiratory tracts is limited by the biological membranes of the lungs and intestines. In order for xenobiotics to have harmful effects on the body, they must first pass through these barriers. Most substances have the ability to do this, resulting in transfer from the place of entry into the blood, which then distributes them throughout the body. The target sites for accumulation of these compounds depend on their affinity for specific tissues in which they accumulate through protein binding, lipid dissolution, or active transport. The site of accumulation can be a target organ for toxicity or a storage tissue where the substance is toxicologically inactive (Lehman-McKeeman, 2008). Therefore, the amount of initial exposure of birds to pollutants in the environment does not necessarily reflect the actual amount of a given xenobiotic that may affect the organism.

For decades the scientific community has been studying the bioaccumulation of the most toxic metals (mercury, lead, cadmium) and persistent organic pollutants (organochlorine pesticides, dioxins and dioxin-like compounds, such as polychlorinated and polybrominated biphenyls) and the related health and lethal effects in birds (Heinz, 1979; Helander et al., 1982; Sileo and Fefer, 1987; Eisler, 1988; Braune and Norstrom, 1989; Sileo et al., 1990; Fry, 1995; Helander et al., 2002; Heinz and Hoffman, 2003; Eagles-Smith et al., 2008; Scheuhammer et al., 2008; Blus, 2011; Harris and Elliott, 2011; Espín et al., 2016; Falkowska et al., 2016; Szumiło-Pilarska et al., 2017; Reindl et al., 2020). However, little is known about the distribution and accumulation of phenol derivatives in seabirds and the associated consequences of exposure. This is important because these xenobiotics are endocrine disrupting compounds (EDCs), which are toxic to organisms and have genotoxic, muta- and teratogenic effects. The main commonly known phenol derivatives are bisphenol A (BPA) and alkylphenols: 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP). The most important reports about their negative impact on organisms concern, among other things, dysfunction of antioxidant defence systems, modulation of gene expression, DNA damage and RNA processing, degenerative organ changes, numerous malformations, changes in mating behaviour, reduced fertility, reproductive fertility and embryonic survival, and the development of genital cancers (Xia et al., 2010; Lam et al., 2011; Senthil Kumaran et al., 2011; Wu et al., 2011; Chaube et al., 2012; Traversi et al., 2014; Won et al., 2014; Bhandari et al., 2015; Sharma and Chadha, 2016; Faheem and Lone, 2017; Li et al., 2017; Moreman et al., 2017; Sharma and Chadha, 2017; Lee et al., 2018). It is therefore clear that phenol derivatives endanger the health and life of organisms at all stages of life. More importantly, they may cause a decline in the number of individuals, and thus threaten the survival of the entire population.

The main application of phenol derivatives is in the production of plastics, e.g. food containers, electronic devices, sports equipment. Alkylphenols and their ethoxylates are present in surfactants such as detergents, stabilisers and emulsifiers, and are found in, among other things, cleaning agents, cosmetics, paints, adhesives and lubricants (Staples et al., 1998; Acir and Guenther, 2018). The consequence of the production, use and processing of products containing phenol derivatives is their emission into both the marine environment and the atmosphere (Saito et al., 2004; Fu and Kawamura, 2010; Liao et al., 2012; Lewandowska et al., 2013; Staniszewska et al., 2014, 2015). In the seas, plastics are also a direct threat to animals, as they degrade into marine litter. These microparticles can then be ingested by the birds and the phenol derivatives released into their organisms (Teuten et al., 2009; Andrady, 2011; Staniszewska et al., 2016a).

Our previous studies showed the presence of phenol derivatives (bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol) in the lungs and intestines of birds living in the Southern Baltic region. Effective transfer of phenols from exposure sites to the bloodstream has also been demonstrated (Bodziach et al., 2021). The question therefore arose - what happens next with phenol derivatives in the organisms of birds? With this in mind, the following research goals were set: (1) to understand the organ distribution of BPA and alkylphenols, taking into account their routes of entry; (2) to identify the main accumulation sites of phenol derivatives and the factors determining their affinity for particular tissues. In order to reflect the fate of phenol derivatives in the organisms of birds, the most important internal tissues were taken into account: the brain, subcutaneous fat, pectoral muscles, liver and kidneys. The study included individuals belonging to three species of waterbirds: goosander (*Mergus merganser*), long-tailed duck (*Clangula hyemalis*) and razorbill (*Alca torda*), wintering in the Southern Baltic. The diet of the long-tailed ducks is based on benthos, mainly mussels. In contrast, goosanders and razorbills feed on fish, mainly pelagic (Cramp and Simmons, 1977). All of the studied bird species are characterised by a biogeographically declining population and are under species protection (BirdLife International, 2021).

2. Sampling area

The Baltic Sea is a small and shallow inland sea in northern Europe with an average depth of about 53 m. Its area is almost four times smaller than that of the catchment area, which is inhabited by over 85 million people. The exchange of water with the North Sea through narrow and shallow straits is approximately 30 years. All of these factors cause the Baltic ecosystem to be exposed to increased chemical pollution and marine litter concentration, 70% of which are plastics (HELCOM, 2018). The Southern Baltic region, which includes waterbodies such as the Gulf of Gdansk, Pomeranian Bay and Szczecin Lagoon, is characterised by the highest population density and, consequently, anthropopressure (HELCOM, 2010). Local sources of pollution include ports, shipyards, the refining industry and an important shipping route. To add to this, the two largest Polish rivers: the Vistula and the Oder, the combined catchment of which covers almost 90% of the territory of Poland, both flow into the Southern Baltic (Kot-Wasik et al., 2003; Pastuszak et al., 2018). These rivers transport pollution from numerous inland industrial centres, urban and agricultural areas.

Due to its saline complexion, the Baltic Sea is ecologically unique and is a resting, wintering, feeding, moulting and breeding place for about 80 species of birds (HELCOM, 2018). The entire Pomeranian Bay with the Szczecin Lagoon, as well as the western part of the Gulf of Gdańsk are areas of special bird protection, belonging to the Natura 2000 network. For water birds in the non-breeding period, they are one of the most important places in the Polish zone of the Baltic Sea (Durinck et al., 1994; Skov et al., 2011). A map of the research area was presented in our previous publication (Bodziach et al., 2021), which this research is a continuation of.

3. Materials and methods

3.1. Biological material for analyses

The research was carried out on dead by-catch birds caught in the winter 2014–2016. Among them were 3 species from 3 different basins: 8 goosanders from the Szczecin Lagoon, 30 long-tailed ducks (15 individuals from the Gulf of Gdansk and 15 from the Pomeranian Bay) and 15 razorbills from the Gulf of Gdansk. No more than 24 h elapsed from the time the birds became caught in the net to the time they were frozen. All specimens dissected and analysed were in good condition with no visible signs of decay, aided by efficient material collection and the low temperatures at which sampling was conducted.

At necropsy, each individual was weighed, and body condition was assessed by intestinal fat and subcutaneous fat content according to the adopted scale (Camphuysen et al., 2007). The pectoral muscles, kidneys, liver, brain and subcutaneous fat were collected and immediately frozen (−20 °C). Prior to analysis, the pectoral muscles, kidneys and livers were freeze-dried and then homogenised. The prepared tissues were stored in borosilicate glass in a desiccator under constant conditions (temp. 20 °C ± 2 °C, humidity 45% ± 5%). Brains and fat tissues were homogenised immediately prior to analysis. In both of these tissues, the humidity was additionally determined in order to convert the results from wet weight to dry weight.

3.2. Extraction of bisphenol A, 4-tert-octylphenol and 4-nonylphenol from biological samples

All used vessels and instruments at the stage of sample preparation and determining were made of glass or metal. Solvents used for analyses (water, acetonitrile, methanol) produced by Merc were of HPLC grade. Ammonium acetate (p.a.) and 70% chloric acid (VII) (p.a.) were produced by POCh. The bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol standards produced by SIGMA-ALDRICH were of high purity (>97%). Working solutions with the following concentrations: 10, 25, 50, 75 and 100 $ng \cdot mL^{-1}$ were prepared in methanol, and a calibration curve was prepared on their basis. The linear correlation coefficient (r) of the analytical standards was >0.999.

3.2.1. Muscles, livers and kidneys

Determination of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol concentrations in previously lyophilised and homogenised muscles, kidneys and livers was carried out according to the method described by Staniszewska et al. (2014, 2018) and Bodziach et al. (2021). Weighed bird tissues (0.1 g ± 10^{-3} g) were immersed in a mixture of the following solvents: 8 mL methanol, 2 mL 0.01 M ammonium acetate and 100 μL 4 M chloric acid (VII), and then extracted in an ultrasonic bath for 10 min at 20 °C. The obtained extracts were purified on Oasis HLB glass columns (200 mg, 5 cc) from Waters. The collected extracts were evaporated to dryness and made up to 0.2 mL with acetonitrile.

3.2.2. Brains and subcutaneous fat

In the case of fat and brains, the homogenised wet tissues (0.2 g ± 10^{-3} g) were extracted twice in acetonitrile (3 mL each for 15 min at 20 °C). The combined extracts were centrifuged. The samples were then purified by shaking with 2 mL of hexane to remove lipophilic impurities (Geens et al., 2012). The collected extracts were concentrated in the same way as described according to the method of Staniszewska et al. (2014, 2018) and Bodziach et al. (2021).

3.3. Chromatographic determinations and validation parameters

Final determinations of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol concentrations were performed using high performance liquid chromatography with a fluorescence detector and a chromatographic column Thermo Scientific HYPERSIL GOLD C18 PAH (250 × 4.6 mm; 5 μm). The generated excitation wavelength was λ = 275 nm, while the emission was measured at λ = 300 nm. The chromatographic separation process was performed under gradient conditions using a mobile phase (water:acetonitrile). The accuracy was determined by the mean recovery, based on the 5-fold measurement of BPA, 4-*t*-OP and 4-NP concentrations in samples with the addition of a known amount of analyte (5, 50, 100 $ng \cdot g^{-1}$). In kidneys, livers and muscles it was 80–82%, while in brains 82–87%, and subcutaneous fat 85–107%. In contrast, the precision of the method expressed as a coefficient of variation was less than 15% in kidneys, livers and muscles, and less than 10% in brains and subcutaneous fat. The limit of quantification of the method was determined as the 10-fold signal-to-noise ratio for every kind of sample with a very low (near the limit of detection) analyte content. In kidneys, livers and muscles it was 2 $ng \cdot g^{-1}$ dw (BPA) and 0.5 $ng \cdot g^{-1}$ dw (4-*t*-OP and 4-NP), while in brains and subcutaneous fat it was 0.4 $ng \cdot g^{-1}$ dw (BPA) and 0.1 $ng \cdot g^{-1}$ dw (4-*t*-OP and 4-NP). The background (lab procedural blanks) was checked every time a new batch of SPE columns was used. The obtained „background" values for BPA and alkylphenols were <LOQ.

3.4. Formulae and statistical analyses

In order to confirm the exposure of birds to phenol derivatives, liver/muscle concentration ratios of BPA, 4-*t*-OP and 4-NP were calculated (Havelkova et al., 2008):

$$\frac{C_{liver}}{C_{muscle}} \tag{1}$$

where:

C_{liver} – the concentration of the compound in the liver [$ng \cdot g^{-1}$ dw];
C_{muscle} – the concentration of the compound in the muscle [$ng \cdot g^{-1}$ dw].

To determine whether the type of food and the trophic position of birds in the trophic chain affect the degree of accumulation of phenol derivatives, the biomagnification factors (BMF) were calculated according to the following formula (Hu et al., 2005):

$$BMF = \frac{C_{predator}}{C_{prey}} \tag{2}$$

where:

$C_{predator}$ – the concentration of the compound in the tissue of the predator [$ng \cdot g^{-1}$ dw];
C_{prey} – the concentration of the compound in the tissue of the prey [$ng \cdot g^{-1}$ dw].

The biomagnification factor was determined only for long-tailed ducks from the Gulf of Gdansk and razorbills, as a result of the availability of data concentrations of phenol derivatives in their diet. To calculate BMF for long-tailed ducks, the concentrations in food were taken as the mean concentrations in the tissue of mussels 43.9 $ng \cdot g^{-1}$ dw (BPA), 30.6 $ng \cdot g^{-1}$ dw (4-*t*-OP) and 74.2 $ng \cdot g^{-1}$ dw (4-NP) (Staniszewska et al., 2017). For the razorbill, the mean concentrations in herring muscle of 105.8 $ng \cdot g^{-1}$ dw (BPA), 161.8 $ng \cdot g^{-1}$ dw (4-*t*-OP) and 109.6 $ng \cdot g^{-1}$ dw (4-NP) were used (Nehring et al., 2018).

Correlations of xenobiotic concentrations between different tissues of animals have been commonly used to track their distribution (Dauwe et al., 2002; Espín et al., 2012; Szumiło-Pilarska et al., 2016; Gómez-Ramírez et al., 2017; Nehring et al., 2018; Grajewska et al., 2019; Bodziach et al., 2021). Therefore, based on the relationship between two variables, in this case the concentrations of phenol derivatives in two different tissues, this paper proposes a circulation of compounds between the tested tissues. When determining the distribution of phenol derivatives in the different organs of the birds, the routes of penetration for these xenobiotics were also taken into account. For this purpose, correlations were determined between concentrations in the intestines, lungs and blood, and concentrations in the muscles, livers, kidneys, fat and brains. The concentrations needed for the calculations in the intestines, lungs and blood were taken from the earlier work by Bodziach et al. (2021) as they came from the same goosander, long-tailed duck and razorbill individuals.

Statistical analyses were performed using the StatSoft STATISTICA12 program, with a level of significance of $p = 0.05$. To test the normality of the data, the Shapiro–Wilk test was used ($p < 0.05$). To assess the significance of statistical differences, the Mann-Whitney *U* test (two variables) and the Kruskal-Wallis test (many variables) were used. To take into account non-linear relationships between the selected parameters (weight, intestinal and subcutaneous fat content of the birds) and the concentrations of BPA, 4-*t*-OP and 4-NP in individual tissues of the birds, Spearman's rank correlations (ρ) were used. The study also took into account some of the correlations that were not statistically significant, but were moderate (0.3–0.5) or strong (>0.5).

4. Results

4.1. Bisphenol A (BPA)

Bisphenol A was measured in almost all muscles and livers (98%), as well as kidneys (96%). A relatively lower detection frequency was obtained for BPA in subcutaneous fat samples (88%), while in brains it was only 16% (Table 1). BPA was characterised by the greatest variability and the highest concentrations in razorbills were in the livers (23.8–318.2 ng·g^{-1} dw). In goosanders and long-tailed ducks, however, they were for the kidneys and fell within respective ranges of 76.6–298.6 ng·g^{-1} dw and <2.0–836.1 ng·g^{-1} dw. In turn, the lowest concentrations of this compound for all bird species were found in fat (<0.4–54.7 ng·g^{-1} dw) and brains (<0.4–37.4 ng·g^{-1} dw). Depending on the tissue and species of the bird, the median BPA concentrations in brains and fat were 2–5 and 3–22 times lower compared to muscles, livers and kidneys, respectively, with the greatest differences between fat, and the livers and muscles of razorbills. BPA concentrations in internal tissues were most often correlated with the concentrations of this compound in the blood and intestines, and the least frequently with those in the lungs (Table 2). The strongest and statistically significant relationships were obtained between BPA concentrations in the kidneys and in the intestines and blood. In turn, the concentrations of this compound in muscles were negatively and statistically significantly correlated with both intestinal and lung concentrations.

4.2. 4-nonylphenol (4-NP)

4-nonylphenol was determined in all muscles, livers and kidneys samples, while concentrations below the limit of quantification were found in 55% and 38% of fat and brains samples, respectively (Table 1). In all bird species, the highest concentrations of 4-NP were determined in the livers (60.7–525.8 ng·g^{-1} dw), muscles (26.0–476.4 ng·g^{-1} dw) and kidneys (29.3–469.2 ng·g^{-1} dw) with the highest medians in the muscles. The lowest concentrations of 4-NP, as in the case of BPA, were determined in the fat (<0.1–57.0 ng·g^{-1} dw) and brains (<0.1–93.2 ng·g^{-1} dw with one extreme value of 272.5 ng·g^{-1} dw for the razorbill) of all bird species. The median concentration of 4-NP in muscles was 2 to 3 times higher in relation to the kidneys and, respectively, an order and two orders of magnitude higher than in the fat and brains. The largest differences in the median concentrations of 4-NP, as much as 68-fold, were obtained between the muscles and fat of the long-tailed duck. The concentrations of 4-NP in internal tissues were correlated with the concentrations of this compound in the intestines, lungs and blood (Table 2). In the case of the intestines, the strongest correlations of 4-NP concentrations occurred with those in fat (negative) and muscles (positive). In turn, the concentrations of this compound in the lungs demonstrated the strongest correlations (in both cases positively) with the concentrations in the livers and muscles. However, the correlations between the concentrations of 4-NP in the blood and those in other internal tissues were the weakest and none of them was statistically significant.

Table 1
Characteristics of the concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in brains, subcutaneous fat, kidneys, livers, and pectoral muscles [ng·g^{-1} dw] of the goosanders, long-tailed ducks and razorbills by-caught in the Southern Baltic region in winter (2014–2016).

Compound		Brains	Fat	Kidneys	Livers	Muscles
Goosander *Mergus merganser*						
BPA	min	<0.4	<0.4	76.6	20.6	23.6
	max*	<0.4	54.7	298.6	131.8	121.3
	x	–	25.6	130.9	87.0	63.6
	md	–	14.5	96.5	102.7	51.9
	SD	–	20.0	71.0	42.7	33.2
4-*t*-OP	min	2.6	0.7	11.5	9.8	4.9
	max*	249.7	173.7	105.1	34.0	24.4
	x	57.6	34.7	29.2	20.1	14.7
	md	25.9	13.7	17.5	20.0	12.2
	SD	75.4	53.9	29.1	7.2	7.0
4-NP	min	<0.1	<0.1	84.3	91.6	142.9
	max*	93.2	10.4	320.5	331.9	372.9
	x	42.3	5.1	173.6	205.1	249.0
	md	50.0	4.0	166.6	197.0	244.7
	SD	32.3	3.3	64.5	79.9	72.6
n		8	8	8	8	7
Long-tailed duck *Clangula hyemalis*						
BPA	min	<0.4	<0.4	<2.0	<2.0	<2.0
	max*	17.5	41.7	836.1	224.2	223.0
	x	13.2	10.8	119.3	85.6	69.7
	md	14.3	7.1	73.1	65.4	60.9
	SD	4.0	9.2	151.5	57.1	37.4
4-*t*-OP	min	5.2	4.4	<0.5	<0.5	<0.5
	max*	257.6	89.7	37.1	698.8	63.2
	x	61.0	19.3	13.9	57.1	15.0
	md	36.9	14.1	13.5	23.4	12.2
	SD	64.0	18.0	7.0	130.5	12.2
4-NP	min	<0.1	<0.1	29.3	60.7	26.0
	max*	88.7	57.0	469.2	525.8	476.4
	x	25.7	13.2	146.5	203.1	266.1
	md	14.0	3.9	117.7	162.3	264.9
	SD	27.5	19.4	100.4	113.7	115.6
n		28	28	30	30	29
Razorbill *Alca torda*						
BPA	min	<0.4	<0.4	39.6	23.8	43.0
	max*	37.4	9.6	123.6	318.2	185.5
	x	24.1	4.3	64.4	125.8	98.6
	md	26.6	4.2	48.6	91.7	94.1
	SD	11.4	2.2	28.5	83.7	41.6
4-*t*-OP	min	15.2	4.1	<0.5	9.9	14.1
	max*	341.1	97.8	32.2	115.9	28.8
	x	89.3	39.1	15.1	27.6	19.3
	md	58.9	31.1	13.7	16.9	19.7
	SD	94.0	29.0	5.6	25.8	3.8
4-NP	min	<0.1	<0.1	33.1	91.2	96.4
	max*	272.5	23.4	213.3	447.3	445.5
	x	75.7	11.6	100.1	227.6	254.2
	md	43.9	10.3	76.6	178.5	246.7
	SD	89.1	8.0	54.2	109.2	110.2
n		14	15	15	15	15

n – number of samples; min – minimum value; max – maximum value; x – mean value; md – median value; SD – standard deviation, dw – dry weight; *maximum values also include outliers and extreme values.

4.3. 4-tert-octylphenol (4-t-OP)

4-*tert*-octylphenol was determined in all brains and fat samples, while in muscles, livers and kidneys, concentrations of this compound were below the limit of quantification in 4%, 6% and 17% of tissues, respectively (Table 1). Moreover, 4-*t*-OP was found to be inversely related to BPA and 4-NP. The highest concentrations of this compound in all bird species were determined in the brains (2.6–341.1 ng·g^{-1} dw),

Table 2
Spearman's correlations between the concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the intestines, lungs and blood, and the concentrations of phenol derivatives in the brains, subcutaneous fat, kidneys, livers and pectoral muscles.

Tissue-tissue	BPA	4-*t*-OP	4-NP
intestine-brain	–	GO: r = 0.68, p = 0.09, n = 7*	–
intestine-fat	RA: r = 0.34, p > 0.05, n = 14*	GO: r = 0.46, p > 0.05, n = 7*	LO: r = −0.75, p = 0.05, n = 7* RA: r = 0.39, p > 0.05, n = 12*
intestine-kidney	GO: r = −0.74, p < 0.05 n = 8 LO: r = 0.42, p < 0.05, n = 27 RA: r = 0.32, p > 0.05, n = 15*	GO: r = 0.32, p > 0.05, n = 7* LO: r = 0.49, p < 0.05, n = 21 RA: r = 0.50, p = 0.07, n = 14*	–
intestine-liver	–	GO: r = 0.32, p > 0.05, n = 7* RA: r = 0.47, p = 0.08, n = 15*	RA: r = 0.44, p = 0.09, n = 15*
intestine-muscle	GO: r = −0.71, p = 0.07, n = 7* RA: r = −0.65, p < 0.05, n = 15	–	GO: r = −0.39, p > 0.05, n = 7* LO: r = − 0.33 p = 0.08, n = 28* RA: r = 0.65, p < 0.05, n = 15
lung-brain	–	–	–
lung-fat	–	–	LO: r = 0.68, p = 0.09, n = 7*
lung-kidney	RA: r = 0.43, p > 0.05, n = 15*	LO: r = 0.32, p > 0.05, n = 22*	GO: r = −0.43, p > 0.05, n = 8*
lung-liver	–	–	RA: r = 0.73, p < 0.05, n = 15
lung-muscle	RA: r = −0.55, p < 0.05, n = 15	–	GO: r = 0.64, p > 0.05, n = 7* LO: r = 0.39, p < 0.05, n = 29 RA: r = 0.39, p > 0.5, n = 15*
blood-brain	–	LO: r = −0.35, p = 0.07, n = 28* RA: r = 0.67, p < 0.05, n = 14	–
blood-fat	GO: r = 0.50, p > 0.05, n = 7*	–	–
blood-kidney	GO: r = −0.71, p < 0.05, n = 8 LO: r = −0.52, p < 0.05, n = 18	GO: r = 0.55, p > 0.05, n = 8*	GO: r = −0.62, p > 0.05, n = 8* RA: r = 0.33, p > 0.05, n = 15*
blood-liver	GO: r = −0.57, p > 0.05, n = 8* LO: r = 0.35, p > 0.05, n = 19* RA: r = −0.36, p > 0.05, n = 15*	–	GO: r = −0.48, p > 0.05, n = 8*
blood-muscle	GO: r = −0.43, p > 0.05, n = 7* RA: r = 0.32, p > 0.05, n = 15*	GO: r = −0.96, p < 0.05, n = 7	GO: r = 0.50, p > 0.05, n = 7* RA: r = 0.38, p > 0.05, n = 15*

r - Spearman's correlation; p - level of significance; n – number of samples; GO – goosander; LO – long tailed duck; RA – razorbill; *correlations moderately strong or strong, but not statistically significant; with correlations close to statistical significance, the exact *p* value was given; for moderately strong or strong, but not statistically significant correlations, p > 0.05 was given; the concentrations in the underlined tissues were published in the study by Bodziach et al. (2021).

then the fat (0.7–173.7 ng·g^{-1} dw) and the livers (<0.5–71.2 ng·g^{-1} dw with three extreme values amounting to 698.8 ng·g^{-1} dw and 191.6 ng·g^{-1} dw in LO, and 115.9 ng·g^{-1} dw in RA). In contrast, the lowest concentrations of 4-*t*-OP were found in muscles (<0.5–63.2 ng·g^{-1} dw) and kidneys (<0.5–37.1 ng·g^{-1} dw with one extreme value of 105.1 ng·g^{-1} dw). Depending on the tissue and species of the bird, the median concentrations of 4-*t*-OP in the brains were 2 to 4 times higher than those of fat, muscles, livers and kidneys, with the greatest differences between the brain and kidneys of the razorbill. The concentrations of 4-*t*-OP in internal tissues were most often correlated with the concentrations of this compound in the intestines and blood (Table 2). In contrast, lung concentrations were only correlated with concentrations in the kidneys. In the case of the intestines, the strongest correlations of 4-*t*-OP concentrations occurred with those in the brain and kidneys. In turn, the concentrations of this compound in the blood were the strongest and were statistically significantly correlated with the concentrations in the brains and muscles.

5. Discussion

5.1. Distribution of bisphenol A (BPA), 4-tert-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) in the internal tissues of birds

The tested birds, i.e. goosanders (GO), long-tailed ducks (LO) and razorbills (RA), live in an environment contaminated by bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) (Bodziach et al., 2021). The exposure of birds to phenol derivatives present in the birds' food and air and the ability of these compounds to penetrate biological barriers results in their transfer into the bloodstream and then distribution to other internal tissues. This is indicated by the many correlations measured between the concentrations of phenol derivatives in the brains, fat, kidneys, livers and muscles, and the concentrations of these compounds in the blood, intestines and lungs (Table 2). These dependencies indicated that the pathway of penetration of phenol derivatives may determine the distribution of these compounds to specific organs. The concentrations of phenol derivatives in the lungs were most often correlated with their concentrations in the kidneys and muscles, and in the case of 4-NP also in the livers. On the other hand, the concentrations of the studied xenobiotics in the intestines were correlated with the concentrations of these compounds in practically all other tissues (Table 2). Therefore, it seems that the penetration of phenol derivatives by inhalation may limit their flow to a smaller number of tissues. While penetration of these compounds by the alimentary route can support their distribution throughout the body.

Although phenol derivatives were distributed throughout the birds' organisms (Table 2), each of the compounds showed a specific affinity for different internal tissues (Fig. 1a, b, c). In each bird species, 4-NP accumulated most in the muscles, followed by the livers and kidneys (Kruskal Wallis test, p < 0.05; Fig. 1c). BPA concentrations showed greater variability and, depending on the species of bird, the greatest accumulation occurred in the livers or kidneys, and then in the muscles (Kruskal Wallis test, p < 0.05; Fig. 1a). On the other hand, a different pattern was observed for 4-*t*-OP, the highest concentrations of which were determined in brains, and then in fat and livers (Kruskal Wallis test, p < 0.05; Fig. 1b). Xenobiotics accumulate selectively in specific tissues as a result of binding to proteins, active transport or dissolution in lipids, which consequently determines their distribution (Lehman-McKeeman, 2008). As discussed earlier, BPA and 4-NP have a greater ability to bind to plasma proteins compared to 4-*t*-OP (Bodziach et al., 2021). One of the functions of plasma proteins is to transport nutrients, but also xenobiotics to organs (Lehman-McKeeman, 2008). Thus, the presence of the highest concentrations of BPA and 4-NP in the blood in the form bound to proteins may explain their distribution to the kidneys, livers and muscles, thus becoming a factor reducing the penetration of lipophilic contaminants into the brains (Lehman-McKeeman, 2008). In contrast, the lower degree of binding of 4-*t*-OP with proteins leads to its dissolution in high fat tissues, i.e. subcutaneous fat and the brains.

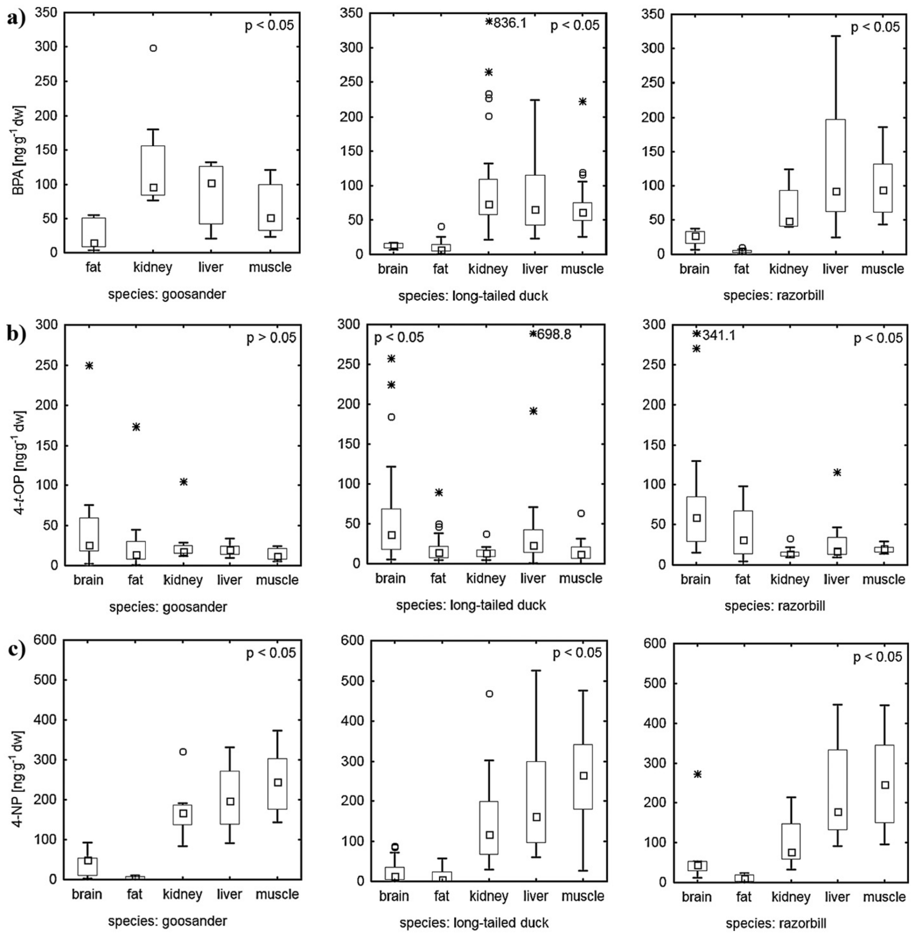

Fig. 1. Box and whiskers plot concentrations of a) bisphenol A (BPA), b) 4-*tert*-octylphenol (4-*t*-OP) and c) 4-nonylphenol (4-NP) between the individual tissues of goosanders, long-tailed ducks and razorbills.

5.2. Phenol derivatives in internal tissues

5.2.1. Liver and kidneys

Concentrations of BPA and 4-*t*-OP in the livers of long-tailed ducks and razorbills were correlated with concentrations in the kidneys (LO: $r = 0.33$, $p = 0.08$, $n = 27$ and RA: $r = 0.72$, $p < 0.05$, $n = 14$ respectively for BPA and 4-*t*-OP). This reflects an important pathway for the transformation and elimination of phenol derivatives from avian organisms. The liver and kidneys possess great ability to bind many substances and concentrate more pollutants than any other organ. The liver is the most metabolically active organ and is responsible for the biotransformation of lipophilic xenobiotics into more polar forms for their subsequent elimination from the body. In turn, the kidneys excrete more pollutants than any other organ (Lehman-McKeeman, 2008). The selective binding of metals and persistent organic pollutants in liver has been described in many studies (Braune and Norstrom, 1989; Kubota et al., 2013; Watanabe et al., 2005). Klaassen and Shoeman (1974) found that 30 min after administration of lead to rats, its concentration in the liver was 50 times higher than that in plasma. It has also been shown that selective sequestration in the liver may limit the subsequent transfer of xenobiotics in birds from the female to the egg (Braune and Norstrom, 1989; Reindl et al., 2019, 2020). Although phenol derivatives accumulated in the liver and kidneys to varying extents, these tissues were among the most burdened. This indicates their selective retention in both organs. Moreover, the livers were the only organs in which the concentrations of the three compounds were positively correlated with each other (4-NP and 4-*t*-OP LO: $r = 0.46$, $p < 0.05$, n = 27; RA: $r = 0.51$, $p < 0.05$, $n = 15$; 4-NP and BPA LO: $r = 0.52$, $p < 0.05$, $n = 29$; RA: $r = 0.70$, $p < 0.05$, n = 15; 4-*t*-OP and BPA RA: $r = 0.43$, $p = 0.1$, n = 15). This shows that the liver is the main and only common storage site for these compounds, thereby underlining its important function in the selective sequestration of the studied EDCs.

Comparison of the concentrations of phenol derivatives in the internal tissues of waterbirds revealed the differential effect of many factors (Fig. 2a, b, c, d, e). Although BPA has the highest production and emission into the environment, the highest concentrations in the livers and kidneys of birds were found for 4-NP (Kruskal Wallis test, $p < 0.05$; Fig. 2c, d). This may be due to the biotransformation of nonylphenol ethoxylates (NP precursors) into the parent compound in the organisms. Part of the 4-NP emitted into the marine environment along with discharged water from wastewater treatment plants is released in the form of its ethoxylates (Ahel et al., 1994a). Ultimately, however, the 4-NP pool in the seas and its components increases through degradation of the ethoxyl chain in waters (Ahel et al., 1994b), but also as a result of biotransformation in organisms (Korsman et al., 2015). The difference in the amounts of accumulation for the phenol derivatives in the livers and kidneys may also indicate varying degrees of elimination from the body, resulting from different affinity to fatty tissue. BPA is moderately lipophilic, while both alkylphenols have a strong and similar tendency to bind to fatty tissue (the log K_{ow} partition coefficient for BPA is 3.3; for 4-*t*-OP 5.3 and for 4-NP 5.9; Grover, 2008). BPA, in relation to alkylphenols, is also the most efficiently eliminated xenobiotic from the organisms of herring gulls (Staniszewska et al., 2014). BPA concentrations in their guano were found to be an order and two orders of magnitude higher, respectively, compared to 4-NP and 4-*t*-OP.

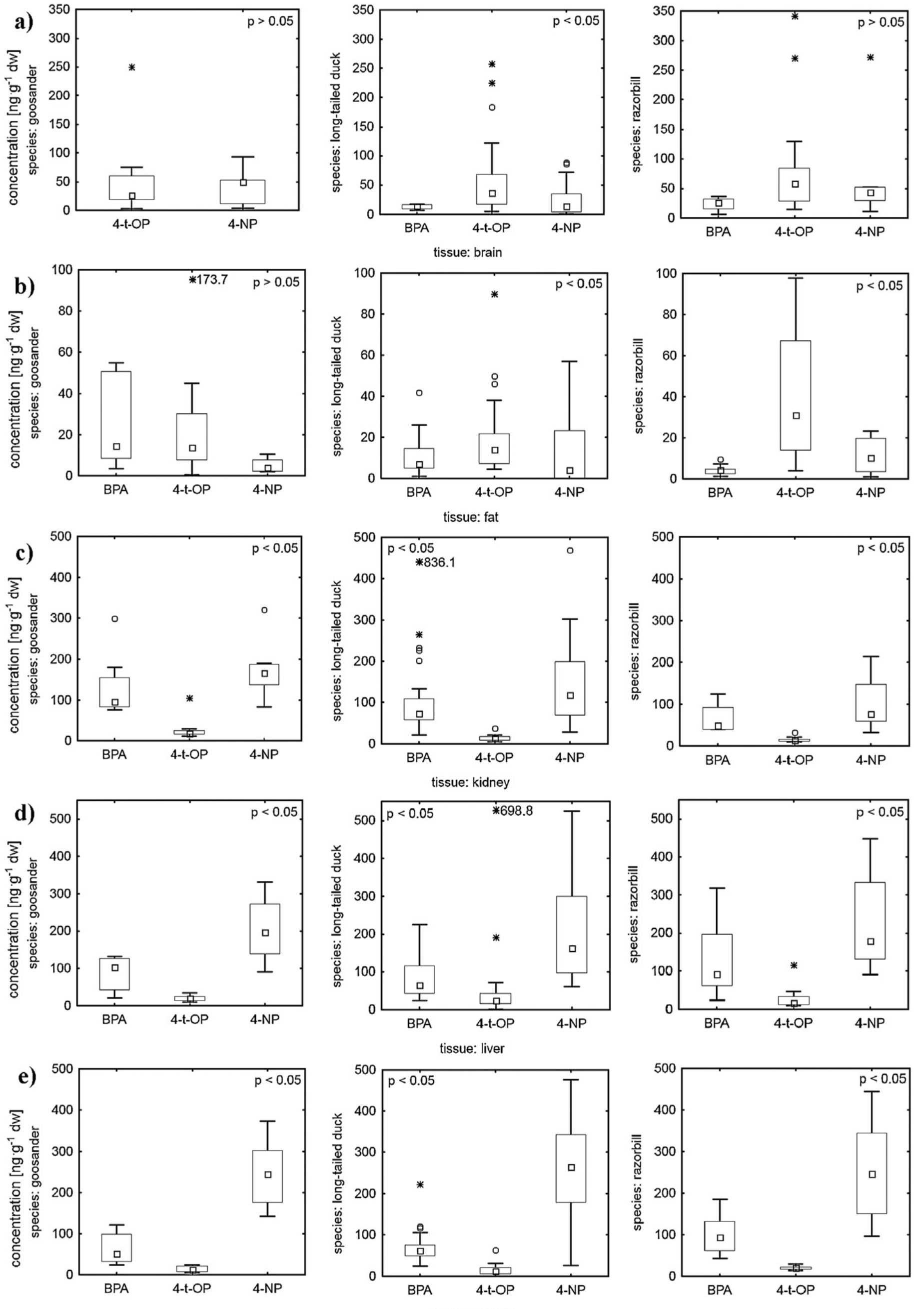

Fig. 2. Box and whiskers plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in a) brains, b) subcutaneous fat, c) kidneys, d) livers and e) pectoral muscles of goosanders, long-tailed ducks, and razorbills.

On the other hand, the accumulation of xenobiotics in livers and kidneys is not always safe for the organisms and may cause chronic toxicity. The negative effect of phenol derivatives on the kidneys and livers of birds is not known to the best of our knowledge, but has been well documented in numerous studies for various species of fish (Traversi et al., 2014; Maradonna et al., 2015; Faheem and Lone, 2017; Sharma and Chadha, 2017). Therefore, potential changes in livers and kidneys caused by high accumulation of the tested xenobiotics in these tissues should be expected, as they have the potential to do so even at environmentally relevant concentrations. This is especially true since phenol derivatives constitute only a fraction of the mixture of xenobiotics taken up by birds with food and air. It is for this reason that the influence of phenol derivatives on some of the most important organs of waterbirds, such as the liver and kidneys, should be thoroughly analysed in the future.

5.2.2. Blood-brain barrier

In the brains, the concentrations of both alkylphenols were correlated with each other (LO: $r = 0.57$, $p < 0.05$, $n = 20$; RA: $r = 0.94$, $p < 0.05$, $n = 6$), indicating a similar distribution pathway for these compounds, probably as a result of similar lipophilicity. The highest concentrations in bird brains were achieved by 4-*t*-OP (Fig. 2a), although the differences between the concentrations of individual compounds were only found to be statistically significant (Kruskal Wallis test, $p < 0.05$) in long-tailed ducks. Despite the previously suggested reason for the greatest distribution of 4-*t*-OP to the brains compared to BPA and 4-NP and other tissues, these results are both unexpected and surprising. Currently 4-*t*-OP tends to be characterised by the lowest concentrations (Staniszewska et al., 2014) in most abiotic and biotic elements of the marine environment (water, phyto- and zooplankton, mussels, fish) in relation to BPA and 4-NP, which results from its lower production and emission into the environment (Ying et al., 2002; Rubin, 2011). Previous studies on the pathways of phenol derivatives to the organisms of birds (Bodziach et al., 2021) have already noted that the concentrations of BPA and 4-NP compared to 4-*t*-OP were at their highest in the intestines and lungs, indicating a greater degree of pollution of the environment with these compounds. On the contrary, in blood, which is the carrier of xenobiotics to various tissues, the highest concentrations occurred with 4-*t*-OP. Kawai et al. (1988) found that the lipid composition of individual tissues may influence the accumulation of organochlorine compounds in striped dolphins, and that some of the contaminants have a clear tendency to bind to phospholipids, while others to triglycerides. The authors also showed that both blood and brain are characterised by a higher content of phospholipids in relation to triglycerides, by 6 and 38 times respectively. It is therefore likely that 4-*t*-OP has a strong affinity for phospholipids, binding to them in the blood, with which it is then transported to the brain. This seems to be confirmed by the statistically significant correlation between the concentrations of 4-*t*-OP in the blood and brains of birds, which was obtained only for this compound (Table 2). The brain is one of the most sensitive and susceptible organs to the action of endocrine disrupting compounds. 4-*t*-OP has been shown to influence the mating behaviour of fish, causing a statistically significant decrease in the number of contacts and a decrease in the mean duration of each contact in male-female pairs (Campbell, 1999). This xenobiotic therefore influences animal behaviour which is crucial for the survival of the population. Furthermore, abnormal brain development and impaired cognitive, social and anxiety behaviours were observed in the offspring of female mice exposed to 4-*t*-OP (Tran et al., 2020). The studies also proved that the estrogenic potential of 4-*t*-OP is comparable to 17β-estradiol, and its potency is several times higher than that of 4-NP (White et al., 1994; Senthil Kumaran et al., 2011; Traversi et al., 2014). Thus, despite the lower production and emission into the environment of 4-*t*-OP, its potential to accumulate in target organs and to induce undesirable effects is greater than expected. In turn, the lower concentrations of BPA and 4-NP in the brain, in spite of their higher production and emission into the environment, indicate that this organ is at least to some extent protected against their negative effects. Our results show that in the future more attention should be paid to 4-*t*-OP which, compared to BPA and 4-NP, seems to have a greater impact on the nervous system of waterbirds.

5.2.3. Subcutaneous fat and pectoral muscles as a storage site

The accumulation of xenobiotics in tissues, i.e. fat and muscles, is a protective function of the organism, as it is believed that the pollutants stored in these places are toxicologically inactive until they are remobilised. Moreover, the concentration of the substance in plasma is thus reduced and, consequently, also at potential negative impact sites (Lehman-McKeeman, 2008). In muscles, the highest accumulation in relation to the other compounds was found in 4-NP (Kruskal Wallis test, $p < 0.05$; Fig. 2e), while the fat of each bird species demonstrated the highest accumulation of the other compound (Fig. 2b). In addition to this, in fat, the concentrations of 4-*t*-OP and 4-NP were positively correlated with each other (LO: $r = 0.79$, $p < 0.05$, $n = 7$; RA: $r = 0.79$, $p < 0.05$, $n = 12$) which, as in the case of the brain, can be explained by the similar lipophilic properties of alkylphenols. In muscles, however, the concentrations of 4-*t*-OP and 4-NP were negatively correlated with each other (LO: $r = -0.47$, $p < 0.05$, $n = 27$) and this probably reflects the previously described different affinity for this tissue, where 4-NP demonstrated the highest potential for bioaccumulation. Nevertheless, all phenol derivatives accumulated to varying degrees in muscle and fat. This may indicate a heavy burden on liver function, as a result of which some phenol derivatives are temporarily transported to storage, i.e. fat and muscles. This seems to be confirmed by the calculated liver/muscle ratios for phenol derivatives being >1. Such values for BPA, 4-*t*-OP and 4-NP were obtained in 53%, 70% and 41% of the tested animals, respectively. They testify to the chronic exposure of birds to phenol derivatives as a result of contamination of the areas where they stayed for a long time. It is therefore possible that the constant load of a mixture of various pollutants from the environment to the organisms of birds results in an increased accumulation of xenobiotics in tissues, i.e. fat and muscles, protecting the organism from being deposited in other target tissues. This is indicated by the case of the highest BPA concentrations compared to alkylphenols in the fat of goosanders (Fig. 2b) and the absence of this compound in the brains of these birds (Fig. 2a). This also seems to be confirmed by the increase in BPA concentration in subcutaneous fat along with its higher content (RA: $r = 0.52$, $p = 0.06$, $n = 14$). It was also found that with decreasing intestinal fat content, the concentration of 4-*t*-OP in the brains of birds increased (RA: $r = -0.61$, $p < 0.05$, $n = 14$). This reflects the protective function of the storage of xenobiotics in fat and small amounts or complete absence may therefore represent greater exposure for sensitive tissues such as the brain. However, compounds accumulated in muscles and fat can be released from them and reintroduced into the systemic circulation when some substances are removed from it (Lehman-McKeeman, 2008). In this study, BPA and 4-NP concentrations in muscles were correlated with those in kidneys (RA: $r = 0.43$, $p = 0.11$, $n = 15$ and RA: $r = -0.56$, $p < 0.05$, $n = 15$ for BPA and 4-NP). In contrast, the concentrations of 4-*t*-OP in the kidneys were only moderately correlated with those in the fat (RA: $r = -0.31$, $p = 0.27$, $n = 14$). This indicates the probable remobilization of phenol derivatives from storage sites in order to undergo further transformations and eventual elimination from the organism.

5.3. Factors determining the amount of phenol derivatives accumulation

5.3.1. Species specific differentiation

Birds being predators at the top of the trophic pyramid are particularly exposed to high concentrations of xenobiotics in their organisms due to the biomagnification of pollutants in the trophic chain (Burger and Gochfeld, 2004). As BPA is a moderately lipophilic compound and alkylphenols are readily lipophilic, dietary habits and position in the trophic chain may determine the final phenol burden of the bird species studied. Based on the analysis of the stable nitrogen isotope (δ15N) in previous studies on the same birds, it was shown that of the three selected species, the highest trophic level was occupied by goosanders feeding on predatory fish (Bodziach et al., 2021). The other two species of birds were found to be on a lower trophic level to goosanders, but relatively similar in relation to each other, with razorbills feeding largely on herring and long-tailed ducks consuming mainly benthic organisms. Unfortunately, due to the lack of data on the concentrations of phenol derivatives in the birds' food, it was only possible to calculate biomagnification factors (BMF) for razorbills and long-tailed ducks from the Gulf of Gdansk. The BMF for both BPA (0.41–2.73) and alkylphenols (4-*t*-OP 0.09–1.03 and 4-NP 0.88–6.42) turned out to be statistically significantly higher in long-tailed ducks than in razorbills (Mann-Whitney *U* test, $p < 0.05$), which can be attributed to the eating habits of both species. Phenol derivatives readily bind to solid particles, therefore high concentrations of these compounds are determined in the sediments of the coastal environment (Koniecko et al., 2014; Staniszewska et al., 2016b), and because they are amphiphiles, they can adsorb to both organic and mineral particles (David et al., 2009). As a result, benthic fauna may contain higher concentrations of BPA and alkylphenols compared to pelagic organisms (Tanabe, 2002; Korsman et al., 2015; Peng et al., 2018), resulting in higher biomagnification in zoobenthos consumers than in pelagic fish.

Among the phenol derivatives, 4-NP demonstrated the highest potential for biomagnification in the trophic chain, while 4-*t*-OP showed the lowest (Kruskal Wallis test, $p < 0.05$; Fig. 3). The results of previous studies with the same birds showed that the concentrations of 4-NP were statistically significantly higher in the intestines of predatory goosanders compared to the other two species (Bodziach et al., 2021), but this study did not distinguish 4-NP in any of the goosander tissues as

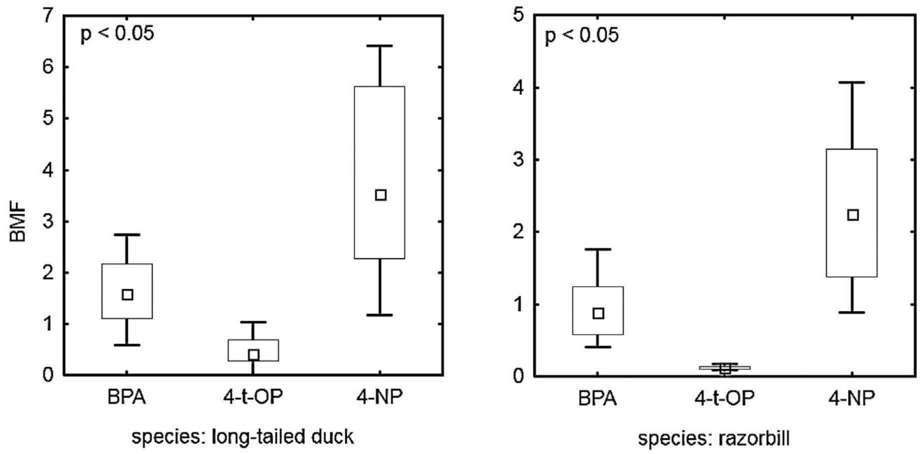

Fig. 3. Biomagnification factors for bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in long-tailed ducks and razorbills.

opposed to long-tailed ducks and razorbills (Kruskal Wallis test, $p > 0.05$). All three phenol derivatives tested returned the highest concentration ranges and/or medians among these three species for the kidneys of goosanders, although these differences were found to be statistically significant only in the case of BPA (Kruskal Wallis test, $p < 0.05$; Fig. 4). Moreover, when summing up the concentrations from all tissues for each species, it turned out that the overall burden on the organism among the different bird species is similar (Kruskal Wallis test, for each compound $p > 0.05$). Since bioaccumulation is a product of intake, metabolism and elimination (Weisbrod et al., 2009), it is likely that elimination played a major role in the absence of differences between these individual bird species feeding at different trophic levels. In this case, the detoxification and elimination mechanisms are more active in goosanders, which are exposed to increased concentrations of phenol derivatives. It has been shown that the activity of xenobiotic metabolising enzymes can vary by up to 100-fold between different bird species (Walker, 1990). This phenomenon is well reflected in our comparison of the concentrations of phenol derivatives in the intestines, lungs (Falkowska et al., 2017), muscles and livers of herring gulls (Staniszewska et al., 2014) with the concentrations of these compounds in the intestines, lungs (Bodziach et al., 2021), muscles and livers of goosanders, long-tailed ducks and razorbills. Herring gulls feeding often in landfills (Kihlman and Larsson, 1974; Meissner and Betleja, 2007) are exposed to extremely high doses of phenol derivatives (Falkowska et al., 2017), compared to birds that eat only marine food. Comparison of the maximum values showed that the intestines and lungs of goosanders, long-tailed ducks and razorbills, depending on the compound and species, were characterised by concentrations from 2 to as much as 1000 times lower than those of herring gulls. A different tendency concerned the livers and muscles, which were characterised by 3 to 20 times higher concentrations of phenol derivatives in the species from the present study compared to herring gulls. The effective elimination of phenol derivatives in herring gulls has been confirmed by high concentrations of these compounds in guano (Staniszewska et al., 2014). Some bird species have a clear tendency to accumulate more pollutants as they lack effective detoxification systems, while omnivorous animals such as gulls are better adapted to their elimination (Fossi et al., 1995).

5.3.2. The condition of birds

During periods of high energy demand of organism, e.g. during migration and breeding, fat which is stored in the body is metabolised, and contaminants are mobilised and transported in the bloodstream (Henriksen et al., 1996; Perkins and Barclay, 1997; Evers et al., 2005). In this study, most of the animals were in good condition, probably due to the accumulation of fat reserves before the spring-summer period of migration and reproduction. However, even in healthy birds, the transformation and distribution of phenol derivatives may be different. Both in the case of BPA and 4-NP, the concentration of these compounds in the livers increased together with the content of intestinal fat (RA: $r = 0.52$, $p < 0.05$, $n = 15$ and RA: $r = 0.54$, $p < 0.05$, $n = 15$ respectively for BPA and 4-NP). Before entering the systemic circulation, xenobiotics transported with blood from the gastrointestinal tract are first directed to the liver (Lehman-McKeeman, 2008). Thus, good bird nutrition resulting in a high intestinal fat content is likely to lead to

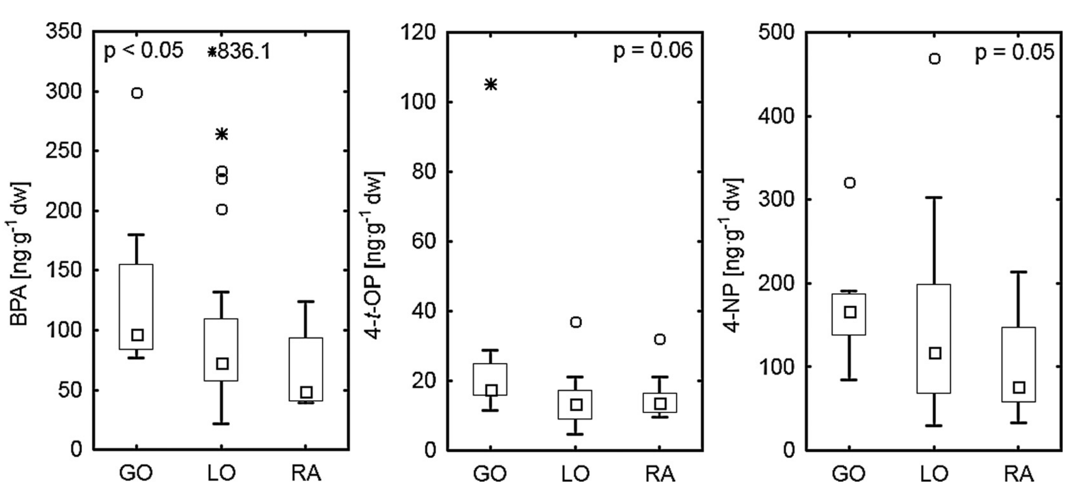

Fig. 4. Differences of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) concentrations in kidneys of goosanders (GO), long-tailed ducks (LO) and razorbills (RA).

more efficient transfer of lipophilic compounds from the gastrointestinal tract to the liver rather than direct excretion from the gut. This may result in a greater burden on liver function and exposure of birds to the undesirable effects of EDCs. The accumulation of alkylphenols was also influenced by the body weight of the birds. The concentration of 4-*t*-OP in muscles (RA: $r = 0.30$, $p > 0.05$, $n = 15$) and 4-NP in livers (RA: $r = 0.31$, $p > 0.05$, $n = 15$) was observed to increase with the weight of the birds, perhaps reflecting the importance of long-term exposure to pollutants (Donaldson et al., 1997; Vorkamp et al., 2004; Szumiło-Pilarska et al., 2016).

6. Conclusions

To the best of our knowledge, we are discussing for the first time the wide distribution of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol in the most important internal tissues of waterbirds including subcutaneous fat, pectoral muscles, livers, kidneys and brains. These tissues are the most sensitive to the negative influence of phenol derivatives and/or are responsible for their storage, biotransformation and elimination. After the ingestion of phenol derivatives into the organisms of birds via the alimentary route, distribution took place practically throughout the organism, while the respiratory exposure limited the distribution of these compounds mainly to the kidneys and muscles. Distribution pathways for the individual xenobiotics differed and were most likely determined by their potential for protein binding and lipid dissolution. On the other hand, the amount of accumulation in individual tissues and the associated exposure of birds to the action of endocrine disrupting phenols in these animals seem to be a complex process dependent, among other things, on: the lipophilicity of these xenobiotics, their affinity to various compounds found in adipose tissue, the condition and eating habits of birds, and their ability to adapt to and remove pollutants.

The greatest biomagnification of BPA and alkylphenols occurred in long-tailed ducks, i.e. birds feeding on benthic organisms, indicating that these organisms may be more contaminated with phenol derivatives than those from the pelagic zone of the open waters of the Southern Baltic region. In turn, among the compounds, 4-NP was characterised by the highest potential for biomagnification, which could be related to the biotransformation of its ethoxylates taking place in birds. However, the biomagnification of phenol derivatives in the tissues of birds may be underestimated and turn out to be higher than the values we presented. This may be especially true in the case of 4-*t*-OP, which accumulated the most in the brains and not in the muscles, for which the biomagnification coefficients were calculated.

Different target sites for the accumulation of the individual phenol derivatives indicated that each of the tested compounds may have different health effects in birds. BPA and 4-NP, those with the highest concentrations in the environment and also the highest concentrations in the livers and kidneys of birds, may have the greatest potential to disturb the proper functioning of these organs. Thus, they have the potential to impair the birds' most important protective systems responsible for metabolism and pollutant removal. On the other hand, 4-*t*-OP, despite low concentrations in the environment, is transported in the organisms of birds directly from the blood to the brain, where it accumulates the most in relation to other compounds. Thus, 4-*t*-OP may possess greater potential to disturb the development and behaviour of these animals, especially individuals with low fat content. However, an increased amount of subcutaneous fat, which contributes to the birds' higher condition, can protect their brains, especially in the case of BPA. Nevertheless the good condition of birds in the non-breeding season, which is also determined by the intestinal fat stores, supports the unfavourable process of taking phenol derivatives from the intestines to the liver, instead of allowing them to be excreted directly from the gastrointestinal tract.

Seabirds experience a lot of stress, which reflects a drastic decline in their world population, and the accumulation and impact of pollutants on their organisms is one of the external stimuli responsible for this decline. As the effects of exposure to phenol derivatives in seabirds are unknown, we hope that the results of this study are an important step towards further exploring this topic. The main sites of phenol derivatives accumulation which we have identified may indicate the direction of research into the potential effects of these xenobiotics on birds. At the same time, knowledge of BPA and alkylphenol concentrations in the individual tissues of these animals forms a good basis for examining the negative effects caused by environmental concentrations of these compounds.

CRediT authorship contribution statement

Karina Bodziach: Resources, Conceptualization, Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Marta Staniszewska:** Resources, Conceptualization, Validation, Writing – original draft, Writing – review & editing, Supervision. **Lucyna Falkowska:** Writing – review & editing. **Iga Nehring:** Investigation, Writing – review & editing. **Agnieszka Ożarowska:** Investigation, Writing – review & editing. **Grzegorz Zaniewicz:** Investigation, Writing – review & editing. **Włodzimierz Meissner:** Investigation, 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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STATEMENTS OF CO – AUTHORS

Bodziach K., Staniszewska M., Falkowska L., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2021. Distribution path of endocrine disrupting phenolic compounds in waterbirds (*Mergus merganser*, *Clangula hyemalis*, *Alca torda*) from southern Baltic. Science of The Total Environment 793, 148556, doi: 10.1016/j.scitotenv.2021.148556.

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- sformułowanie problemu badawczego,
- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- analizę statystyczną wyników,
- graficzne i statystyczne przedstawienie wyników pochodnych fenolu,
- interpretację pozyskanych wyników pochodnych fenolu w świetle posiadanej wiedzy oraz zgromadzonego przeglądu literatury przedmiotowej,
- tworzenie manuskryptu,
- pełnienie funkcji autora korespondencyjnego.

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- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną na każdym etapie tworzenia manuskryptu, w szczególności: w interpretacji wyników i redagowaniu manuskryptu.

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- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną.

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- przygotowanie materiału biologicznego do analiz chemicznych.

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- przygotowanie materiału biologicznego do analiz chemicznych.

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- pomoc w interpretacji wyników i redagowaniu manuskryptu.

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Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic

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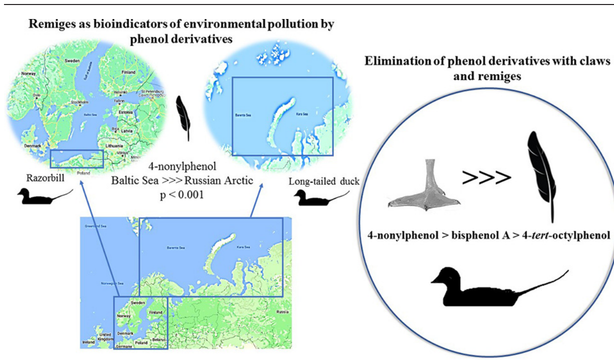
HIGHLIGHTS

- Claws and remiges are an important route of 4-NP removal from bird systems.
- 4-*t*-OP is the most poorly removed phenol derivatives from claws and remiges.
- In birds, there is greater phenol derivatives elimination via claws than remiges.
- 4-NP and bird remiges have potential for environmental pollution studies.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper investigates the effectiveness of phenol derivatives removal from bird organisms via claws and remiges, and performs a preliminary assessment of the usefulness of these epidermal products for environmental biomonitoring and estimating bird exposure levels. Concentrations of bisphenol A (BPA) and alkylphenols: 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) were determined in claws and remiges of long-tailed ducks *Clangula hyemalis* and razorbills *Alca torda*, obtained during a by-catch in the winter period (2014–2016) in the Southern Baltic region. For razorbills, the Baltic is a permanent habitat, while long-tailed ducks are migratory and stay in the Southern Baltic only during the non-breeding season. Their remiges are replaced in the Arctic seas of Siberia.

The removal of phenol derivatives, depending on the compound and the epidermal product, ranges from 12 % to 34 %. Among these compounds, in both bird species, the highest degree of elimination was observed for 4-NP in remiges (<0.1–656.0 $ng \cdot g^{-1}$ dw) as well as claws (<0.1–338.6 $ng \cdot g^{-1}$ dw). On the other hand, the least removed compound in both the long-tailed duck and the razorbill was 4-*t*-OP. The removal of phenol derivatives from claws in both bird species was at the same level. However, 4-NP concentrations were found to be statistically significantly higher in razorbill remiges compared to those of the long-tailed duck ($p < 0.05$).

Comparison of concentrations in the remiges of the long-tailed duck and the razorbill, moulted in two different environments with different levels of pollution and distances from sources, indicated that the Baltic Sea is approximately 3 times more polluted with 4-NP than the marine areas of the Russian Arctic. This demonstrates the potential for the use of 4-NP and remiges as indicators of environmental pollution with phenol derivatives.

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1. Introduction

Bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) are synthetic compounds, classified as phenol derivatives, and are used primarily in the production of plastics and surfactants. They are found in, among other things, packaging, the lining of tins and cans, dental fillings, medical and sports equipment, CDs, detergents, cosmetics, adhesives, paints and lubricants. As a result of the production, use and processing of the above, phenol derivatives are released into the environment (Corrales et al., 2015; Ghazali and Johari, 2015; Acir and Guenther, 2018). Transported by rivers to the seas and oceans (Ahel et al., 1994; Staniszewska et al., 2015a) and emitted into the atmosphere (Ying et al., 2002; Xie et al., 2006; Vasiljevic and Harner, 2021), phenol derivatives eventually accumulate in sediments (Koniecko et al., 2014) and in the trophic chain (Staniszewska et al., 2014, 2015b, 2016a, 2016b, 2017; Graca et al., 2021), birds being the organisms with the highest concentrations found so far (Falkowska et al., 2017; Bodziach et al., 2021a, 2021b).

Bisphenol A and alkylphenols are endocrine disrupting phenolic compounds, which imitate hormones and disrupt the proper functioning of organisms by e.g. reduced fertility, sex inversion, damage to organs and embryos, development of congenital malformations and genital cancers (Levy et al., 2004; Oehlmann et al., 2006; Arslan et al., 2007; Lam et al., 2011; Traversi et al., 2014; Bhandari et al., 2015; Faheem and Lone, 2017; Lee et al., 2018). Our previous work has shown that phenol derivatives accumulate in high concentrations in birds, especially in the tissues and organs which are particularly sensitive to their negative effects, such as the brain, liver or kidneys (Bodziach et al., 2021a). Therefore, it is important to assess whether birds are capable of removing the excess of these harmful compounds from their bodies. Both feathers and claws have been shown to be important pathways for removing many contaminants, including persistent organic pollutants, which mostly accumulate in adipose tissues (Jaspers et al., 2006; Behrooz et al., 2009; Burger et al., 2009; Espín et al., 2012; Szumiło-Pilarska et al., 2017; Grajewska et al., 2019). In the case of mercury, estimates show that feathers contain up to 90 % of the total body load due to the strong bond of this element to keratin (Braune and Gaskin, 1987; Agusa et al., 2005).

Birds have been a subject of intensive environmental research since the 1960s (Erwin and Custer, 2000), with scientists focusing mostly on the use of feathers as indicators of environmental pollution and successfully linking high concentrations of xenobiotics in feathers to local sources of these pollutants (Appelquist et al., 1985; Thompson et al., 1992; Dauwe et al., 2002; Adout et al., 2007; Burger et al., 2009; Jaspers et al., 2009; Gómez-Ramírez et al., 2017; González-Gómez et al., 2020). The potential of feathers as a non-invasive tool for assessing the pollutant load in birds has also been investigated. Several studies have in fact linked the concentrations of chemicals in bird feathers to concentrations in internal tissues (Agusa et al., 2005; Jaspers et al., 2006; Kim and Koo, 2008; Meyer et al., 2009). However, various methods of handling feathers were used, including different washing procedures (Hoff Brait and Antoniosi Filho, 2011; Jaspers et al., 2011), as well as testing various types and parts of feathers (Dauwe et al., 2003; Adout et al., 2007; Jaspers et al., 2011; Szumiło-Pilarska et al., 2017). All of these approaches produce different results and information and lead to varying interpretations. On the other hand, as far as the number of studies performed is concerned, the role of bird claws in removing pollutants remains overshadowed by feathers.

The objectives of this study were 1) to assess the potential of feathers and claws for removing bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol from birds, and 2) to determine whether feathers and claws can successfully serve as indicators of environmental contamination and/or body load with bisphenol A and alkylphenols. Free (unconjugated) forms of the compounds were determined in the study. The selected type of feathers was remiges because the studied species do not fly while these are being replaced. As a result, the concentrations assayed in these feathers can be linked with the specific area where the birds moult (Viain et al., 2014). The research was based on two bird species: the long-tailed duck (*Clangula hyemalis*) and the razorbill (*Alca torda*), both of which replace remiges in a simultaneous manner, thereby avoiding the problem of uneven distribution of concentrations which may be seen in successively growing feathers (Furness et al., 1986; Altmeyer et al., 1991). Each of the species moults remiges in a very different region with different environmental conditions and distances from potential sources of pollution.

2. Materials and methods

2.1. Characteristics of the studied species

The research was carried out on dead birds from a by-catch, collected from fishing nets in the winter periods of 2014–2016 in the Southern Baltic region. The birds were kept in the nets between 24 and 48 h with no visible signs of decomposition. Among them were 29 long-tailed ducks (15 from the Gulf of Gdansk and 14 from the Pomeranian Bay) and 15 razorbills from the Gulf of Gdansk. For razorbills, the Baltic is a permanent habitat, while long-tailed ducks are only found in the Southern Baltic during the non-breeding period, from October to April, and breed along the shores of the Arctic seas of Europe and Asia (Cramp and Simmons, 1977; Karwinkel et al., 2020). Following the breeding period, the long-tailed ducks moult completely in freshwater reservoirs which are located in the vicinity of the Arctic seas, and only afterwards do they arrive at their Baltic wintering grounds (Cramp and Simmons, 1977; Karwinkel et al., 2020). During the non-breeding season, long-tailed ducks mainly feed on zoobenthos (predominantly mussels), while razorbills feed exclusively on fish (Cramp, 1985; Stempniewicz, 1995).

In the post-breeding period, from August to October, adult birds of both species undergo complete moulting which involves the replacement of their entire set of feathers (i.e. all over their bodies). The flight feathers - remiges - are moulted simultaneously. Young birds replace feathers that cover the body before the coming of winter, but not the remiges, which are not moulted until the following year (Cramp, 1985; Cramp and Simmons, 1977). The wintering population of the long-tailed duck in the Baltic Sea declined by 65 % between 1992/1993 and 2007/2009 (Skov et al., 2011) and has been reclassified by the International Union for Conservation of Nature (IUCN) from 'Least Concern' to 'Vulnerable' (BirdLife International, 2022). The razorbill revealed significant increase in the Baltic and in its global population (Ottvall et al., 2009; BirdLife International, 2022) and it is not considered to be an endangered species receiving the "Least Concern" category from IUCN (Lavers et al., 2020).

2.2. Bird moulting areas

2.2.1. Baltic Sea

The semi-enclosed Baltic Sea in Northern Europe is small in volume, with a low water exchange rate (approx. 30 years) and a large drainage area. The average depth of the Baltic Sea is approx. 53 m, and its area, which is almost four times smaller than the catchment, is inhabited by over 85 million people (HELCOM, 2018). The South Baltic Sea is characterised by the highest human population density and anthropopressure (HELCOM, 2018). The Vistula and Oder rivers flow into this part of the sea, transporting pollutants from drainage areas which account for almost 90 % of Poland (Kot-Wasik et al., 2003; Pastuszak et al., 2018). The River Vistula terminates in the Bay of Gdańsk, while the River Oder flows into the Pomeranian Bay. These two water basins are special bird protection areas within the Natura 2000 network. In the non-breeding period, they are two of the most important places for birds in the Polish zone of the Baltic Sea (Durinck et al., 1994; Skov et al., 2011). According to regional reports issued by the Environmental Protection Inspectorates in Poland, the chemical condition at all of the test stations located within these reservoirs has been described as substandard (GIOŚ, 2020a, 2020b). The local sources of pollution in the Southern Baltic include, among others, pharmaceutical, textile, paper, chemical and refining industries, and leaks from dumped munitions (Bełdowski et al., 2016).

2.2.2. Arctic region of Europe and Asia

The Arctic region of Europe and Asia stretches from Norway and the Barents Sea in the west to the eastern part of Russia and the Sea of Okhotsk. The Arctic territory is dominated by the three main river systems of Russia: the Yenisei, the Lena and the Kolyma, which flow into the Kara Sea, the Laptev Sea and the East Siberian Sea, respectively (Stein, 2008). These areas are sparsely populated and barely urbanised. Depending on information sources and adopted borders, the population inhabiting the entire Arctic is estimated at 4–10 million (Andrew, 2014), with the Russian part amounting to approx. 2 million (https://www.thearcticinstitute.org/countries/russia/). However, this region is rich in crude oil and natural gas, as well as in fish and seafood, which is why mining and fishing industries are dynamically developed here. Local sources of pollution include, among others, dumped radioactive waste and parts of military infrastructure, industrial activities on the Kola Peninsula, mines, oil spills and outdated local sewage treatment plants. In addition, pollutants are transported from distant areas via currents, rivers and the atmosphere (AMAP, 1997, 2010, 2016). Arctic areas are important breeding sites for many bird species (Ganter and Gaston, 2013), as well as places where the replacement of feathers can take place before migrating to wintering grounds (Cramp and Simmons, 1977; Cramp, 1985).

2.3. Reagents

The solvents used for analyses (water, acetonitrile, methanol) were HPLC graded (Merck). Ammonium acetate (p.a.) and chloric acid (VII) were 70 % solutions (POCh). High purity (> 97 %) bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol standards were produced by SIGMA-ALDRICH. All vessels and instruments used for the preparation and labeling of samples were made of either glass or metal.

2.4. Preparing the biological material for analyses

The material collected for analysis consisted of primary flight feathers selected randomly from the birds, and all of the claws separated from the skin and tissue. The biological material was immediately frozen (−20 °C). Prior to analysis, both epidermal formations were washed in acetone using ultrasound for 10 min at 20 °C. Next, the biological material was freeze-dried and homogenised. The feathers and claws thus prepared were stored in borosilicate glass in a desiccator under constant conditions (temperature 20 °C ± 2 °C, humidity 45 % ± 5 %). The samples were then treated according to the method used in the works of Staniszewska et al. (2014), Nehring et al. (2017) and Bodziach et al. (2021b). Weighed samples of claws (0.1 g ± 10^{-3} g) and feathers (0.2 g ± 10^{-3} g) were extracted in an ultrasonic bath for 10 min at 20 °C in the following mixture: 8 cm^3 methanol, 2 cm^3 0.01 M ammonium acetate and 100 μcm^3 4 M chloric acid (VII). The extracts were then purified on Oasis HLB glass columns (200 mg, 5 ml) produced by Waters, then evaporated to dryness and topped up to 0.2 cm^3 with acetonitrile.

2.5. Chromatographic determinations and validation parameters

Concentrations of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol were determined using a high performance liquid chromatography method with a fluorescence detector and a Thermo Scientific HYPERSIL GOLD C18 PAH chromatographic column (250 × 4.6 mm; 5 μm). The excitation wavelength generated was λ = 275 nm and the emission was measured at λ = 300 nm. The chromatographic separation process was performed under gradient conditions using a mobile phase (water: acetonitrile).

The linearity of the method was >0.999 %, within the standard curve for solutions of 10–100 ng·cm^{-3} concentrations. However, the precision, expressed as a coefficient of variation, was <15 %. The accuracy was determined on the basis of the mean recovery, based on a 5-fold measurement of BPA and alkylphenol concentrations, in samples with a known amount of the analyte (5, 50, 100 ng·g^{-1}). Depending on the compound, the recovery ranged from 86 % for 4-NP to 99 % for BPA. The limit of quantification of the method was defined as the 10-fold signal-to-noise ratio for each sample type with very low (close to the detection limit) content of the analyte. That was 1.0, 0.3 and 0.1 ng·g^{-1} dw for BPA, 4-*t*-OP, and 4-NP, respectively. Whenever a new batch of SPE columns was used, the background was checked (procedural laboratory blank tests) and the BPA, 4-*t*-OP and 4-NP values were <LOQ each time.

2.6. Statistical analyses and additional data sources

Statistical analyses were performed using the STATISTICA12 program (StatSoft Inc.). The level of significance was adopted at $p < 0.05$. The data distribution was significantly different than the normal distribution (Shapiro-Wilk Test, p < 0.05). The Mann-Whitney *U* test and the Kruskal-Wallis test were used to determine the relationship between the selected parameters and the concentrations of BPA, 4-*t*-OP and 4-NP, respectively for two variables and multiple variables. Spearman rank correlations were determined between phenol derivative concentrations in claws and remiges and the concentrations of these compounds in blood and internal tissues (intestines, lungs, kidneys, liver, muscles, fat, brain). The effectiveness of BPA and alkylphenols elimination from the long-tailed duck and razorbill bodies was also assessed. For this purpose, it was assumed that the average concentrations in all the tissues examined so far (brain, fat, intestine, kidney, liver, lung and muscle), together with blood, claws and remiges, constitute 100 % of the contamination with these compounds in birds. The concentrations used for calculating and determining the correlations had been obtained as part of an earlier publication using the same long-tailed duck and razorbill specimens (Bodziach et al., 2021a, 2021b).

3. Results

3.1. Concentrations in remiges

The highest concentrations in remiges were found for 4-NP, which was assayed in 98 % of the samples (Table 1). The concentrations of this compound in the razorbill ranged from 65.4 to 656.0 ng·g^{-1} dw, and in the long-tailed duck from <0.1 to 202.5 ng·g^{-1} dw. As for the long-tailed duck, BPA and 4-*t*-OP were respectively determined in 72 % and 62 % of the feather samples. In this species, these compounds were characterised by similar concentrations ranging from <1.0 to 119.9 ng·g^{-1} dw with a median of 10.7 ng·g^{-1} dw (BPA) and from <0.3 to 113.3 ng·g^{-1} dw with a median of 10.9 ng·g^{-1} dw (4-*t*-OP). For the razorbill, both BPA

Table 1
Characteristics of the concentrations of bisphenol A (BPA), 4-tert-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) in claws and flight feathers [ng.g^{-1} dw] of long-tailed ducks and razorbills by-caught in the Southern Baltic region in winter (2014–2016).

		Long-tailed duck		Razorbill	
		Remiges	Claws	Remiges	Claws
BPA	Min	<1.0	<1.0	<1.0	9.8
	Max	119.9	809.5	86.8	271.6
	x	28.7	84.4	48.1	70.4
	Md	10.7	39.4	46.5	45.2
	SD	35.1	164.2	27.9	64.8
4-*t*-OP	Min	<0.3	<0.3	<0.3	<0.3
	Max	113.3	460.2	23.1	76.3
	x	22.2	56.4	10.5	34.2
	Md	10.9	27.5	10.0	30.0
	SD	26.9	91.5	5.9	22.5
4-NP	Min	<0.1	<0.1	65.4	<0.1
	Max	202.5	338.6	656.0	310.4
	x	87.5	151.9	300.9	165.6
	Md	77.7	153.7	269.6	180.6
	SD	48.2	95.1	153.2	84.1
	n	29	29	15	14

n – number of samples; min – minimum value; max – maximum value; x – mean value; md – median value; SD – standard deviation.

and 4-*t*-OP were determined in 60 % of the remiges samples, with 4-*t*-OP concentrations at the lowest level (<0.3–23.1 ng·g^{-1} dw). The median of BPA concentrations in the remiges of this species was almost 5 times higher than for 4-*t*-*OP* and 27 times lower than for 4-NP.

3.2. Concentrations in claws

The only compound found in all of the razorbill claws was BPA, while 4-*t*-OP and 4-NP were determined in 71 % and 64 % of the samples, respectively. On the other hand, phenol derivatives were quantifiable in 76–79 % of long-tailed duck claws. The highest concentrations in claws were of 4-NP and ranged <0.1–338.6 ng·g^{-1} dw (long-tailed duck) and <0.1–310.4 ng·g^{-1} dw (razorbill), with two extreme values marked in the long-tailed duck, one of 809.5 ng·g^{-1} dw (BPA) and the other of 460.2 ng·g^{-1} dw (4-*t*-OP). The lowest concentrations were determined in razorbill claws within a range of <0.3–76.3 ng·g^{-1} dw and 9.8–271.1 ng·g^{-1} dw for 4-*t*-OP and BPA, respectively. On the other hand, the lowest medians were for long-tailed duck claws and were 27.5 ng·g^{-1} dw (4-*t*-OP) and 39.4 ng·g^{-1} dw (BPA) (Table 1).

3.3. Correlations of phenol derivative concentrations in the products of the epidermis, blood and tissues

There were 5 statistically significant correlations between the concentrations of phenol derivatives in feathers and claws with those in internal tissues, but none was obtained for blood (Table 2). Most of the correlations were obtained for the long-tailed duck (4 out of 5) and for remiges (4 out of 5). They were mostly weak and moderate correlations, with only one instance of a strong one. Among the phenol derivatives, most of the strongest correlations concerned 4-NP, while BPA and 4-*t*-OP had only one statistically significant, moderately strong correlation.

3.4. Feathers/claws share in the elimination of phenol derivatives

The elimination of phenol derivatives via claws and feathers, depending on the compound and the species, accounted for 12 to 34 % of the total known load of these compounds in avian organisms (Fig. 1). In the long-tailed duck, more phenol derivatives accumulated in the claws (13–17 %) compared to the remiges (4–8 %). In the razorbill, BPA and 4-*t*-OP also accumulated more in claws (9–11 %), while 4-NP accumulated more in remiges (22 %). Moreover, in razorbills the best eliminated compound via both claws and remiges was 4-NP (34 %), while in the long-tailed duck it was 4-*t*-OP (24 %). On the other hand, the compounds that were the least effectively removed with epidermal formations were 4-*t*-OP (12 %) and BPA (17 %) respectively in razorbills and long-tailed ducks. The weaker elimination of 4-*t*-OP via claws and feathers in razorbills corresponded to greater bioaccumulation of this compound in the brain and fat, which together accounted for 1/3 of the total load. In turn, the weaker elimination of BPA via the epidermal formations in long-tailed ducks corresponded to a greater bioaccumulation of this xenobiotic in their intestines and kidneys, accounting for almost half (43 %) of the total known load (Fig. 1).

Table 2
Spearman's correlations between the concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the claws and remiges and the concentrations of phenol derivatives in the internal tissues.

Bisphenol A (BPA)	4-*Tert*-octylphenol (4-*t*-OP)	4-Nonylphenol (4-NP)
Remiges-lungs LO **$r = -0.47, p = 0.036$, $n = 20$**	**Claws-muscles LO** **$r = -0.46, p = 0.031$, $n = 22$**	**Remiges-intestines LO** **$r = 0.41, p = 0.034$, $n = 27$**
Claws-liver RA $r = -0.47, p = 0.088$, $n = 14$	Claws-muscles RA $r = 0.59, p = 0.074$, $n = 10$	**Remiges-kidney LO** **$r = -0.38, p = 0.044$, $n = 28$**
Claws-lungs LO $r = -0.36, p = 0.097$, $n = 22$	Claws-liver LO $r = 0.36, p = 0.115$, $n = 20$	**Remiges-fat RA** **$r = -0.76, p = 0.004$, $n = 12$**
Remiges-blood RA $r = -0.52, p = 0.154$, $n = 9$	Remiges-blood RA $r = -0.52, p = 0.154$, $n = 9$	Remiges-muscles RA $r = -0.49, p = 0.064$, $n = 15$
	Claws-intestines RA $r = -0.53, p = 0.117$, $n = 10$	Remiges-blood LO $r = -0.35, p = 0.069$, $n = 28$
	Claws-kidney RA $r = -0.53, p = 0.139$, $n = 9$	Remiges-lungs LO $r = -0.31, p = 0.110$, $n = 28$
		Claws-blood LO $r = -0.35, p = 0.113$, $n = 22$

r - Spearman's correlation; p - level of significance; n – number of samples; LO – long tailed duck; RA – razorbill; statistically significant correlations are in bold; the concentrations in blood, intestines and lungs were published in an earlier study (Bodziach et al., 2021b), as were the concentrations in the fat, kidneys, livers and muscles (Bodziach et al., 2021a).

4. Discussion

4.1. Indicators of environmental pollution with phenol derivatives

Birds, and especially their feathers, are well-known indicators of environmental pollution (Dauwe et al., 2002; Adout et al., 2007; Burger et al., 2009; Jaspers et al., 2009; Gómez-Ramírez et al., 2017; Table 3). This is due to the fact that the transport of xenobiotics to feathers via the blood is available only during their growth, which takes an average of several weeks. After the feather has grown, the path for blood closes down completely, leaving it inaccessible for further transfer (Goede and de Bruin, 1984; Burger and Gochfeld, 1995; García-Fernández et al., 2013). In this study, remiges were used to assess environmental pollution. Both of the tested species moult all of their remiges at the same time and do not fly during the moulting season, eating only local food. As a result, the accumulated xenobiotics in these feathers can with high probability be associated with the region where birds replaced their remiges. Long-tailed ducks replace their flight feathers in the Russian Arctic and razorbills moult in the Baltic region (Cramp and Simmons, 1977; Karwinkel et al., 2020).

Two out of the three tested compounds, i.e. bisphenol A and 4-nonylphenol, were characterised by higher concentrations in razorbill remiges compared to those of long-tailed ducks, although only in the case of 4-NP were these differences statistically significant (Mann-Whitney *U* test, $p < 0.001$, Fig. 2). This indicates a different degree of contamination with phenol derivatives in the respective regions where the studied bird species replaced their remiges. The fact that statistically significant differences were noted only in the case of 4-NP is consistent with up-to-date findings, which have shown that out of the three studied compounds 4-NP is characterised by the highest (and statistically significant) bioaccumulation in tissues, i.e. muscles, liver and kidneys, of the long-tailed duck and razorbill (Bodziach et al., 2021a). In this case, it seems that among phenol derivatives, 4-NP may be the most representative in terms of environmental biomonitoring studies using bird feathers.

On the basis of the obtained results, it can be assumed that the Baltic region is characterised by a greater influx of phenol derivatives compared to the environment of the Russian Arctic. The greater pollution of the Baltic Sea is favoured by its hydrological and geographical features. This is reflected in numerous studies confirming the presence of phenol derivatives in Baltic organisms from all trophic levels (Staniszewska et al., 2014, 2015b, 2016b, 2017; Graca et al., 2021), and the highest concentrations so far have been found in bird tissue (Falkowska et al., 2017; Bodziach et al., 2021a, 2021b). Phenol derivatives are introduced into the environment mainly through rivers as a result of incomplete disposal from wastewater treatment plants (Ahel et al., 1994; Staniszewska et al., 2015a, 2015b). An important source of these compounds is also the combustion of products containing phenol derivatives, which is to be observed especially in urbanised areas (Fu and Kawamura, 2010). Previous studies on the same long-tailed duck and razorbill specimens showed that in the Southern Baltic Sea region, both the alimentary and respiratory tracts

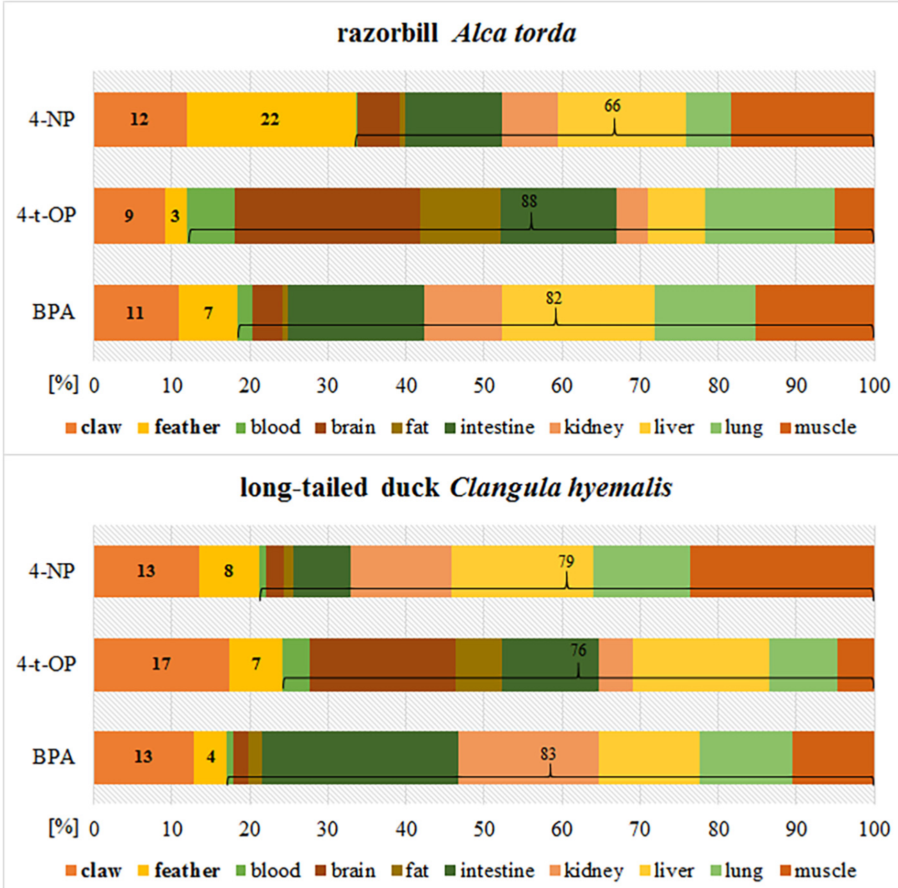

Fig. 1. Percentage share of remiges and claws against the background of accumulation sites for bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the bodies of long-tailed ducks and razorbills; the concentrations in the blood, intestines and lungs were previously published in the study by Bodziach et al. (2021b); the concentrations in the fat, kidneys, livers and muscles were previously published in another study by the aforementioned authors (Bodziach et al., 2021a).

play an important role in the transfer of phenol derivatives from the environment to the organisms of birds (Bodziach et al., 2021b).

The Russian Arctic sector covers about half of the Arctic Ocean coastline and remains poorly researched from an environmental point of view. The levels of organic pollutants in the western part of the Russian Arctic are the least known, as the region has remained inaccessible to environmental research for many years (de Wit et al., 2006, 2010; Lebedev et al., 2018). In the present study, the lipophilic concentrations of 4-NP were >3 times lower in long-tailed ducks compared to razorbills (Table 1), which indicates less phenol derivatives pollution in the Russian Arctic environment. According to available data, the average concentration of ΣNPs in surface water from the Svalbard region located in the Arctic, at only 10.0 ng·dm^{-3}, is 3 times lower compared to the average concentration of 4-NP alone in subsurface coastal water from the Gulf of Gdansk, at 32.2 ng·dm^{-3} (Staniszewska et al., 2015a, 2015b; Ademollo et al., 2021). The Arctic's distance from industrialised centres and agricultural regions clearly and significantly reduces the transfer of pollutants to this ecosystem. However, as the present study shows, all three phenol derivatives were present at measurable levels in the long-tailed duck remiges (Table 1), showing that the Arctic is not completely free from the effects of anthropogenic activity. The presence of phenol derivatives has also been documented in the muscles and livers of Greenland sharks (Ademollo et al., 2018). The highest concentrations detected were those of 4-NP and nonylphenol ethoxylate, which is formed as a result of 4-NP decomposition (NP1-2EO). The presence of floating marine macro litter (FMML) in the Arctic seas has also been uncovered (Pogojeva et al., 2021) and it has been shown that it occurs mainly in the waters of Atlantic origin, i.e. in the western part of the Russian Arctic. Plastics present in seawater may be an important source of phenol derivatives (Staniszewska et al., 2016c), also for the long-tailed duck (Morkūnas et al., 2021). Research to date shows that the Arctic, although relatively clean itself, acts as a sink for global pollutants. Long-distance transport via the atmosphere, oceans and rivers is considered to be the main source of pollution in this part of the world. In addition, some xenobiotics may come from local sources, which are villages and settlements in regions lacking modern wastewater treatment plants (AMAP, 2017).

In the case of 4-*t*-OP concentrations, no differences were found between the remiges of the two studied bird species, although higher values were determined for the long-tailed duck (Mann-Whitney U test, $p = 0.411$; Fig. 2). It seems, therefore, that xenobiotics deposited in the tissues of long-tailed ducks while wintering in the Baltic Sea, could have been remobilised from fat reserves during the remiges replacement that took place in the Arctic, even before leaving the Arctic regions. The elimination of lipophilic compounds from the tissues takes place during lipid mobilisation, which is one of the key factors disturbing the relative level of xenobiotic accumulation in feathers and internal tissues and depends on the time at which the samples are taken (Burger, 1993; Espín et al., 2012; García-Fernández et al., 2013).

Furthermore, the present study used long-tailed ducks from two regions of the Baltic Sea, and, as noted in the previous work based on the same specimens, each group lived in waters with a different degree of 4-*t*-OP pollution. The long-tailed ducks from the Pomeranian Bay had statistically significantly higher concentrations of 4-*t*-OP in their intestines compared to birds from the Gulf of Gdansk (Bodziach et al., 2021b). The authors noticed that due to the contamination of the Pomeranian Bay sediments and the affinity of 4-*t*-OP for binding with the organic matter in sediments, individuals of this species feeding on benthos are particularly exposed to

Table 3
Summary of selected studies using bird claws and feathers.

Xenobiotic/group of xenobiotics	Matrix	Species	Region/tissue correlations	Type of study	References
Organic compounds					
BPA, 4-*t*-OP, 4-NP	Body feathers	Herring gull	Gdynia harbour, Vistula estuary, fishing port, municipal waste dumps (N Poland)	Environmental biomonitoring	Nehring et al., 2017
PCB, PBDE, OCP	Body feathers	Greater rhea	Pampas grasslands in Argentina, four sites with different land uses	Environmental biomonitoring	Lèche et al., 2021
PFAS	Body feathers	White-tailed eagle, northern goshawk	Blood plasma	Indicators of body contamination	Gómez-Ramírez et al., 2017
PCB, PBDE, OCP	Body feathers	White-tailed eagles	Blood plasma, preen oil	Indicators of body contamination	Eulaers et al., 2011a
organochlorine pesticides	Primary wing feathers	Razorbill	Southeastern Spain	Environmental biomonitoring	Espín et al., 2012
PBDEs, PCBs, DDE	Tail feathers	Common magpies	Belgium, Antwerp: urban and rural areas	Environmental biomonitoring	Jaspers et al., 2009
PBDEs, PCBs, DDT	Tail feathers	Common buzzards	Liver, muscle	Indicators of body contamination	Jaspers et al., 2006
PBDEs, PCBs, OCPs, OPPs, PAHs, PYRs	Body feathers	Feral pigeons	8 urban areas in NW Spain	Environmental biomonitoring	González-Gómez et al., 2020
PFOS	Outermost tail feathers	Grey heron, herring gull, eurasian sparrowhawk, eurasian magpie, eurasian collared dove	Liver	Indicators of body contamination	Meyer et al., 2009
Metals					
Zn, Cu, Cd, Cr, Ni, As, Pb,	Primary and breast feathers	White-breasted waterhen, common moorhen	Tissue (heart, liver, kidney) and bones (sternum and femur)	Indicators of body contamination	Mukhtar et al., 2020
Mg, Al, Mn, Cu, Zn, Rb, Mo, Cd, Ba, Hg, Pb	Distal part of primary feathers	Pigeons and ravens	Israel: urban environment in the city of Beer Sheva, rural area in the Nahal Ashan farm, industrial environment and the open natural environment of Borot Lotz	Environmental biomonitoring	Adout et al., 2007
Cd, Cu, Pb, Zn	Outermost tail feathers	Great and blue tits	Belgium, Antwerp: polluted site near a metallurgic factory and a reference site, 4 km east of the polluted site, Cd and Pb: liver, kidney and muscle	Environmental biomonitoring, indicators of body contamination	Dauwe et al., 2002
As, Cd, Cr, Cu, Se, Sr	Claws	Yellow-bellied slider	wetlands located on the Savannah River Site (Aiken, SC, USA): comparison between area contaminated by coal combustion residues and uncontaminated reference area	Environmental biomonitoring	Haskins et al., 2017
Cd, Cu, Pb, Zn, Mn, Fe	Breast feathers	Black-tailed gull	Hongdo Island, Korea liver and stomach contents	Environmental biomonitoring, indicators of body contamination	Kim and Oh, 2014
Cd, Pb	Breast feathers	Kentish plovers, mongolian plovers, dunlins, great knots, terek sandpipers	Liver	Indicators of body contamination	Kim and Koo, 2008
Hg	Fifth primary feather of the left wing	Black, common and brünnich's guillemot	The Baltic, Kattegat, Faroe Islands and Greenland	Environmental biomonitoring	Appelquist et al., 1985
Hg	Outermost primary, innermost primary, rectrices, contour and down feathers	Herring gull, common gull, black-headed gull, great black-backed gull	Liver, kidney, muscles, heart, lungs, brain	Indicators of body contamination	Szumiło-Pilarska et al., 2017
Hg	Claws	Herring gulls	Liver, kidney, lung, muscle, heart, brain, blood, intestine	Indicators of body contamination	Grajewska et al., 2019

the accumulation of this alkylphenol. This may therefore explain the lack of significant differences in the concentration of this compound between the remiges of the long-tailed duck and the razorbill, the latter of which came only from the Gulf of Gdansk.

It has been demonstrated that both the intestines and the lungs of birds show promise as indicators of pollution by phenol derivatives in the environment and its components (Bodziach et al., 2021b). On the other hand, subsequent research has ruled out the possibility that tissues such as muscles, liver, kidneys, brain or subcutaneous fat could be used for environmental research in a similar way (Bodziach et al., 2021a). Also in this study no statistically significant differences were found in the concentrations of phenol derivatives between the claws of the long-tailed ducks from the Gulf of Gdansk and from the Pomeranian Bay (Mann-Whitney *U* test, BPA $p = 0.901$, 4-*t*-OP $p = 0.089$, 4-NP $p = 0.598$; Fig. 3). Nevertheless, the concentrations of 4-*t*-OP were clearly higher in the claws of the long-tailed ducks from the Pomeranian Bay (Fig. 3).

These results are consistent with previous findings of higher pollution in this basin on the basis of studies on phenol derivatives in the intestines (Bodziach et al., 2021b). Bird claws grow conically (in length and in width) in a continuous manner, which requires a constant supply of blood along with nutrients and contaminants. Moreover, in adult migratory songbirds the average claw growth rate of 0.03–0.05 mm·d^{-1} is slow enough for the stable isotopes assayed therein to reflect both the winter and breeding environments of these birds (Hahn et al., 2014). Thus, long-tailed duck claws accumulate phenol derivatives from both the Baltic and the Arctic as well as from places where they stop during their migration. This is confirmed by the large dispersion of the concentrations of all three phenol derivatives in long-tailed duck claws (Table 1). This suggests that the claws

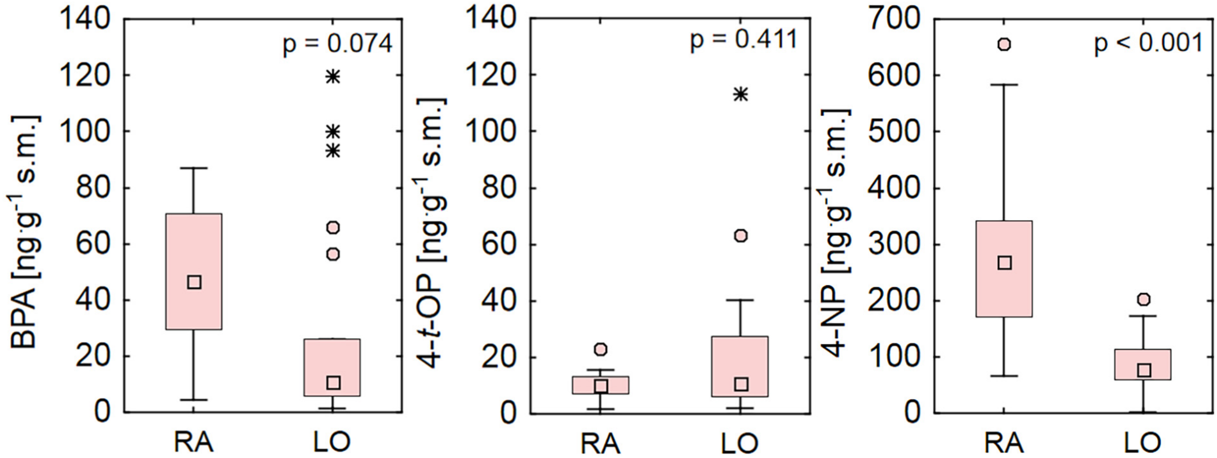

Fig. 2. Box and whiskers plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in remiges of razorbills (RA) and long-tailed ducks (LO); o – outliers; * - extreme values.

of migratory species may not be a reliable indicator of phenols contamination in a particular place.

4.2. Indicators of phenol derivatives load in organisms

In order to establish whether feathers and/or claws show promise as indicators of xenobiotics in birds, it is important to determine whether the concentrations found in them can be linked in terms of quantity with the levels accumulated in internal tissues (García-Fernández et al., 2013). In the case of organic pollutants (e.g. PBDEs, PCBs, PFOS, DDT) and trace elements, correlations have been obtained for concentrations between feathers and e.g. the liver, kidneys and pectoral muscles (Dauwe et al., 2002; Jaspers et al., 2006; Agusa et al., 2005; Kim and Koo, 2008; Meyer et al., 2009; Table 3). However, a few studies (Dauwe et al., 2005; Espín et al., 2010; Eulaers et al., 2011a; Abbasi et al., 2017) suggest that the time between moulting and sampling may be one of the key factors restricting the use of feathers as a tool to assess the load in bird organisms. This effect can be bypassed by collecting feathers while they are still growing or shortly after their completion (Eulaers et al., 2011b). On the other hand, Grajewska et al. (2019) suggested that because claws, unlike feathers, grow continuously, requiring a constant blood supply (Lucas and Stettenheim, 1972; Braune and Gaskin, 1987), they may be better at reflecting the xenobiotic load in the body. The authors successfully linked the concentrations of mercury in claws with the concentrations of this metal in seven different tissues and in blood, obtaining statistically significant positive correlations (Table 3). In view of these findings, the present study expected to obtain a greater number of stronger correlations between BPA and alkylphenols concentrations in tissues and in claws, which grow continuously. However, the relationship of phenol derivatives concentrations between tissues and feathers could have been disturbed by the passage of several months from the time of their replacement to the time of collection and analysis. An additional obstacle for obtaining such correlations in long-tailed ducks could be the migratory nature of this species of bird. Depending on the season, long-tailed ducks live in two different areas with different degrees of environmental pollution with phenol derivatives (chapter 4.1). This species changes remiges in remote regions of the Arctic, while the bioaccumulation of phenol derivatives in internal tissues probably originates mainly from the Southern Baltic region (Bodziach et al., 2021b).

However, contrary to the assumptions, 4 out of 5 of the statistically significant correlations obtained for phenol derivative concentrations between tissues and epidermal products were determined for the long-tailed duck and for remiges that had been cut off from the bloodstream for several months (Table 2). This suggests that phenol derivatives accumulated in long-tailed duck feathers originate not only from the place where the birds changed the feathers, but also from the xenobiotic pollution of the Baltic Sea, as a result of their remobilisation from tissues during the feather replacement process. The small number of statistically significant

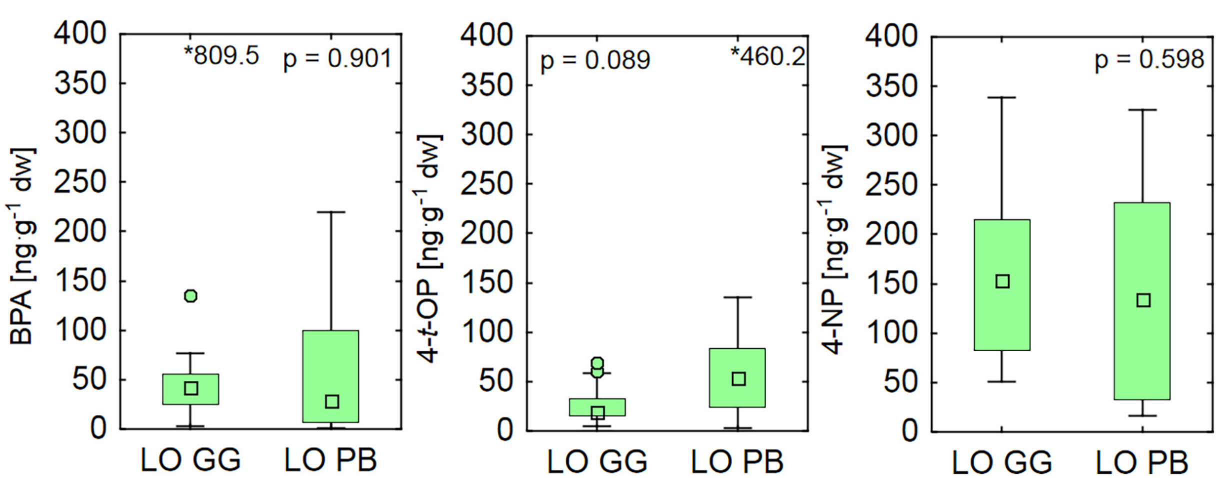

Fig. 3. Box and whisker plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in claws of long-tailed ducks from the Gulf of Gdansk and the Pomeranian Bay; LO GG – long tailed duck from Gulf of Gdansk, LO PB – long-tailed duck from Pomeranian Bay; o – outliers; * - extreme values.

correlations obtained suggests that remiges and claws may reflect the phenol derivative load of bird organisms to a small extent. However, they have potential for further research, particularly concerning 4-NP and remiges (Table 2).

4.3. Phenol derivatives elimination

Birds have several ways of removing pollutants from their bodies, i.e. elimination via guano, feathers, claws and, in the case of females, also into the egg during breeding (Burger et al., 1994; Burger et al., 2009; Espín et al., 2012; Grajewska et al., 2015; Grajewska et al., 2019; Staniszewska et al., 2020). In the case of metals such as Sb and Hg, it has been noticed that the vast majority of them, sometimes constituting in excess of 70 % of the bird's total body load, is distributed to feathers, favouring elimination. By contrast, lead is mainly bioaccumulated, and only 32 % is removed with feathers (Agusa et al., 2005). In the present study, despite some simplification and not considering all tissues or removal via guano, it becomes apparent that in razorbill and long-tailed duck organisms, bioaccumulation processes prevail over elimination. The latter constitutes from 12 to 34 %, depending on the compound and the bird (Fig. 1). Most likely, this results from the pollution of the environment with phenol derivatives and their lipophilic nature. This indicates that endocrine-active alkylphenols and BPA may endanger the proper functioning of birds due to possible bioaccumulation with age. This is confirmed by previous studies of phenol derivatives involving the same birds, in which they revealed, the potential of BPA and 4-NP for biomagnification in the organisms of long-tailed ducks and razorbills. The BMF values for BPA and 4-NP ranged from 0.41–2.73 and 0.88–6.42, respectively (Bodziach et al., 2021a).

Due to the fact that phenol derivatives were not assayed in all the matrices used by bird organisms to remove pollutants, it is impossible to assess which route contributes the most to the purification of their organisms. In the case of mercury, feathers have been shown to contain up to 90 % of the total body load due to the strong binding of this element to keratin (Braune and Gaskin, 1987; Agusa et al., 2005). It has also been found that the levels of mercury concentrations in feathers and claws are similar (Grajewska et al., 2019). However, when comparing the concentrations of phenol derivatives in feathers and claws, it was found that claws had a greater share in the removal of phenol derivatives from the organisms of birds (Fig. 1), although statistically significant differences were found only for alkylphenols (Mann-Whitney *U* test, $p < 0.05$; Fig. 4).

This is most likely due to the fact that the penetration of xenobiotics to claws is constant, while feathers are available only for a short period during their growth (Lucas and Stettenheim, 1972; Braune and Gaskin, 1987; Grajewska et al., 2019). There was one exception to this, where the concentrations of 4-NP in razorbill feathers were higher in comparison with its claws (Mann-Whitney U test, $p = 0.034$; Figs. 1, 4). This may have been caused by the razorbill replacing remiges in an area where its main food was more contaminated with 4-NP. Due to the fact that the birds do not fly when changing their remiges, their choice of food is limited by location. Another possible reason for the higher concentrations of 4-NP in the remiges of this razorbill is the general diet for this species, which is mostly fish. Due to the lipophilic nature of 4-NP and its wide distribution in the environment, 4-NP may bioaccumulate in the trophic chain

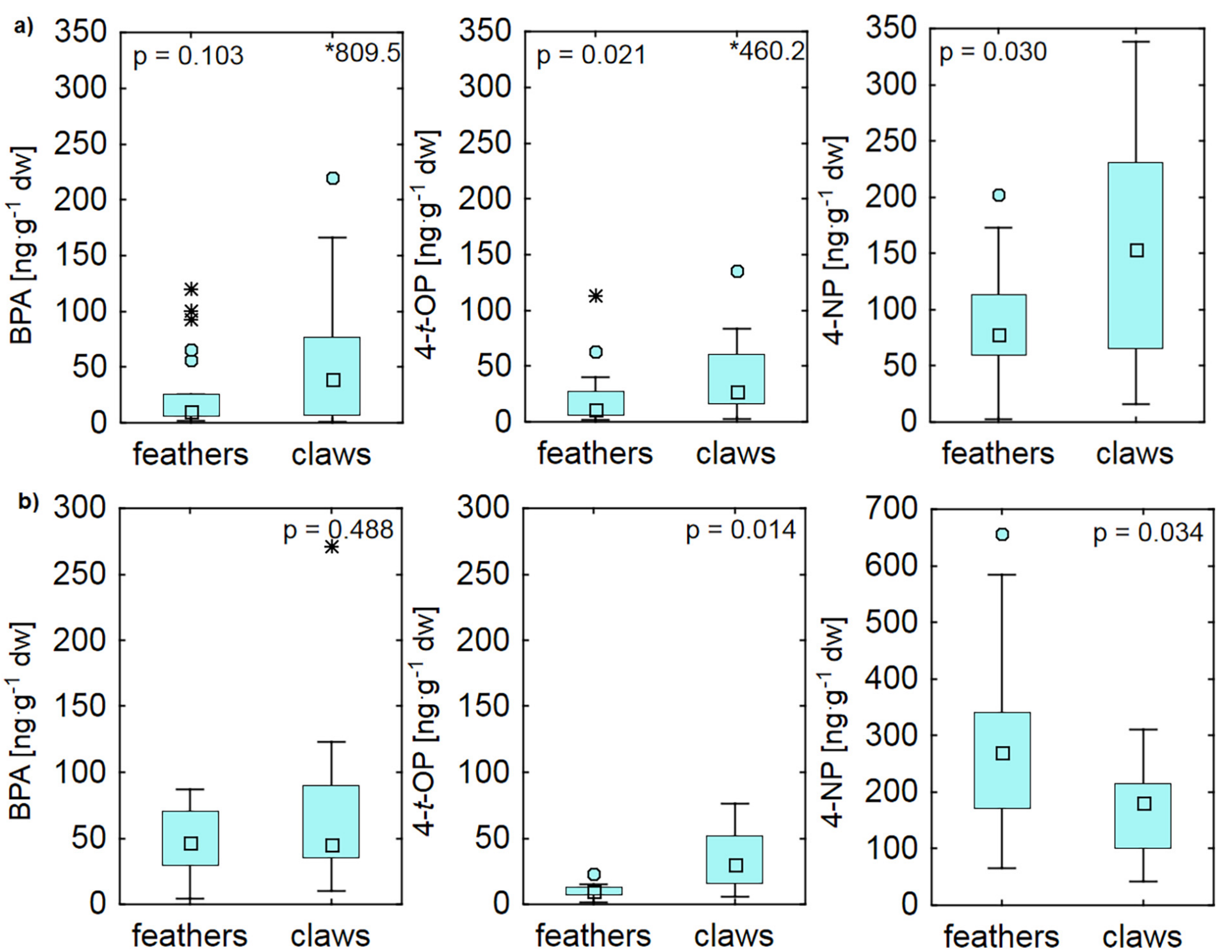

Fig. 4. Box and whisker plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) between the remiges and claws of a) long-tailed ducks and b) razorbills; o – outliers; * - extreme values.

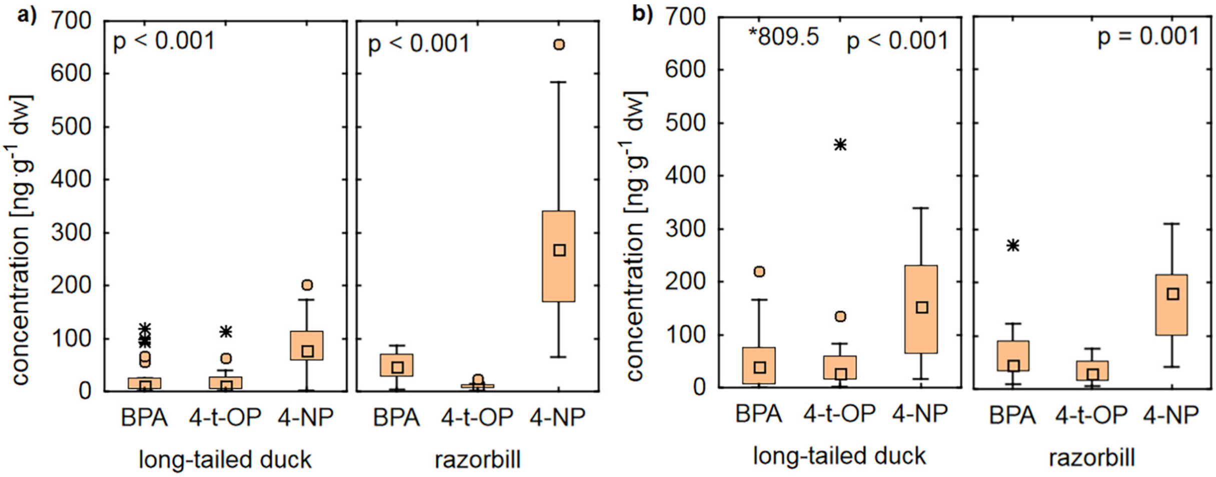

Fig. 5. Box and whisker plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in a) remiges and b) claws of long-tailed ducks and razorbills; o – outliers; * - extreme values.

(Diehl et al., 2012). However, previous studies did not confirm higher 4-NP concentrations in the tissues of the piscivorous razorbill compared to the long-tailed duck feeding on benthos (Bodziach et al., 2021a). Thus, it is possible that there is no greater diet-related accumulation of 4-NP in the razorbill because a large proportion of this compound is eliminated, as reflected in the remiges (Fig. 1).

In both bird species, out of the three phenol derivatives, 4-NP was the most effectively removed with both remiges and claws (Kruskal-Wallis test, $p < 0.05$; Fig. 5). That is also consistent with its greatest accumulation in muscles, livers and kidneys of the same individuals (Bodziach et al., 2021a). However, studies of phenol derivatives in the lungs and intestines of the long-tailed duck and the razorbill have shown that birds are exposed to high bioaccumulation of both 4-NP and BPA (Bodziach et al., 2021b). Therefore it seems that the two compounds with the highest emissions to the environment and accumulation in birds are eliminated from their bodies in different ways, possibly resulting from the lower lipophilicity of BPA compared to 4-NP. It has been shown that seagull guano can contain extremely high BPA concentrations, reaching 2701.9 $ng{\cdot}g^{-1}$ dw., which was 9 times higher than the concentration of 4-NP and as much as 160 times higher than that of 4-*t*-OP (Staniszewska et al., 2014, 2020). On the other hand, in the long-tailed duck, almost half of the accumulated BPA was found in the intestines and kidneys (Fig. 1), which may confirm that this xenobiotic is removed mainly with the guano.

4-*t*-OP was particularly poorly removed with feathers and claws in razorbills, probably owing to the high degree of accumulation in their brains and fat, accounting for 1/3 of the total body load (Fig. 1). Lipophilic contaminants such as 4-*t*-OP accumulate in tissues with high triglyceride content, like the brain and fat (García-Fernández et al., 2013; Bodziach et al., 2021a). On the other hand, feathers are characterised by a lower content of lipids and thus contain fewer lipophilic contaminants (García-Fernández et al., 2013). Moreover, the obtained correlations (Table 2) indicate that phenol derivatives are removed from almost all tissues studied so far. The exception was the brain, for which no correlation of 4-*t*-OP concentrations with claws or feathers was obtained. It therefore appears that this alkylphenol of a branched structure is more difficult to remove from this organ. As the elimination of lipophilic compounds takes place during lipid mobilisation (Burger, 1993; Perkins and Barclay, 1997; Espín et al., 2012; García-Fernández et al., 2013) this suggests that the blood-brain barrier may be more easily crossed from blood to brain than vice versa. In this case, the brain could be good tissue for observing the bioaccumulation of 4-*t*-OP with the age of the birds.

5. Conclusions

Long-tailed ducks and razorbills eliminate phenol derivatives from their bodies by incorporating them into epidermal formations, i.e. feathers and claws. The xenobiotic that was most effectively removed via both of these routes was 4-NP. On the other hand, for the majority of compounds and birds, it was claws that contributed the most to elimination. Taking into account only these two ways of phenol derivative removal, it was found that in the birds studied so far the level of elimination is lower than the accumulation in internal tissues. However, this degree of elimination appears to be effective enough to prevent possible bioaccumulation with age and biomagnification in birds feeding on organisms from higher trophic levels. Phenol derivatives are most likely removed from all, or at least most, internal tissues, although the brain may be more resistant to their elimination. The difficulty in removing 4-*t*-OP from this important organ may turn out to be responsible for the possible bioaccumulation of this xenobiotic with age in long-living bird species.

The analysis of long-tailed duck and razorbill remiges and claws as biomonitoring tools showed the possibility of using them for preliminary, approximate assessment of environmental pollution with phenol derivatives and the exposure levels of birds. The most promising indicators in both cases were remiges and 4-NP, especially for monitoring two different environments with different levels of pollution. The usefulness of remiges in this study most likely result from the fact that in both species these feathers are moulted simultaneously and that the birds do not fly while replacing them. As was assumed based on the distance from potential sources of phenol derivatives, their concentrations in the remiges indicated a greater influx of these xenobiotics into the Baltic environment compared to the Russian Arctic. However, in order to be able to use remiges in the monitoring of environmental pollution with phenol derivatives in the future, subsequent studies should confirm the usefulness of these feathers in other sample areas. It is also necessary to investigate the effects of external pollution and its possible origin, as well as the distribution of phenol derivative concentrations in a single feather and its parts, and their accumulation in other types of feathers.

Nevertheless, caution should be exercised when estimating the exposure of birds to phenol derivatives when using feathers. Based on the few statistically significant correlations obtained for concentrations between epidermal formations and tissues, their usefulness at estimating the exposure levels of birds cannot be unequivocally stated. They do however constitute a premise for further research, mainly due to the fact that the obtained correlations were inconsistent with the expected results and because they evoked more questions than they answered. First, it is not known why most of the statistically significant correlations of derivative compound concentrations were obtained between tissues and remiges, which had been cut off from the blood supply for many months; and also why only one of these correlations concerned claws, which are continuously supplied with substances via blood. It is also thought-provoking to consider why all of the statistically significant correlations were obtained for the long-tailed duck, which is a migratory species, and none for the razorbill which inhabits the Baltic region all year round. Many factors, i.e. the time that elapsed from the time of remiges replacement to their collection and analysis, remobilisation of phenol derivatives from the tissues with fat reserves, the age of the birds, their unknown condition at the time of moulting, unknown external pollution, insufficient number of samples for testing and the ecology of the studied bird species, could have potentially affected the concentration levels of phenol derivatives and distorted the picture. It seems, however, that none of these factors can fully explain the obtained correlation of concentrations between the remiges and the internal tissues of the long-tailed duck. This suggests that a further search for possible causes is necessary to understand the usefulness of epidermal products in estimating the exposure of birds to phenol derivatives.

CRediT authorship contribution statement

Karina Bodziach: Resources, Conceptualization, Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Marta Staniszewska:** Resources, Conceptualization, Validation, Writing – original draft, Writing – review & editing, Supervision. **Iga Nehring:** Investigation, Writing – review & editing. **Agnieszka Ożarowska:** Investigation, Writing – review & editing. **Grzegorz Zaniewicz:** Investigation, Writing – review & editing. **Włodzimierz Meissner:** Investigation, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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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STATEMENTS OF CO – AUTHORS

Bodziach K., Staniszewska M., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641.

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Gdynia, 22.05.2023 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Bodziach K., Staniszewska M., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the southern Baltic", stanowił około **55%** całości i obejmował:

- sformułowanie problemu badawczego,
- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- analizę statystyczną wyników,
- graficzne i statystyczne przedstawienie wyników pochodnych fenolu,
- interpretację pozyskanych wyników pochodnych fenolu w świetle posiadanej wiedzy oraz zgromadzonego przeglądu literatury przedmiotowej,
- tworzenie manuskryptu.

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OŚWIADCZENIE

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Bodziach K., **Staniszewska M.**, Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **20%** całości i obejmował:

- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną na każdym etapie tworzenia manuskryptu, w szczególności: w interpretacji wyników i redagowaniu manuskryptu,
- pełnienie funkcji autora korespondencyjnego.

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OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Bodziach K., Staniszewska M., **Nehring I.**, Ożarowska A., Zaniewicz G., Meissner W., 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the southern Baltic", stanowił około **10%** całości i obejmował:

- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną.

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Gdańsk, 18.05.2023 r.

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie niżej wymienionej publikacji naukowej:

Bodziach K., Staniszewska M., Nehring I., **Ożarowska A.**, Zaniewicz G., Meissner W., 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **5%** całości i obejmował:

- przygotowanie materiału biologicznego do analiz chemicznych.

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Gdańsk, 18.05.2023 r.

OŚWIADCZENIE

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Bodziach K., Staniszewska M., Nehring I., Ożarowska A., **Zaniewicz G.**, Meissner W., 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **5%** całości i obejmował:

- przygotowanie materiału biologicznego do analiz chemicznych.

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OŚWIADCZENIE

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Bodziach K., Staniszewska M., Nehring I., Ożarowska A., Zaniewicz G., **Meissner W.**, 2022. Elimination of endocrine disrupting phenolic compounds via feathers and claws in seabirds moulting in the Baltic and Russian Arctic. Science of the Total Environment 853, 158641, doi: 10.1016/j.scitotenv.2022.158641,

wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **5%** całości i obejmował:

- pomoc w interpretacji wyników i redagowaniu manuskryptu.

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PUBLICATION 4

Bodziach K., Staniszewska M., Nehring I., Ożarowska A., Zaniewicz G., Meissner W., 2023. Endocrine disrupting bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol in gonads of long-tailed ducks *Clangula hyemalis* wintering in the southern Baltic (under review in the Science of the Total Environment).

Own contribution: 55%

IF: 10.754, 5-year IF: 5.291, MSHE points: 200

Endocrine disrupting bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol in gonads of long-tailed ducks *Clangula hyemalis* wintering in the southern Baltic

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Abstract

This paper focuses on determining the concentrations of phenol derivatives in the gonads of seabirds and examining the potential factors (age, sex and region) affecting the degree of their bioaccumulation. The study involved assays of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the gonads of long-tailed ducks taken as bycatch from the Southern Baltic region in 2015-2016. The concentration levels of phenol derivatives in the birds' gonads were similar to the levels which had been observed to have negative endocrine effects in other authors studies. This shows that the studied xenoestrogens can interfere with the reproduction and development of birds. Among phenol derivatives, 4-NP was found to reach the highest concentrations in the gonads of long-tailed ducks, and its concentrations were in the range of < 0.1 – 717.5 $ng{\cdot}g^{-1}$ dw. The concentrations of BPA and 4-*t*-OP were similar and amounted to < 0.4 – 181.6 $ng{\cdot}g^{-1}$ dw and < 0.1 – 192.4 $ng{\cdot}g^{-1}$ dw respectively. Moreover, mature long-tailed ducks had higher concentrations of phenol derivatives compared to immature ones, possibly resulting from long-term bioaccumulation, as well as from diverse pollution in their respective habitats. Particularly in the case of 4-NP, the median concentrations in mature gonads were 2-fold higher than in immature ones. In turn, among adult long-tailed ducks, phenol derivatives were characterized by higher concentrations in males than in females, with almost 3 times and approx. 3.5 times higher median concentrations of BPA and 4-*t*-OP, respectively. Lower concentrations of phenol derivatives in female gonads may result from the additional elimination of pollutants from their organisms through the transfer of pollutants from mother to egg. The results obtained in

this study show the need for further research on phenol derivatives in the gonads of birds, focusing on their impact on the reproductive system and early development.

Keywords: gonads, birds, xenoestrogens, sex, age

1. **Introduction**

Bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) are compounds whose activity has a disruptive effect on the endocrine system (EDCs). The activity of these compounds results from their structural similarity to endogenous hormones, which enables them to bind to receptors, modulating, inducing or blocking the responses of the endocrine system (Sonnenschein & Soto, 1998). The toxic effects of phenol derivatives towards reproduction and development are of particular importance, and the majority of reports on this topic concern fish. It has been demonstrated that in fish, exposure to 4-NP induces vitellogenin synthesis and inhibits testicular growth (Jobling et al., 1996), as well as affecting reproductive hormones, causing histopathological changes in the gonads (Shirdel et al., 2020) and changes in gene expression linked to, among other things, estrogen signalling (Won et al., 2014). Researchers observed a dose-dependent drop in the hatching rate, and there was a decrease in the survival rate of hatched larvae with increasing vitellogenin concentration and 4-NP exposure (Lee et al., 2018). The toxic effects of 4-NP also included: reduced fertilization rate, increased embryo mortality, developmental defects in larvae, e.g. vertebral deformities, axial deformities, skull defects with an undeveloped head, and visual impairment (Chaube et al., 2012). Developmental malformations after the exposure of fish to BPA have also been observed, including cardiac edema, vertebral malformations and craniofacial deformities (Moreman et al., 2017). Similarly to 4-NP, BPA caused changes in the histological structure of the testicular tissue, abnormalities in the expression levels of genes involved in testicular steroidogenesis, but also reduced motility of males during courtship and a decreased number of courtship behaviors towards females (Li et al., 2017). Alarming symptoms were observed in the *Oryzias latipes* fish: exposure to BPA effected a significant drop in the fertilization rate of the offspring two generations later (F2) and reduced embryo survival in the offspring three generations later (F3) (Bhandari et al., 2015). Researchers have also described the possible behavioral and transgenerational effects of 4-*t*-OP on reproductive success and embryo-larval development of the second generation of *Oryzias latipes* (Gray et al., 1999). Those results show that the exposure of wild animals to xenoestrogens can result in harmful developmental and reproductive consequences, leading to reproductive impairment even in subsequent generations and eventual reduction of the population of the species. However, little is known about the impact of phenol derivatives on the reproduction and development of birds, although the few available studies indicate the possibility of disorders similar to those observed in fish (Oshima et al., 2012; Cheng et al., 2017; Mentor et al., 2020). So far no information has been presented even on the environmental concentration levels of phenol derivatives in the gonads of wild birds.

Phenol derivatives constitute ubiquitous components of synthetic materials. Bisphenol A is a monomer used for the synthesis of polycarbonate, a material of important economic importance. All phenols are used as additives in the production of various types of plastics, e.g. packaging, CDs, tyres, sport equipment, toys or dental fillings. Alkylphenols are also components of non-ionic surfactants (Staples et al., 1998; Acir and Guenther, 2018). Phenol derivatives can be released from these products into the environment at various stages of their life, during production, use, processing, and later when they become waste in the environment. The main recipients of these xenoestrogens are the seas and oceans, to which BPA and alkylphenols are mainly transported by rivers (Ahel et al., 1994; Staples et al., 1998; Staniszewska et al., 2015a; Nehring et al., 2023). However, birds, including long-tailed ducks (*Clangula hyemalis*), are exposed to phenol derivatives not only through their diet, but also through the air (Bodziach et al., 2021a). In their bodies, the compounds are subsequently distributed to tissues, where they undergo bioaccumulation and/or biotransformation (Bodziach et al., 2021b) and also to epidermal products, via which they can be eliminated from the system (Bodziach et al., 2022). Recent reports on the presence of microplastics in various tissues of water birds (Wang et. al, 2021) also indicate an important route of exposure of birds to phenol derivatives through the migration of these compounds from plastics ingested by birds and accumulated in their bodies (Tanaka et al., 2015; Staniszewska et al., 2016a). In the case of birds, there are many literature reports raising the importance of the problem of confusing plastic with food. Pieces of plastic of various sizes, sometimes even as small as nano- or picoplankton, can be found in sea water in large numbers (Teuten et al., 2009; Ivar do Sul & Costa, 2014).

Located in the southern part of the Baltic Sea, the Gulf of Gdańsk and the Pomeranian Bay are important sites of resting, feeding, moulting and breeding for about 80 species of birds (HELCOM, 2018). These reservoirs are included in the European Natura 2000 network as special bird protection areas. For the long-tailed duck, these two areas have been among the most important in the Polish zone of the Baltic Sea for many years (Durinck et al., 1994; Skov et al. 2011; Wardecki et al. 2021). While wintering in the southern part of the Baltic Sea, long-tailed ducks are exposed to increased concentrations of marine litter and chemical pollution, including phenol derivatives (Bodziach et al., 2021a, Bodziach et al., 2022). The reason for this is the nature of the basin itself combined with strong anthropopressure, especially in its southernmost part. The Baltic Sea is a relatively small and shallow body of water, and its area is almost four times smaller than the catchment area, which is inhabited by over 85 million people. Moreover, the rate of water exchange with the North Sea through the narrow and shallow straits is about 30 years (HELCOM, 2018). The main transporters of pollutants to the southern part of the Baltic Sea are two rivers: the Vistula and the Oder, whose combined catchment area covers almost 90% of Poland (Kot-Wasik et al., 2003; Pastuszak et al., 2018). Numerous sources of pollution exist in the Polish coastal zone due largely to two agglomerations, the Tri-City and Szczecin, both of which feature industrial plants (pharmaceutical, textile, paper, chemical), as well as ports and shipyards.

The aim of the present study was to determine whether bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol have the ability to bioaccumulate in the gonads of long-tailed ducks and to identify groups of individuals that are potentially more exposed to these compounds in the process of reproduction. In this study, we analysed the influence of factors such as age, sex, region of habitat and its pollution on the concentration of BPA and alkylphenols in the gonads. The research was carried out on dead long-tailed ducks (n = 47) collected as bycatch in the winter of 2014 – 2016 in the Southern Baltic region. These birds feed mainly on benthos (Cramp & Simmons, 1977). Long-tailed ducks are a protected species and their wintering population in the Baltic Sea is described as declining (BirdLife International, 2023).

2. Materials and methods

2.1. Biological material for analyses

During dissection of the long-tailed ducks, their gonads were collected and immediately frozen (-20^{o} C). Prior to analysis, the gonads were lyophilized and then homogenized. Thus prepared, the tissues were placed in a borosilicate glass vessel in a desiccator under constant conditions (temp. 20^{o} C ± 2^{o} C, humidity 45% ± 5%). During dissection of the birds, their age was also determined based on plumage (Baker, 2016). Sex was determined by the appearance of the gonads.

2.2. Extraction of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol from the gonads

All vessels and instruments used at the stage of sample preparation and assay were made of either metal or glass. The solvents used for the analysis, i.e. water, acetonitrile and methanol, all of gradient purity for HPLC, were manufactured by Merck. The 70% ammonium acetate (PA) and 70% chloric acid (VII) were produced by POCh, while the high purity standards of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol (>97%) were produced by SIGMA-ALDRICH. The calibration curve was prepared on the basis of working solutions prepared in methanol with the following concentrations: 10, 25, 50, 75 and 100 $ng \cdot cm^{-3}$.

Bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol concentrations were determined according to the method described by Staniszewska et al. (2014; 2018). The lyophilized, homogenized and weighed gonads of the long-tailed ducks ($0.1 g \pm 10^{-3} g$) were immersed in a mixture of the following solvents: 8 cm^3 of methanol, 2 cm^3 of 0.01M ammonium acetate and 100 μcm^3 of 4M chloric acid (VII). The samples were then subjected to a 10-minute extraction in an ultrasonic bath at 20^{o} C. The obtained extracts were purified on Oasis HLB glass columns (200 mg, 5 cm^3) produced by the Waters company, then evaporated to dryness and topped with acetonitrile to reach 0.2 cm^3.

2.3. Chromatographic determinations and validation parameters

The final assays of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol concentrations were carried out using high-performance liquid chromatography with a fluorescence detector and a Thermo Scientific HYPERSIL GOLD C18 PAH chromatography column (250×4.6 mm; 5 µm). The length of the generated excitation wave was $\lambda = 275$ nm, while the emission was measured at a wavelength of $\lambda = 300$ nm. The chromatographic separation process was performed under gradient conditions using a mobile phase (water:acetonitrile). The recovery was determined in samples with the addition of a known amount of analyte, based on five measurements of the concentrations of BPA, 4-*t*-OP and 4-NP. The precision of the method was expressed as a coefficient of variation. The limit of quantification for the method was determined for a sample with a small analyte content as a tenfold signal-to-noise ratio. For each compound, the average recovery in gonads was above 80% and the precision of the method was below 15%. The limit of quantification of the method was 0.4 $ng \cdot g^{-1}$ dw for BPA and 0.1 $ng \cdot g^{-1}$ dw for 4-*t*-OP and 4-NP.

2.4. Statistical analyses

Statistical analyzes were performed using the STATISTICA 12 program (StatSoft Inc.), where the significance level was assumed to be $p = 0.05$. As the distribution of data for all three compounds deviated from a normal distribution (Shapiro-Wilk test, $p < 0.05$), the non-parametric Mann-Whitney U test was used when comparing two trials, and the Kruskal-

Wallis test together with the Dunn post-hoc test were used when there were more than two trials. This paper compares the concentrations of particular phenol derivatives (BPA, 4-*t*-OP and 4-NP) in the gonads of long-tailed ducks. We also determined the relations between selected parameters (sex, age) and the concentrations of BPA, 4-*t*-OP and 4-NP in the gonads of birds. Due to the scarcity of immature individuals (n = 8), the influence of sex was tested only in mature birds (n = 39). In addition, the concentrations of phenol derivatives in the gonads of the long-tailed ducks were analyzed in comparison to the concentrations of these xenobiotics in other tissues collected from the same specimens. For this purpose, data concerning the concentrations of phenol derivatives found in the muscles, kidneys, liver, fat and brain was taken from the authors' previous work (Bodziach et al., 2022).

3. **Results**

Among the phenol derivatives, the highest concentrations in the gonads of all long-tailed ducks were found for 4-NP (Kruskal-Wallis test, $p < 0.001$). The comparison of mean ranks in three groups showed that only the concentrations of 4-NP differed statistically significantly from the concentrations of BPA (Dunn post-hoc test, $p < 0.001$) and 4-*t*-OP (Dunn post-hoc test, $p = 0.001$). 4-NP was present at measurable levels in 98% of the samples, with concentrations ranging from < 0.1 $ng.g^{-1}$ dw to 717.5 $ng.g^{-1}$ dw (Table 1), with a median of 85.7 $ng.g^{-1}$ dw. The concentrations of BPA and 4-*t*-OP in the gonads of all long-tailed ducks were similar to each other (Mann-Whitney U test, $p = 0.177$): < 0.4 - 181.6 ng. g^{-1} dw, with a median of 29.8 ng. g^{-1} dw for BPA, and < 0.1 – 192.4 ng. g^{-1} dw, with a median of 15.4 ng. g^{-1} dw for 4-*t*-OP. The determination level of BPA in the gonads was as high as that of 4-NP, while 4-*t*-OP was determined in 3/4 of the samples.

Table 1 Concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the gonads [$ng{\cdot}g^{-1}$ dw] of the long-tailed ducks by-caught in the Southern Baltic region in winter in the years 2014-2016

n – number of samples; min – minimum value; max – maximum value; md – median value; x – mean value;
SD – standard deviation, dw – dry weight
*maximum values also include outliers and extreme values

Mature birds were characterized by higher concentrations of BPA (< 0.4 – 181.6 $ng.g^{-1}$ dw) and 4-*t*-OP (< 0.1 – 192.4 $ng.g^{-1}$ dw) compared to immature ones, in the gonads of which the concentrations of BPA and 4-*t*-OP were within a range of 0.4 – 49.3 $ng.g^{-1}$ dw and < 0.1 – 17.4 $ng.g^{-1}$ dw, respectively (Table 1). These differences were not statistically significant (Mann-Whitney U test, $p = 0.401$ for BPA and $p = 0.204$ for 4-*t*-OP). On the other hand, the median BPA and 4-*t*-OP concentrations in mature and immature specimens were at the same level, whereas concentrations of 4-NP in the gonads of mature birds, ranging from < 0.1 to 717.5 $ng.g^{-1}$ dw, were statistically significantly higher compared to immature specimens (Mann-Whitney U test, $p = 0.024$), with concentrations within a range of 17.1 - 149.1 $ng.g^{-1}$ dw. Moreover, the median for 4-NP concentrations in the gonads of mature birds was twice as high as in immature long-tailed ducks.

In mature long-tailed ducks, the BPA concentations in males were found to be statistically significantly higher than in females, with almost 3 times higher median concentrations (< 0.4 - 126.9 $ng.g^{-1}$ dw), compared to the opposite sex (0.5 - 99.5 $ng.g^{-1}$ dw, with one extreme of 181.6 $ng.g^{-1}$ dw) (Mann-Whitney U test, $p = 0.048$). 4-*t*-OP also reached higher concentrations in the gonads of mature males (< 0.1 - 175.1 $ng.g^{-1}$ dw) compared to mature females (< 0.1 - 76.1 $ng.g^{-1}$ dw, with one extreme value of 192.4 $ng.g^{-1}$ dw). The differences of 4-*t*-OP concentrations between the representatives of both sexes, however, were not statistically significant (Mann-Whitney U test, $p = 0.126$), but the median concentrations between them differed by about 3.5 times. In turn, 4-NP concentrations in mature males were within a range of < 0.1 – 717.5 $ng.g^{-1}$ dw, with a median of 121.4 $ng.g^{-1}$ dw, while in mature females they ranged from 26.3 $ng.g^{-1}$ dw to 551.8 $ng.g^{-1}$ dw with a median of 93.1 $ng.g^{-1}$ dw. However, as was the case with 4-*t*-OP, these differences were not found to be statistically significant (Mann-Whitney U test, $p = 0.503$).

The concentrations of all three phenol derivatives were statistically significantly different between the following tissues of long-tailed ducks: gonads, muscle, kidneys, liver, fat and brain (Kruskal Wallis test, $p < 0.001$ Fig. 1). When comparing BPA concentrations in the gonads of long-tailed ducks to other tissues of this species, statistically significant differences occurred only between the gonads and kidneys (Dunn post-hoc test, $p = 0.010$) and gonads and livers (Dunn post-hoc test, $p = 0.005$). In turn, the concentrations of 4-NP in the gonads were statistically significantly different from the concentrations of this alkylphenol in the muscles (Dunn post-hoc test, $p = 0.018$) and in the brain (Dunn post-hoc test, $p = 0.043$). However, the concentrations of 4-*t*-OP in the gonads did not differ statistically significantly from the concentrations of this xenobiotic in other previously examined tissues (Dunn post-hoc test, $p > 0.05$ in all cases).

Fig. 1. Box and whiskers plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the gonads of long-tailed ducks compared to other tissues; concentrations in muscle, kidneys, liver, fat and brain were published in Bodziach et al., 2021b; the arrow indicates groups that are statistically significantly different from each other according to the Dunn post-hoc test

4. Discussion

4.1 Bioaccumulation of phenol derivatives in the gonads

Every day, human activity contributes to the release of a whole spectrum of endocrine disrupting compounds into the environment. Seas and oceans are a particularly abundant reservoir of various types of xenoestrogens. As a consequence, marine fauna and flora are exposed to the bioaccumulation of these xenobiotics and the resultant health effects. Studies conducted in the Gulf of Gdańsk show high seasonal, geographical and vertical variability of concentrations of phenol derivatives in seawater (Staniszewska et al., 2015a). The contamination of the surface water microlayer with these compounds reached up to 3,659.6 $ng \cdot dm^{-3}$ in the port area (4-NP), while in near-bottom offshore water it was only 56.4 $ng \cdot dm^{-3}$ (4-*t*-OP) (Table 2). High concentrations of BPA and alkylphenols were also found in fish, as well as in organisms from lower trophic levels – phytoplankton and zooplankton (Table 2). The wide distribution of anthropogenic pollution in the marine environment has also affected animals at the top of the trophic chain. It was shown that birds, i.e. the long-tailed duck, the razorbill and the goosander, are exposed to the penetration of bisphenol A, 4-*tert*-octylphenol and 4-nonylphenol into their bodies with food, as well as with air (Bodziach et al., 2021a). A subsequent study revealed the presence of phenol derivatives in the most important organs of birds, i.e. the brain, liver and kidneys, and in other bioaccumulative tissues, i.e. muscles and subcutaneous fat, which may be a secondary source of exposure (Bodziach et al., 2021b). The present research confirmed our hypothesis that the gonads of long-tailed ducks, an important reproductive gland, are also exposed to the bioaccumulation of endocrine active BPA, 4-t-OP and 4-NP (Table 1).

Table 2 Concentration of bisphenol A (BPA), 4-*tert*-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) in the water and trophic chain of the Gulf of Gdansk

[a] – concentration in water [$ng \cdot dm^{-3}$], [b] – concentration in biological sample [$ng \cdot g^{-1}$ dw]

The highest concentrations in long-tailed duck gonads were found for 4-NP, while the concentrations of the other two compounds, i.e. BPA and 4-*t*-OP, were similar to each other (Fig. 2). 4-NP is a lipophilic compound, characterized by a wide distribution in the environment and high bioaccumulation in organisms (Table 2). As shown in previous studies, in the waters of the Gulf of Gdańsk, the highest concentrations were of 4-NP, especially in the surface water microlayer collected from the port area (Table 2). Due to the smaller amount of food available for birds in the Baltic Sea in winter, the port and the vicinity of the approach channels to the port may prove of interest to them in terms of obtaining food during that time. The presence of birds in ports contaminated with petroleum derivative substances may thus be a factor increasing their exposure to 4-NP. In the organisms of the bird species studied so far, the tissues such as muscle, liver and brain, were characterized by the highest concentrations of alkylphenols. However, in terms of the particular routes of penetration of phenol derivatives, the highest concentrations found in the intestines were for BPA, and in the lungs for 4-NP. Differences were also found in the epidermal products of birds tested for the presence of phenol derivatives, with the highest concentrations of 4-NP in feathers and BPA in claws (Table 2). Bioaccumulation is the outcome of uptake, metabolism and elimination (Weisbrod et al., 2009). Among the three phenol derivatives, 4-NP shows the greatest affinity to animal fat, which may be favourable to its bioaccumulation in the trophic chain (Diehl et al., 2012; Gautam et al., 2015; Korsman et al., 2015; Lee et al., 2015). The log Kow partition coefficient for individual phenol derivatives is 3.3 (BPA), 5.3 (4-*t*-OP) and 5.9 (4-NP) (Grover, 2008). The biomagnification factors of phenol derivatives calculated for the long-tailed duck are as follows: 0.41 - 2.73 (BPA), 0.09 - 1.03 (4-*t*-OP) and 0.88 - 6.42 (4-NP) (Bodziach et al., 2021b). Furthermore, the distribution of xenobiotics to individual organs occurs as a result of binding to proteins, active transport or dissolution in lipids. As a consequence, these mechanisms determine the affinity of xenoestrogens to specific tissues (Lehman-McKeeman, 2008) and the concentration levels they reach there (Bodziach et al., 2021b). In the example of the fish species *Coregonus lavaretus*, it has been shown that gonads can contain almost three times more lipids than muscles (Dörücü, 2000). All these factors may jointly result in a particularly high bioaccumulation of 4-NP in the gonads, compared to BPA and 4-*t*-OP.

Fig. 2. Box and whiskers plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the gonads of long-tailed ducks

4.2 **Risk of potential reproductive and endocrine effects**

To the best of our knowledge, so far there have not been any studies on the concentration levels of phenol derivatives accumulated in the gonads of birds as a result of environmental exposure. However, when comparing the concentrations of these xenoestrogens in the gonads to the previously examined tissues of the same long-tailed ducks, it can be seen that the gonads were not the main tissue of phenol derivative bioaccumulation (Fig. 1). The concentrations of all three compounds in the gonads were lower compared to their concentrations in the liver, it being an organ responsible for the metabolism of xenobiotics. Moreover, the concentrations of BPA and 4-NP in the gonads were higher than in fat, but lower than in muscle tissues. This indicates that these compounds are partially deposited in places where they remain at least temporarily neutral for the body's functions. In addition, for the tested compounds, there are no similarities in the distribution of their concentrations to individual tissues of birds. This suggests complex mechanisms of distribution, potentially determined by a number of different factors, i.e. the lipophilicity of the xenobiotic and its potential for bioaccumulation and biomagnification, the concentration of the substance in the environment, the ability to bind to proteins or dissolve in fats, tissue composition, as well as individual characteristics of the organism (Kawai et al. al., 1988; Fossi et al., 1995; Burger & Gochfeld, 2004; Lehman-McKeeman, 2008; Diehl et al., 2012; Szumiło-Pilarska et al., 2016; Bodziach et al., 2021a, Bodziach et al., 2021b).

However, considering the organs that would be the most sensitive to the effect of endocrine disrupting phenol derivatives, BPA and 4-NP showed a greater tendency to accumulate in the gonads of long-tailed ducks, while 4-*t*-OP was prevalently bioaccumulated in their brains (Fig. 1). The gonads and the brain are target organs for EDCs. That is because these organs are particularly sensitive to xenoestrogens, which negatively affect all the key functions for the survival of the population, such as the behavior, reproduction and development of the animals (Gray et al., 1999; Seki et al., 2003; Chaube et al. al., 2012; Bhandari et al., 2015; Li et al., 2017; Shirdel et al., 2020; Tran et al., 2020). Previous studies have shown that the tissue most contaminated with 4-*t*-OP in birds was the brain (Bodziach et al., 2021b). In contrast, the present study has revealed the affinity of BPA and 4-NP with gonads. These results show that endocrine disrupting phenols can impair the functioning of birds on various levels, leading to a cumulative effect on the organism. It should also be emphasized that in the classification of EDCs, BPA is placed in the highest category, i.e. *Proven impact causing hormonal disruptions in animals and negative impact on human health* (Revised report to DG Environment, 2007). As for the studied alkylphenols, they were both included on the list of 33 hazardous substances or groups of substances of special importance for the Baltic Sea (EC, 2001).

Although there is much information about the negative impact of phenol derivatives on fish, the effect of these xenoestrogens on the organisms of birds, including their reproduction and endocrine system, is still poorly investigated. In addition, most of the research conducted so far has focused on embryonic exposure of birds. That early phase of life, during which the gonads, the brain and behavior udergo sexual differentiation, is probably the most sensitive period for birds. Exposure to relatively small amounts of xenoestrogens during the critical periods for sexual differentiation can result in severe and permanent changes in gonadal and/or nerve structure and subsequently in adult reproductive behavior (Cutting et al., 2012). In birds, the very early stage of development is characterized by the absence of sexual differentiation. At this stage, the undifferentiated gonads are identical and can develop either way. The female is heterogametic (ZW) while the male is homogametic (ZZ). The sexual differentiation of the reproductive system in birds is driven by estrogens (Woods et al., 1975). Estrogen, which is a differentiating hormone, causes the embryo to change from the default to the target sex. Males produce very low levels of estrogen, and it is believed that this lack of female estrogen levels allows testes to develop from undifferentiated gonads and induce sperm formation from Wolffian ducts while the Müllerian ducts regress (Scheib et al., 1985). It is therefore possible to cause a change of sex to female during embryonic exposure, e.g. by exposing a genetically male egg to xenoestrogens during a critical period of embryonic development (Cutting et al., 2012).

In the case of a chicken embryo, it was shown that BPA administered to an egg at a dose of 510 nmol. g^{-1} for 14 days, caused increased mortality and feminization of the testes (Mentor et al., 2020). Feminization was also observed in Japanese quail embryos to which a dose of 200 ng/egg of 4-NP and 20 ng/egg of BPA was administered for 14 days (Oshima et al., 2012). In other studies, 4-NP was administered at concentrations of 0.1 $\mu g \cdot dm^{-3}$, 1.0 $\mu g \cdot dm^{-3}$, 10 $\mu g \cdot dm^{-3}$ and 100 $\mu g \cdot dm^{-3}$ to quails in drinking water (Cheng et al., 2017). Reduced fertilization rates and impaired spermatogenesis were noted in all groups. Reduced hatchability occurred in groups where 4-NP concentrations of 10 $\mu g \cdot dm^{-3}$ and 100 $\mu g \cdot dm^{-3}$ were administered. Disturbing observations also included reduced survival of embryos after 14 days of 4-NP administration at concentrations of 1.0 $\mu g \cdot dm^{-3}$, 10 $\mu g \cdot dm^{-3}$ and 100 $\mu g \cdot dm^{-3}$. It should be emphasized that the effects of exposure of birds to phenol derivatives are similar to those observed so far in fish and to those caused by other xenoestrogens in birds (Berg, 2000). Therefore, when estimating the possible risk of exposure of birds to phenol derivatives, a synergistic effect with other xenoestrogens should be taken into consideration. Thus, the current pollution of the Baltic Sea environment with endocrine disrupting compounds indicates that adverse effects of the exposure of the reproductive and endocrine systems of long-tailed ducks are likely to occur. These birds seem to be a species particularly sensitive to anthropogenic factors, including contamination of their environment with xenoestrogens. In recent years, the long-tailed duck population has declined by as much as 65% (Skov et al., 2011). By way of comparison, the decline in the global population of seabirds is estimated at approx. 70% over the last 60 decades (Paleczny et al., 2015). In turn, in the Baltic Sea region, the population of water birds decreased by approximately 30% and 20%, respectively in the breeding and wintering seasons in 2011-2016 (Helcom, 2018). These decreases are attributed to, among other factors, industrialization, which has led to pollution, overfishing of the food base or degradation and occupation of bird habitats by humans (Croxall et al., 2012).

4.3 Age and sex differentiation

Owing to the wide distribution of phenol derivatives in the marine environment, their bioaccumulation in the gonads of long-tailed ducks, as well as the disturbing reports quoted here on their impact on reproduction and proper development of birds at environmentally relevant concentrations, an important aspect of this study was to determine which groups of birds may be potentially more vulnerable to negative effects of xenoestrogens. The obtained results indicate that mature long-tailed ducks are exposed to higher concentrations of phenol derivatives than immature ones (Fig. 3). The long-term bioaccumulation of phenol derivatives in mature specimens may not be the only cause of these differences however (Donaldson et al., 1997; Vorkamp et al., 2004; Szumiło-Pilarska et al., 2016). The long-tailed duck is a species that spends winter in the Southern Baltic region, but breeds in Arctic Russia (Cramp & Simmons, 1977; Karwinkel et al., 2020). As confirmed by our previous studies of phenol derivatives in bird feathers, the latter region is relatively less polluted by phenol derivatives than the Baltic Sea, which remains under constant anthropopressure (Bodziach et al., 2022). It is probable that the immature long-tailed ducks, which arrived to wintering grounds in the Southern Baltic for the first time, had not been exposed to these compounds to the same extent as adults, both due to the length of their lives and the characteristics of the environment where they had lived. Moreover, statistically significantly higher concentrations of phenol derivatives in the gonads of mature long-tailed ducks compared to immature ones were determined only for 4-NP (Fig. 3). Out of the three phenol derivatives assayed in bird feathers, it was also only 4-NP that was characterized by statistically significantly higher concentrations in razorbills (*Alca torda*) which exchange their flight feathers in the Baltic region, compared to the long-tailed duck moulting in the Russian Arctic (Bodziach et al., 2022). On one hand, breeding long-tailed ducks in relatively clean areas may promote their proper reproduction and protect the most sensitive phase of embryonic development. On the other hand, however, the long-distance spring migrations of long-tailed ducks from the Southern Baltic, polluted with phenol derivatives, to breeding sites, during which they lose fat reserves and therefore also xenobiotics accumulated in these places (Henriksen et al., 1996; Perkins and Barclay, 1997), may favor their transfer from mothers to offspring (Ackerman et al., 2016; Nehring et al., 2018; Reindl et al., 2019).

Fig. 3 Box and whiskers plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in the gonads of adults (ad) and immature (imm) long-tailed ducks

Adult male long-tailed ducks showed higher concentrations of phenol derivatives than females (Fig. 4), which may be due to females having an additional way of getting rid of xenobiotics from their bodies. Both in males and females, the main route of removing pollutants, including phenol derivatives, is excretion with guano (Staniszewska et al., 2014; 2020). In addition, birds have the ability to eliminate phenol derivatives from their bodies along with epidermal products, such as feathers and claws (Bodziach et al., 2022). However, females, unlike males, have an additional way to remove xenobiotics by transferring some of the contamination of their body to their offspring and eggshells (Ackerman et al., 2016; Nehring et al., 2018; Reindl et al., 2019). It should be emphasized that although the direct effect of phenol derivatives in connection with the exposure of males to these xenoestrogens will target their endocrine system, the indirect effect may also reach females and embryos, which in turn may affect the entire population.

Fig. 4 Box and whiskers plot concentrations of bisphenol A (BPA), 4-*tert*-octylphenol (4-*t*-OP) and 4-nonylphenol (4-NP) in gonads of male (M) and female (F) adult long-tailed ducks

5. **Conclusions**

Seabirds, and especially long-tailed ducks, are under a lot of stress today, as is reflected in the drastic decline in their global population. Since the effects caused by exposure to phenol derivatives in the reproductive system of seabirds are not recognized, it is all the more important to determine whether xenoestrogens present in their environment contribute to the current poor state of the world's bird population, including long-tailed ducks. To the best of our knowledge, we are documenting for the first time the presence of bisphenol A (BPA), 4-*tert*-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) in seabird gonads. This important gland is one of the most sensitive organs to the endocrine disrupting compounds. The results of this study, combined with the previously proven effects of exposure at environmentally relevant concentrations, suggest that phenol derivatives may be a factor disrupting the proper reproduction and development of long-tailed ducks. Among the phenol derivatives, 4-NP was characterized by the greatest bioaccumulation potential in the gonads of long-tailed ducks, which could be related to the concentration of the substance in the environment, its lipophilicity, ability to bioaccumulate and biomagnify, as well as binding to proteins and dissolving in fats. At the same time, however, based on the measured concentrations, it seems that male and adult long-tailed ducks are the most exposed to phenol derivatives. The greater exposure of males to the health effects caused by the bioaccumulation of phenol derivatives in the gonads may result from fewer means to get rid of pollutants from their bodies. The higher exposure of adults to phenol derivatives may also be associated with both long-term bioaccumulation and their annual stay in the southern Baltic region, which is characterized by increased anthropopressure and chemical pollution. However, taking into account the above, the influence of phenol derivatives on the development of embryos in the sensitive period of growth, when concentrations of xenobiotics, as well as other nutrients, can be transferred from the mother to the egg cannot be ruled out. In addition, the specific functions for which the reproductive system is responsible mean that its disorder in one organism can trigger a chain of effects in other individuals, as well as in the entire population.

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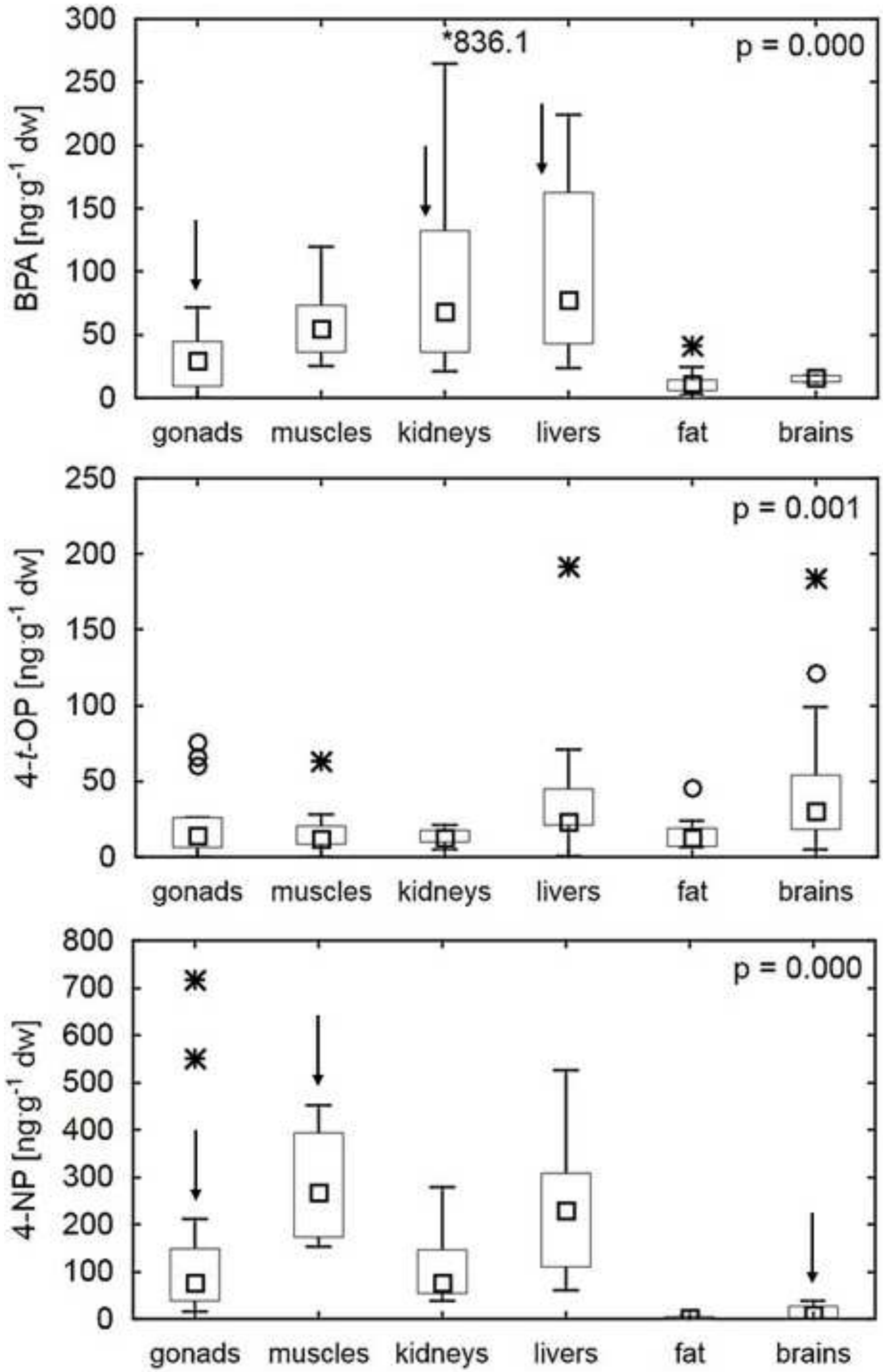
	BPA	**4-*t*-OP**	**4-NP**
	all birds (n = 47)		
min	<0.4	<0.1	<0.1
max	181.6	192.4	717.5
x	37.0	36.0	131.1
md	29.8	15.4	85.7
SD	35.6	49.0	134.5
	adult male (n = 19)		
min	<0.4	<0.1	<0.1
max	126.9	175.1	717.5
x	48.2	60.6	168.5
md	49.6	48.0	121.4
SD	32.5	58.5	168.6
	adult female (n = 20)		
min	0.5	<0.1	26.3
max	181.6	192.4	551.8
x	32.1	29.2	127.2
md	17.8	14.1	93.1
SD	40.7	44.1	113.2
	adult (n=39)		
min	<0.4	<0.1	<0.1
max	181.6	192.4	717.5
x	39.7	41.1	146.2
md	29.8	15.7	96.2
SD	37.9	52.3	142.9
	immature (n = 8)		
min	0.4	<0.1	17.1
max	49.3	17.4	149.1
x	24.3	11.1	61.4
md	29.6	12.3	47.1
SD	15.9	4.9	38.2

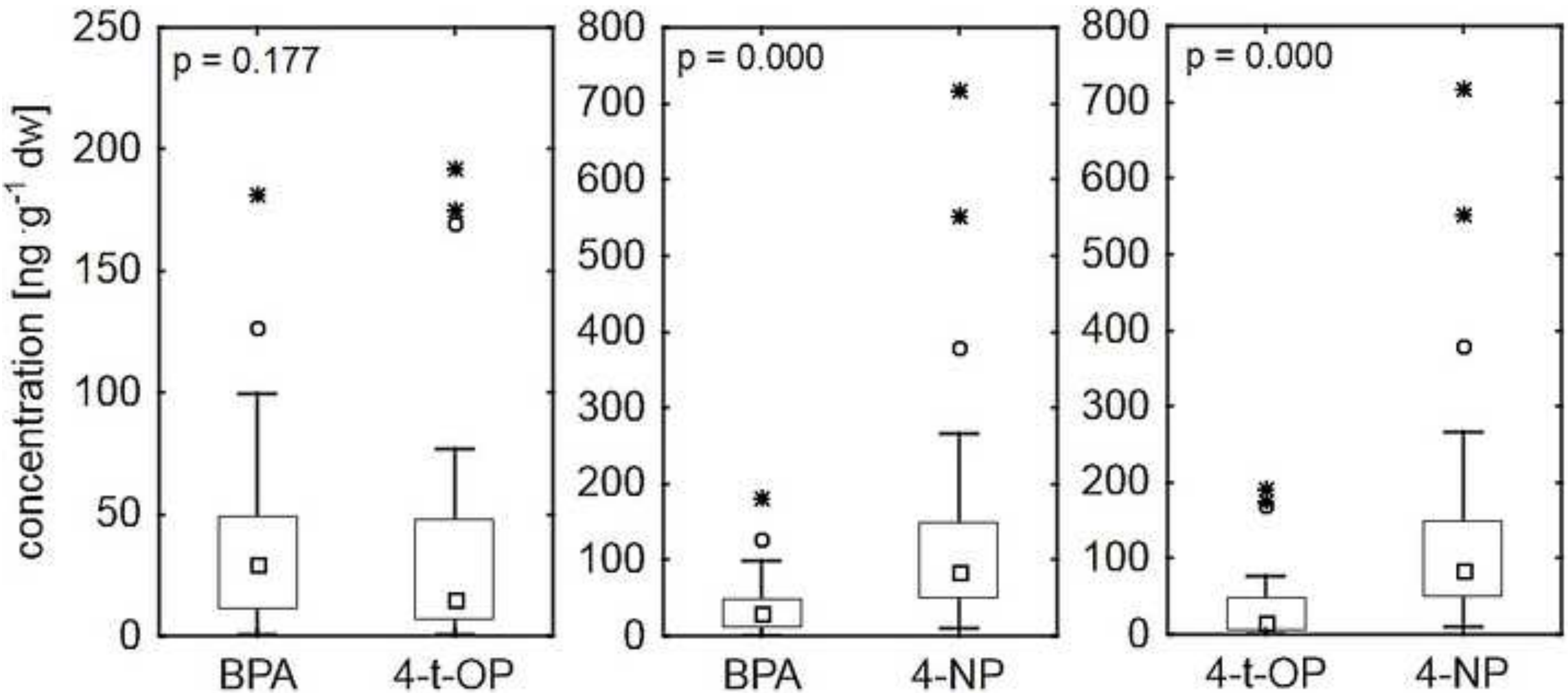
matrix	BPA (min – max)	4-*t*-OP (min – max)	4-NP (min – max)	literature
surface microlayer[a]	31.6 – 713.9	< 1.0 – 177.9	< 4.0 – 3659.6	Staniszewska et al., 2015a
coastal sea water[a]	< 5.0 – 154.3	< 1.0 – 63.0	< 4.0 – 156.8	Staniszewska et al., 2015a
open water < 4 m depth[a]	20.8 – 198.6	< 1.0 – 65.9	< 4.0 – 104.8	Staniszewska et al., 2015a
near-bottom water[a]	< 1.0 – 152.6	< 1.0 – 56.4	< 4.0 – 172.1	Staniszewska et al., 2015a
fitoplankton[b]	20.3 – 968.3	0.8 – 486.9	11.5 – 643.1	Staniszewska et al., 2015b
zooplankton[b]	9.6 – 769.2	< 0.8 – 67.6	< 1.0 – 263.7	Staniszewska et al., 2016b
mussels[b]	< 0.8 – 273.6	0.8 – 176.1	< 1.0 – 263.8	Staniszewska et al., 2017
fish[b]	19.7 – 798.4	< 0.8 – 89.2	5.4 – 41.9	Staniszewska et al., 2014
birds muscles[b]	< 2.0 – 223.0	< 0.5 – 63.2	26.0 – 476.4	Bodziach et al., 2021b
birds livers[b]	< 2.0 – 318.2	< 0.5 – 698.8	60.7 – 525.8	Bodziach et al., 2021b
birds brains[b]	< 0.4 – 37.4	2.6 – 341.1	< 0.1 – 272.5	Bodziach et al., 2021b
birds intestines[b]	6.6 – 1176.2	< 0.5 – 233.6	6.7 – 524.5	Bodziach et al., 2021a
birds lungs[b]	< 2.0 – 331.7	< 0.5 – 135.7	33.9 – 399.7	Bodziach et al., 2021a
birds claws[b]	< 0.1 – 809.5	< 0.3 – 460.2	< 0.1 – 338.6	Bodziach et al., 2022
birds feathers[b]	< 0.1 – 119.9	< 0.3 – 113.3	< 0.1 – 656.0	Bodziach et al., 2022

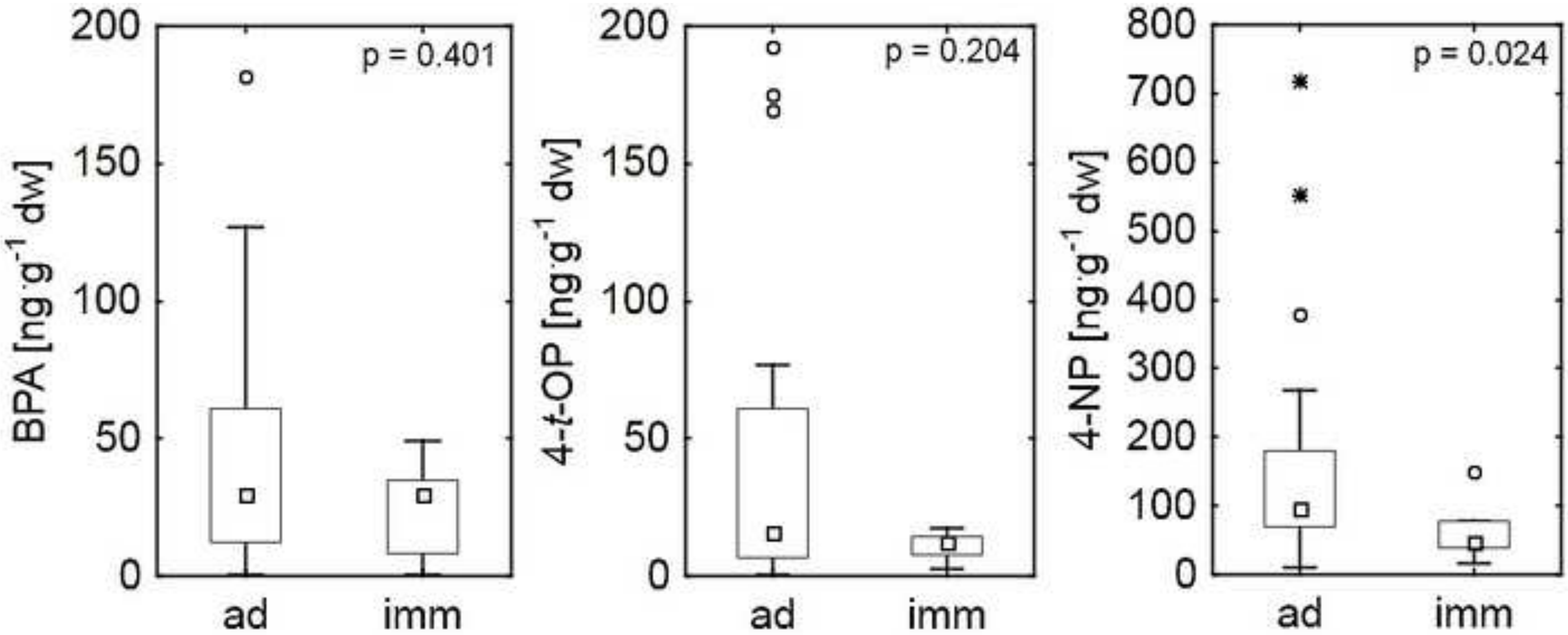
[a] – concentration in water [ng·dm^{-3}], [b] – concentration in biological sample [ng·g^{-1} dw]

Figure

Click here to access/download;Figure;Fig.1.jpg

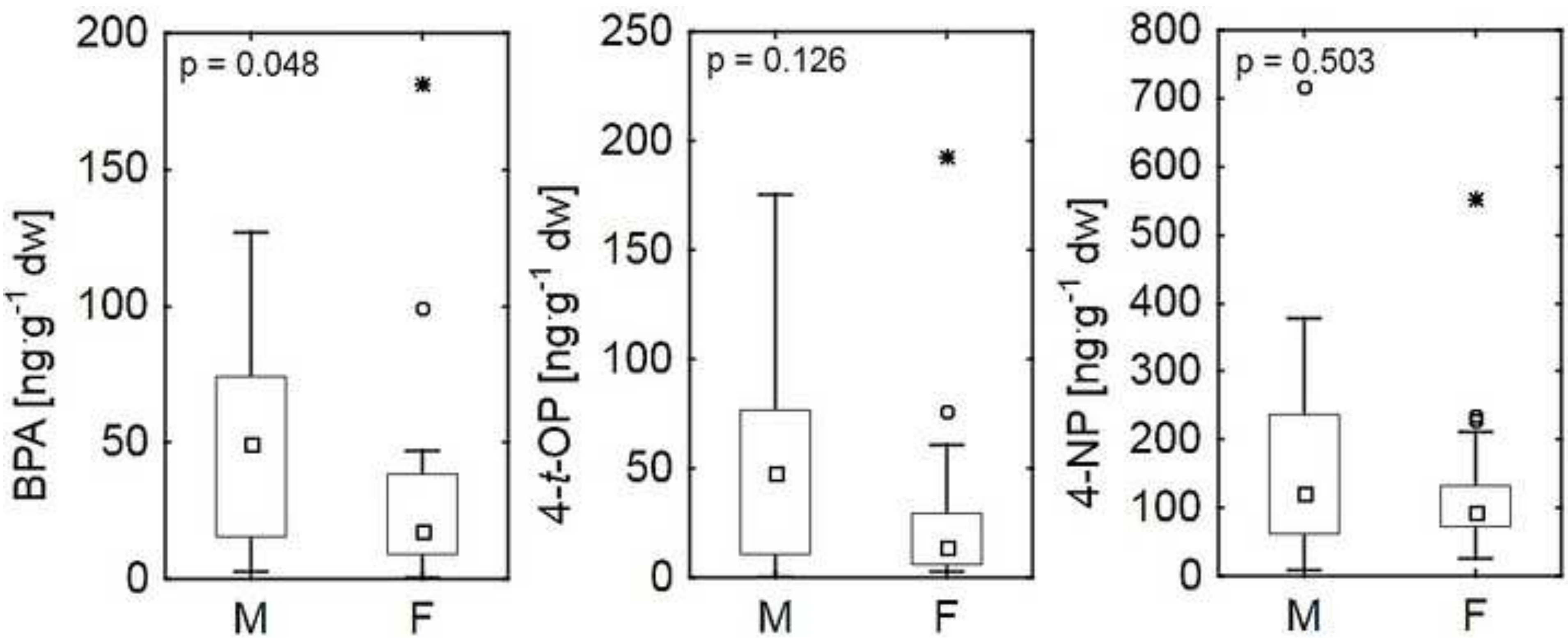



concentration [ng g-1 dw]
250
200
150
100
50
0
p = 0.177
BPA
4-t-OP
800
700
600
500
400
300
200
100
0
p = 0.000
BPA
4-NP
800
700
600
500
400
300
200
100
0
p = 0.000
4-t-OP
4-NP


BPA [ng·g-1 dw]
p = 0.401
200
150
100
50
0
ad
imm
4-t-OP [ng·g-1 dw]
p = 0.204
200
150
100
50
0
ad
imm
4-NP [ng·g-1 dw]
p = 0.024
800
700
600
500
400
300
200
100
0
ad
imm

Figure

Click here to access/download;Figure;Fig.4.jpg



Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Karina Bodziach: Resources, Conceptualization, Investigation, Validation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing
Marta Staniszewska: Resources, Validation, Writing - Original Draft, Writing - Review & Editing, Supervision
Iga Nehring: Investigation, Writing - Review & Editing
Agnieszka Ożarowska: Investigation
Grzegorz Zaniewicz: Investigation
Włodzimierz Meissner: Investigation, Writing - Review & Editing

STATEMENTS OF CO – AUTHORS

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wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the southern Baltic", stanowił około **55%** całości i obejmował:

- sformułowanie problemu badawczego,
- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- analizę statystyczną wyników,
- graficzne i statystyczne przedstawienie wyników pochodnych fenolu,
- interpretację pozyskanych wyników pochodnych fenolu w świetle posiadanej wiedzy oraz zgromadzonego przeglądu literatury przedmiotowej,
- tworzenie manuskryptu.

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- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną na każdym etapie tworzenia manuskryptu, w szczególności: w interpretacji wyników i redagowaniu manuskryptu,
- pełnienie funkcji autora korespondencyjnego.

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- przygotowanie materiału biologicznego do analiz chemicznych,
- oznaczenia stężeń pochodnych fenolu w materiale biologicznym,
- opiekę merytoryczną.

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- przygotowanie materiału biologicznego do analiz chemicznych.

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- przygotowanie materiału biologicznego do analiz chemicznych.

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wchodzącej w skład rozprawy doktorskiej pt. „Uptake, accumulation and elimination of endocrine disrupting phenolic compounds in selected waterbirds from the sourthern Baltic", stanowił około **5%** całości i obejmował:

- pomoc w interpretacji wyników i redagowaniu manuskryptu.

....

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