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