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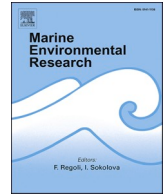
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The effect of environmental pollution on gene expression of seabirds: A review

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ABSTRACT

One of the biggest challenges for ecotoxicologists is to detect harmful effects of contaminants on individual organisms before they have caused significant harm to natural populations. One possible approach for discovering sub-lethal, negative health effects of pollutants is to study gene expression, to identify metabolic pathways and physiological processes affected by contaminants. Seabirds are essential components of ecosystems but highly threatened by environmental changes. Being at the top of the food chain and exhibiting a slow pace of life, they are highly exposed to contaminants and to their ultimate impacts on populations. Here we provide an overview of the currently available seabird-related gene expression studies in the context of environmental pollution. We show that studies conducted, so far, mainly focus on a small selection of xenobiotic metabolism genes, often using lethal sampling protocols, while the greater promise of gene expression studies for wild species may lie in non-invasive procedures focusing on a wider range of physiological processes. However, as whole genome approaches might still be too expensive for large-scale assessments, we also bring out the most promising candidate biomarker genes for future studies. Based on the biased geographical representativeness of the current literature, we suggest expanding studies to temperate and tropical latitudes and urban environments. Also, as links with fitness traits are very rare in the current literature, but would be highly relevant for regulatory purposes, we point to an urgent need for establishing long-term monitoring programs in seabirds that would link pollutant exposure and gene expression to fitness traits.

1. Introduction

The rapid growth of the human population size and the development of human industries, and their indirect consequences (e.g. climate change, habitat modifications, pollution and invasive alien species) are causing an increasing anthropogenic pressure on ecosystems (Bowler et al., 2020; Cohen, 2010). Many chemical pollutants, such as poly-aromatic organic pollutants and metals, have well-known negative effects on animals and the environment (e.g., Tan et al., 2009) leading to changes in the functioning of ecosystems (Johnston et al., 2014). In case of chronic exposure to low concentrations of contaminants (including cocktails of different toxicants) these changes are detected with a time lag. This creates a challenge for ecotoxicologists even during annual monitoring programs; not to mention failing to notify decision makers, on time, about possible negative effects on animals' and ecosystems' health. Therefore, it is both challenging and urgent to detect harmful,

sublethal effects of anthropogenic pollutants on biota early in time, before they have caused significant harm to natural populations (Rodríguez-Romero et al., 2021).

Similarly to the August Krogh principle: "For many problems there is an animal on which it can be most conveniently studied" (Krebs, 1975), one can expand and paraphrase it accordingly to biomarkers and model organisms. For example, viviparous eelpouts (*Zoarces viviparus*) have proved to be irreplaceable in monitoring early developmental disorders (Strand et al., 2004), and similarly water fleas (*Daphnia magna*) in experimental dose-response toxicology (Okamoto et al., 2015). Seabirds present an opportunity to detect the effects of chronic exposure to contaminants as well as acute exposure by using a wide array of molecular biomarkers. Seabirds are a group of globally distributed marine top-predators with an extremely slow life history, which is to say that they have a comparatively high survival rate (annual survival rates of well above 90% for many species; Jouventin and Mouglin 1981) and low

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fecundity (Bennett and Owens, 2002). Seabirds have been proposed as a key model group in avian ecotoxicology (Hao et al., 2021): they are exposed to high levels of contaminants originating from land, sea and the atmosphere, transported locally or over long distances, and magnifying along food webs. Accordingly, seabirds are considered as powerful indicators of the state of the marine ecosystems (Burger and Gochfeld, 2004). Seabirds are essential components of marine and terrestrial ecosystems, acting as ecosystem engineers and nutrient transporters (Hentati-Sundberg et al., 2020). Due to their long lifespan (e.g. up to 60 years for southern royal albatross, *Diomedea epomophora* (Schreiber and Burger, 2002)) and slow life history, their capacity to adapt to rapid environmental changes is limited, and even a small decrease in survival can have huge effects on their lifetimes reproductive success (Wooller et al., 1992). Therefore, being aware of a seabirds' health status has conservation implications (Hoffmann and Sgrò, 2011). More than half of the seabird species, for which population trends are known, are in decline (Phillips et al., 2023), highlighting the need to further understand underlying mechanisms, including sublethal effects of pollutants.

Molecular biomarkers, such as expression of pollution-affected genes, might prove to be a valuable tool, yet knowledge of the applicability and usefulness of this approach needs to be carefully considered. Genomics has been suggested to be a useful tool in ecotoxicological research since the beginning of 2000s (Snell et al., 2003; Schirmer et al., 2010). This approach seems appealing, as it allows analyzing thousands of genes representing hundreds of pathways, making it possible to identify potential health effects of individual toxicants and environmentally existing pollutant cocktails. Analyzing gene expression allows not only to assess how much individual pathways are affected by pollutant exposure, but also to look into the unknown health effects, by highlighting genomic pathways that activate as a result of exposure, either as upregulations of defense mechanisms or cellular damage on the level of gene expression (Prunicki et al., 2021; Lemaire-Gony et al., 2020; Favé et al., 2018).

In this review paper, we discuss the 37 currently available studies (including 10 *in vitro* studies with cell cultures) on seabird gene expression patterns in response to contaminants, focusing on the affected metabolic pathways. The questions we address here are: 1) to what extent has the expression of pollution-related genes in seabirds been studied? 2) What kind of physiological pathways can be linked to pollutant exposure on the gene expression level? 3) What limitations must be taken into account when conducting gene expression studies in free-living seabirds? We start with a review table indicating studied species, life-history stages, chemical contaminants, tissues and genomic pathways, specifying the used methodologies. We divide the studied physiological effects into *xenobiotic metabolism* (aimed at removing harmful substances from the organism) and *other physiological pathways* (physiological changes resulting from the negative effects of contaminants, or indicating the organismal effort to prevent these negative effects from taking place).

1.1. Representativeness of studies conducted so far

Out of 37 reviewed articles several publications originate from the same working groups/labs, generating a significant bias in materials and methods of the field. Therefore, it could be suggested that there is a lack of seabird gene expression studies to cover the full diversity of the topic. Below we have pointed out some key aspects in this matter.

From the studies published so far, only two have used non-lethal sampling methodologies, analyzing changes in gene expression from blood (Kim et al., 2013; Gibson et al., 2014). The rest of the studies have taken samples from liver tissue, which is the primary organ for pollutant metabolism, and some studies have also been conducted on brain samples (Table 1). For analyzing gene expression from liver tissue, hepatocytes have been grown *in vitro* in 11 studies (Table 1), which allows for more controlled conditions for studying gene expression, but at the same time are more challenging to interpret in ecological contexts. To be able

to conduct studies on a wider range of species, and for studying endangered species and populations, future studies would need to develop more non-invasive protocols. When birds survive the sampling, it is possible to carry out long-term monitoring, to be able to link changes in gene expression patterns to fitness effects.

So far, equally comprehensive representation of all the main environmental contaminant classes (metals, polycyclic aromatic hydrocarbons (PAHs) and organohalogenes) are seen in studies. Moreover, amongst those 37 papers, adult birds, chicks, and embryos (eggs) are uniformly covered. Indeed, as fat-soluble pollutants tend to accumulate in eggs, but also in the tissues of long-lived birds, very young and relatively old age classes seem to be the most vulnerable to contaminant effects (Fig. 1). While we are so far missing studies on pollution accumulation, gene expression and senescence in long-lived birds, a study on known-aged common gulls (*Larus canus*), using the whole transcriptome approach, indicated that gene expression is affected by the age of the birds, with older birds showing both higher vulnerability and stronger defense mechanisms against cancer (Meitern et al., 2020). A similar approach could be used in known-aged colonies for understanding the age-specific effects of pollutants (bioaccumulation in organisms' tissues) on gene expression. Interestingly, one of the two studies not looking at a pre-selected set of genes was conducted on hatchlings, indicating mainly the upregulation of defense mechanisms to avoid cellular damage and to maintain homeostasis. This suggests that protecting the developing tissues and organs against pollution effects is a crucial physiological need, and hatchlings can flexibly respond to this need, but whether this comes with a cost in terms of reduced growth or lifespan remains to be studied.

Different species might be differently exposed to contaminants because of the trophic ecology and habitats. So far, different ecologies (coastal and oceanic birds, different feeding ecology, Table S2) have been investigated, but the focus has mainly been on piscivorous species (cormorants and gulls), missing planktivorous species, species feeding on bivalves, and those feeding at very high trophic levels (predating on other birds). Similarly, we can see that studies have been geographically biased: eight of the thirteen bird species studied breed in the Arctic and subarctic regions (black-legged kittiwake (*Rissa tridactyla*), northern fulmar (*Fulmarus glacialis*), thick-billed murre (*Uria lomvia*), black guillemot (*Cephus grille*), double-crested cormorant, herring gull (*Larus argentatus*), ring-billed gull (*Larus delawarensis*), sanderling (*Calidris alba*); Table 1). Local pollution in the Arctic is low, although increasing due to the development of industries. However, due to global long-range transportation of contaminants towards polar regions, the Arctic environment still suffers from pollution and high environmental contaminant levels (AMAP, 2021; AMAP 2021). In addition, long lifespan and high fat stores contribute to the high bioaccumulation of lipophilic contaminants in organisms living in cold environments (Dietz et al., 2019), which makes the Arctic biota more susceptible to pollution. Adding studies from known high-contamination areas would improve our understanding of the effects of different exposure levels. From this viewpoint, the study conducted by Técher et al. (2016) on ring-billed gulls in the urbanized Montreal area offers an interesting insight, although the focus of this study was quite narrowly on pre-selected thyroid-related genes, such as thyroid hormone receptor (TR) genes, transthyretin (TTR) and deiodinase (D) genes.

1.2. Preselected genes versus genomics approach

Several approaches have been used to study the potential modifications of seabirds' gene expression in polluted habitats. Most studies conducted so far on wild species have analyzed the expression of specific preselected genes, using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The pre-selected gene approach can be successfully used when we know, very well, which kind of physiological pathway should be affected by a certain type of pollutant, as with PCBs and thyroid function (Técher et al., 2016), or air pollution and the tumor-suppressing P53 pathway (Wallace et al., 2017). However, with

Table 1
Overview of the studied species and methods used in research that investigated effects of pollutants on gene expression in seabirds.

Species	Pollutant	Region	Targeted genes	Mol. Method	Life stage	Tissue	Studied physiological pathway ^a	Possible biomarker genes ^b		Reference
								Upregulated:	Downregulated:	
Black-tailed gull (<i>Larus crassirostris</i>)	metals, metalloids and non-metals: aluminium (Al), cadmium (Cd), manganese (Mn), lead (Pb), chromium (Cr), iron (Fe), copper (Cu), zinc (Zn), selenium (Se), total mercury (THg), arsenic (As)	Temperate (South Korea)	1	RT-qPCR	chicks	blood	xenobiotic metabolism	metallothionein (MT)	NA	Kim et al. (2013)
Herring gull (<i>Larus argentatus</i>)	Hg	Subarctic (Great lakes area, Canada)	1	RT-qPCR	adults	brain	nervous system	NA	NA	Rutkiewicz et al., 2010
Ring-billed gull (<i>Larus delawarensis</i>)	halogenated flame retardants (HFR)	Subarctic (Montreal area, Canada)	1	RT-qPCR	adults	liver	endocrine system	type 1 deiodinase (D1)	D1	François et al. (2016)
Common cormorant (<i>Phalacrocorax carbo</i>)	dioxin and related compounds (DRCs): 2,3,7,8-tetrachlorodibenzofuran (TCDF), 3,3',4,4'-tetrachlorobiphenyl (PCB77)	Subtropical (Lake Biwa, Japan)	2	RT-qPCR and sequencing	NA	liver	xenobiotic metabolism	CYP1A4, CYP1A5	NA	Kubota et al. (2006)
Herring gull (<i>Larus argentatus</i>)	TCDD	Subarctic (Great lakes area, Canada) and temperate (Kent island, USA)	2	RT-qPCR	eggs	liver (hepatocytes)	xenobiotic metabolism	CYP1A	NA	Head and Kennedy (2019)
Herring gull (<i>Larus argentatus</i>)	TCDD	Subarctic (Chantry island, Canada)	2	RT-qPCR	eggs	liver (hepatocytes)	xenobiotic metabolism	CYP1A4, CYP1A5	NA	Head and Kennedy (2007)
Herring gull (<i>Larus argentatus</i>)	TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), TCDF	Subarctic (Lake Huron, Canada)	2	RT-qPCR	eggs	liver (hepatocytes)	xenobiotic metabolism	CYP1A4, CYP1A5	NA	Hervé et al. (2010)
Black-footed albatross (<i>Phoebastria nigripes</i>)	TCDD	North Pacific ocean	2	RT-qPCR	adults	liver	xenobiotic metabolism	aryl hydrocarbon receptor (AHR1)	NA	Mol et al. (2012)
Common cormorant (<i>Phalacrocorax carbo</i>), Mallard (<i>Anas platyrhynchos</i>)	metals and non-metals (Ag, Cd, Co, Rb, Cu, Mo, Hg, Mn, Se, Zn)	Subtropical (Lake Biwa, Japan)	2	RT-qPCR and sequencing	adults	liver, kidney, brain, muscle, heart, lung, spleen, pancreas, gonad, and eyeball	xenobiotic metabolism	MT1, MT2	NA	Nam et al. (2007)
Black-legged kittiwake (<i>Rissa tridactyla</i>), Northern fulmar (<i>Fulmarus glacialis</i>)	polychlorinated biphenyls (PCB)	Arctic (Svalbard, Norway)	3	RT-qPCR	chicks	liver, brain	xenobiotic metabolism	CYP1A4, CYP1A5 and glutathione S-transferase (GST) as general biotransformation associated genes.		Helgason et al. (2010)
Common cormorant (<i>Phalacrocorax carbo</i>)	PCBs, dichlorodiphenyltrichloroethane (DDT), perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), perfluorooctanesulfonamide (PFOSA), dioxins	Subtropical (Lake Biwa, Japan)	4	RT-qPCR and sequencing	adults and juveniles	liver	xenobiotic metabolism	NA	CYP2C45, CYP2J25	Kubota et al. (2011)

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Table 1 (continued)

Species	Pollutant	Region	Targeted genes	Mol. Method	Life stage	Tissue	Studied physiological pathway ^a	Possible biomarker genes ^b		Reference
								Upregulated:	Downregulated:	
Herring gull (<i>Larus argentatus</i>)	polybrominated diphenyl ethers (PBDE), incl penta-BDE mixture DE-71, and 3,3',5'-triiodo-L-thyronine (T3)	Subarctic (Great Lakes area, Canada)	4	RT-qPCR	adults and eggs	brain	endocrine system	thyroid hormone receptors (TR α and β)	transthyretin (TTR)	Crump et al. (2008)
Double-crested cormorant (<i>Nannopterum auritum</i>)	THg, methyl mercury (Me-Hg)	Subarctic (Great Lakes area, Canada)	5	RT-qPCR	adults	blood	xenobiotic metabolism, glutathione metabolism	glutathione peroxidase 3 (GPX3); glutathione S-transferase μ 3 (GSTM3)	NA	Gibson et al. (2014)
Herring gull (<i>Larus argentatus</i>)	halogenated organic compounds (HOC): e.g. PCBs, PBDEs, DDT, BDEs.	Arctic (Norway)	5	RT-qPCR	chicks	liver, brain	xenobiotic metabolism	CYP1A4, CYP1A5, CYP3A (reduced food)	NA	Routti et al. (2013)
Herring gull (<i>Larus argentatus</i>)	perfluorinated compounds (PFC)	Subarctic (Chantry island, Canada)	5	RT-qPCR	eggs	brain cells	endocrine system	octamer motif-binding factor (OCT-1), neurogranin (RC3)	NA	Vongphachan et al. (2011)
Common cormorant (<i>Phalacrocorax carbo</i>)	TCDD	Subtropical (Lake Biwa, Japan)	6	RT-qPCR	adults, juveniles, and eggs	liver	xenobiotic metabolism	CYP1A4, CYP1A5	NA	Iwata et al. (2010)
Red knot (<i>Calidris canutus</i>)	metals, metalloids and non-metals (Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Zn)	Atlantic Coast of France (Ré Island, Yves Bay, and Marennes-Oléron Bay)	7	RT-qPCR	adults, juveniles	liver, kidneys, muscle and feathers	xenobiotic metabolism, glutathione metabolism (and other antioxidant enzymes)	catalase (CAT) (in the liver); acetyl-CoA carboxylase (ACC), CAT, cytochrome <i>c</i> oxidase subunit 1 (COX1), malic enzyme (ME), superoxide dismutase (SOD1), SOD2 (in the kidney); ACC (in the muscle)	NA	Lucia et al. (2012a)
Northern fulmar (<i>Fulmarus glacialis</i>)	flame retardants (FR), including PBDEs, pentabromoethylbenzene (PBEB), dechlorane 602 (Dec-602), and dechlorane plus (DP)	Subarctic (Faroe island)	8	RT-qPCR	adults	liver	endocrine system	UDP-glucuronyltransferase-1 (UGT1), hepatic thyroid hormone transporter (ABCC2), albumin (ALB)	UGT1, ALB, iodothyronine deiodinase 1 (DIO1)	Mortensen et al. (2022)
Black-tailed godwit (<i>Limosa</i>)	metals, metalloids and non-metals (Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Zn)	Atlantic Coast of France (Ré Island, Yves Bay, and Marennes-Oléron Bay)	9	RT-qPCR	adults, juveniles	liver, kidneys, muscle and feathers	xenobiotic metabolism, glutathione metabolism (and other antioxidant enzymes)	NA	NA	Lucia et al. (2012b)
Sanderling (<i>Calidris alba</i>)	polycyclic aromatic hydrocarbons (PAH)	Subarctic (Canada)	9	RT-qPCR	adults	liver	xenobiotic metabolism, lipid metabolism, glutathione metabolism	NA	liver basic fatty acid binding protein 1 (LBFABP), hepatic lipase (LIPC)	Bianchini et al. (2021)
Ringed-billed gull (<i>Larus delawarensis</i>)	brominated flame retardants (BFR): e.g. PBDEs; HOCs: e.g. PCBs, DDT, HCB	Subarctic (Montreal area, Canada)	14	RT-qPCR	adults	thyroid, brain, liver	endocrine system	sodium-iodide symporter and thyroid hormone receptor α (TR α) (in the liver). Organic anion transporter protein (1C1), TTR, TR α , TR β , D2 (in the brain)	D3, thyroid peroxidase, thyroid hormone receptor β (TR β) (in the thyroid gland)	Técher et al. (2016)
Double-crested cormorant (<i>Nannopterum auritum</i>)	PAHs	Subarctic (Great Lakes area, Canada)	14	RT-qPCR	chicks	liver, lung	cell growth and death	DNA repair and cellular apoptosis genes: P53R2, BAX, GADD45 α	p53 regulation genes: MDM2	Wallace et al. (2017)
Ring-billed gull (<i>Larus delawarensis</i>)	HFRs	Subarctic (Montreal area, Canada)	14	RT-qPCR	chicks	liver	endocrine system	deiodinases type 1, 2, 3, sodium iodide symporter,	T4, total T4/T3 (in the liver)	Técher et al. (2018)

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Table 1 (continued)

Species	Pollutant	Region	Targeted genes	Mol. Method	Life stage	Tissue	Studied physiological pathway ^a	Possible biomarker genes ^b		Reference
								Upregulated:	Downregulated:	
Common cormorant (<i>Phalacrocorax carbo</i>)	TCDD, PCB126	Subtropical (Lake Biwa, Japan)	15	RT-qPCR	cells	liver	xenobiotic metabolism	thyroglobulin (in the thyroid gland) CYP1A4, CYP1A5	NA	Nakayama et al. (2008b)
Double-crested cormorant (<i>Nannopterum auritum</i>)	mix of chemicals: bisphenol S (BPS), bisphenol A (BPA), tris(methylphenyl) phosphate (TMPP), tris(2-butoxyethyl) phosphate (TBOEP), triethyl phosphate (TEP), tris(2,3-dibromopropyl) isocyanurate (TBC), allyl 2,4,6-tribromophenyl ether (ATE), 1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane (DBE-DBCH), PCB 126, BDE 209, tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz); tris(1-chloro-2-propyl) phosphate (TCIPP), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), TCDD	Subarctic (Lake Huron, Canada)	27	“ToxChip” analysis	eggs	liver (hepatocytes)	xenobiotic metabolism, immune system, endocrine system, lipid metabolism	CYP1A4, UGT1A9, fibroblast growth factor 19 (FGF19)	IGF1	Crump et al. (2016)
Double-crested cormorant (<i>Nannopterum auritum</i>)	PAHs	Subarctic (Lake Huron, Canada)	27	“ToxChip” analysis	eggs	liver (hepatocytes)	xenobiotic metabolism, endocrine system	CYP1A4, pyruvate dehydrogenase kinase 4 (PDK4)	THRSP, IGF1, TTR	Crump et al. (2017)
Herring gull (<i>Larus argentatus</i>)	organohalogen flame retardants (OFRs)	Subarctic (Lake Huron, Canada)	27	“ToxChip” analysis	eggs	liver (hepatocytes)	xenobiotic metabolism, endocrine system, lipid metabolism, mineral absorption	CYP3A37, CYP1A4, UGT1A9, SULT2B1, DIO1, VTG2, MT4, thioredoxin (TXN)	IGF1, THRSP	Porter et al. (2013)
Thick-billed murre (<i>Uria lomvia</i>), Black guillemot (<i>Cephus grylle</i>)	PAHs, metals, metalloids and non-metals (THg, As, Cd, Pb, Se)	Arctic (Baffin Island, Canada)	31	“ToxChip” analysis	adults	liver	xenobiotic metabolism, immune system, endocrine system, genetic information processing	NA	NA	Zahaby et al. (2021)
Double-crested cormorant (<i>Nannopterum auritum</i>)	OFRs	Subarctic (Lake Huron, Canada)	43	“ToxChip” analysis	eggs	liver (hepatocytes)	xenobiotic metabolism, endocrine system, lipid metabolism	ALAS1, CYP1A4	IGF1	Pagé-Larivière et al. (2018)
Double-crested cormorant (<i>Nannopterum auritum</i>)	PAHs	Subarctic (Lake Huron, Canada)	44	“ToxChip” analysis	eggs	liver (hepatocytes)	xenobiotic metabolism	CYP1A4, CYP3A37	NA	Mundy et al. (2019)
Double-crested cormorant (<i>Nannopterum auritum</i>)	BPA and similar toxicants	Subarctic (Lake Huron, Canada)	44	“ToxChip” analysis	eggs	liver (hepatocytes)	xenobiotic metabolism, endocrine system, lipid metabolism	MT4, fibroblast growth factor 19 (FGF19), cytochrome P450 A 37 (CYP3A7), Aldehyde dehydrogenase 1 family member A1 (ALDH1A1)	LIPC, DNA polymerase kappa (POLK), frizzled-related protein (FRZB), LBFABP, TTR	Sharin et al. (2021)
Double-crested cormorant (<i>Nannopterum auritum</i>)	PCB, PBDE	Subarctic (Great Lakes area, Canada)	47	“ToxChip” analysis	embryo	liver	xenobiotic metabolism, lipid metabolism, mineral absorption	Overall variability of genes of xenobiotic metabolism (CYP1A4), LBFABP, and MT4		Xia et al. (2020)

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Table 1 (continued)

Species	Pollutant	Region	Targeted genes	Mol. Method	Life stage	Tissue	Studied physiological pathway ^a	Possible biomarker genes ^b		Reference
								Upregulated:	Downregulated:	
Common cormorant (<i>Phalacrocorax carbo</i>)	PFCs; DRCs	Subtropical (Lake Biwa, Japan)	1061	custom microarray for 1061 mRNA probes followed by 4 target gene RT-qPCR	adults	liver	xenobiotic metabolism, glutathione metabolism, immune system, energy metabolism, carbohydrate metabolism, genetic information processing	glutathione peroxidase 1 (GPX1), glutathione S-transferase alpha 3 (GSTA3)	heat shock 70-kDa protein 8, tumor rejection antigen 1	Nakayama et al. (2008a)
Common cormorant (<i>Phalacrocorax carbo</i>)	polychlorinated dibenzo-p-dioxins (PCDD), furans, PCBs, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane and its metabolites (DOTS), hexachlorocyclohexane isomers (HCHs), chlordane compounds (CHLs), butyltins, and BPA	Subtropical (Lake Biwa, Japan)	1061	custom microarray for 1061 mRNA probes followed by 10 target gene RT-qPCR	NA	liver	xenobiotic metabolism, glutathione metabolism	CYP 1A4, CYP1A5	GPX3, glutathione S-transferase μ (GSTM)	Nakayama et al. (2006)
Common cormorant (<i>Phalacrocorax carbo</i>)	TCDD toxic equivalent (TEQ), PFOS	Subtropical (Lake Biwa, Japan)	1061	custom microarray for 1061 mRNA probes	adults	liver	xenobiotics metabolism, immune system, mineral absorption (iron binding)	CYP1A4, CYP1A5	hemopexin, nine genes encoding molecular chaperones	Nakayama et al. (2008c)
Laughing gull (<i>Larus atricilla</i>)	Me-Hg	Wild (unspecified)	12 000	suppression subtractive hybridization (SSH) to find diff. exp. genes, followed by sequencing and 8 target gene RT-qPCR	eggs and chicks	liver	xenobiotic metabolism, mineral absorption, endocrine system	cytochrome C oxidase subunit I	cytochrome C oxidase subunit II, transferrin, methionine adenosyltransferase	Jenko et al. (2012)
Double-crested cormorant (<i>Nannopterum auritum</i>)	Organophosphate pesticide (Chlorpyrifos)	Subarctic (Lake Huron, Canada)	21 547	whole transcriptome	eggs	liver	main affected pathways: xenobiotic metabolism, endocrine system, lipid metabolism	NA	NA	Desforges et al. (2021)

^a KEGG categories.

^b Described as significantly different in minimum three publications.

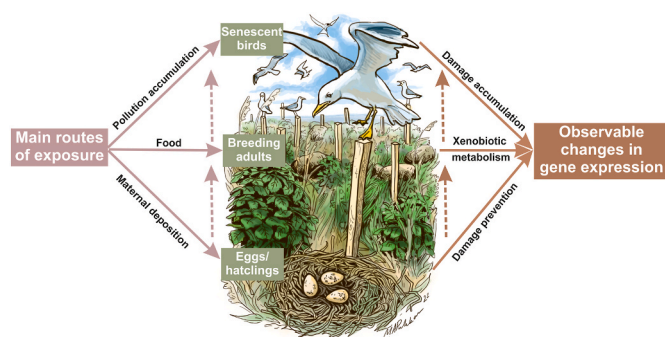


Fig. 1. Exposure routes for chemical pollution in seabirds of different age classes, and expected main effects on genomic pathways.

less studied pollutants and pollutant cocktails, the risk of the target gene approach is to miss the harmful effects of pollutants on physiological pathways not targeted by the study, thereby concluding no effects of pollutants, when there might actually be quite strong (sublethal, but fitness-affecting) effects on organismal homeostasis.

Microarray technology (Heller, 2002) has also been used, relying on a predefined set of oligonucleotide sequences representing genes of interest. In recent years, due to the availability and affordability of genomic analyses, whole genome methods have become more and more popular in ecotoxicological studies (Hrdlickova et al., 2017; Malone and Oliver, 2011). As opposed to looking at the expression pattern of single genes, using microarrays or whole transcriptome sequencing allows broader coverage of the expressed genes, and allows for the identification of the genes, metabolic pathways, physiological processes, or molecular biomarkers affected by an exposure to contaminants with consequences on the organism's health (Hao et al., 2021).

Indeed, genomics has been suggested to be a useful tool in ecotoxicological research covering thousands of genes representing hundreds of pathways (Snell et al., 2003; Schirmer et al., 2010). Although, as suggested by the low number of studies on seabird gene expression published so far, there are also factors that hinder this field of research.

- First, challenges due to the absence of reference genomes. This is probably one of the reasons why most studies published so far have focused on specific pre-selected genes for which well-conserved primer sequences are available.
- Second, caution has been suggested for interpreting gene expression results in a fitness context: as genes with large impacts on fitness are rare, a large change in gene expression is not necessarily equating to a large effect on fitness, and mRNA abundance is an unreliable indicator of protein activity (Evans, 2015). Accordingly, transcriptomics is considered more useful as a means for revealing candidate mRNAs for further study than revealing actual health effects.
- Third, gene expression analyses have been expensive so far for ecologists, limiting the possibility to use and generalize this approach. This is likely to change, as genomic analyses have become increasingly affordable over recent years.

Studies in poultry species (e.g. O'Brien et al., 2009; He et al., 2020) could be used as reference material for some research questions related to other bird taxa, but with their low genetic diversity and captive set-up, they likely do not reflect very well the ecological settings and physiological and life-history trade-offs affecting seabirds. An interesting example here is a field exposure study with chickens (*Gallus domesticus*), showing correlations between environmental dichlorodiphenyltrichloroethane (DDT) exposure and mRNA expression of several genes involved in immunity and metabolism (Thompson et al., 2018). Caution should be used in cross-species comparisons, as comparative studies of pollutant sensitivity on the gene expression level

suggests species-specific responses in detoxification genes and toxicant sensitivities (Nam et al., 2007; Helgason et al., 2010; Lucia et al., 2012a; Pagé-Larivière et al., 2018; Bianchini and Morrissey, 2020; Desforges et al., 2021). As an example, a comparison of a poultry species and a seabird species, Sharin et al. (2021) described different gene-expression responses in double-crested cormorant (*Phalacrocorax auritus*) compared to primary embryonic hepatocytes of chicken following exposure to several bisphenol A (BPA) replacement compounds. However, as long as we are lacking full reference genomes for most seabird species, model species serve as useful proxies for downstream ortholog mapping. Finally, while it may be challenging to analyze the gene expression of non-model animals due to the absence of reference genomes, several tools are frequently developed that aim to facilitate this process (Fallon et al., 2023; Shrestha et al., 2022; Meyer et al., 2011).

Although care must be taken in the interpretation of gene expression results, in accordance with the recommendation by Evans (2015), most studies published so far add biochemical analyses to support the results on gene expression analysis (e.g. Jenko et al., 2012; Técher et al., 2016). As these two approaches indeed seem to support each other's findings, gene expression analysis seems to be applicable for understanding the sub-lethal effects of pollution exposure on seabirds. Therefore, we encourage researchers to apply the whole genome transcriptome approach that has not been used so far, for a better understanding of the physiological processes being affected by chemical pollution.

1.3. Xenobiotic metabolism pathways

The first level of defense against the negative effects of chemical pollution exposure is xenobiotic metabolism, aimed at removing the toxicants from the organism through biotransformation. Organic pollutants such as PAHs are lipophilic and known to bioaccumulate in the fatty tissues. These contaminants are metabolized by biotransformation enzymes, which convert lipophilic contaminants into more water-soluble excretable compounds. However, during the course of biotransformation, highly reactive intermediate metabolites can be even more damaging than the original contaminant, e.g. leading to carcinogenesis via DNA adduct formation (Stegeman and Lech, 1991; Willett et al., 2006). In comparison to PAHs, synthetic organic chemicals like dioxins and polychlorinated biphenyls (PCB) are only partly metabolized (Grimm et al., 2015). Yet, PCB intermediates can have similar effects, such as mimicking seabird thyroid hormones and binding more strongly to hormone transporters than the endogenous hormones themselves (Ucán-Marín et al., 2009).

The main family of biotransformation genes are cytochrome P450, or CYP genes (Nelson et al., 2013). CYP genes play a major role in the relationship between environmental pollutants and animal health and have also been shown to be affected by contaminant exposure in seabirds. Most studies described in Table 1 indeed show some kind of effect of pollutants on biotransformation pathways, especially on CYP-pathway genes CYP1A4 and CYP1A5 (but also CYP3A and CYP2 genes) from the oxidative phase of biotransformation. Indeed, out of 37 studies, 16 described upregulation of CYP1A genes. For example, a study in great cormorants (*Phalacrocorax carbo*) described a positive association between the levels of dioxins in the liver and the levels of cytochrome gene expression (CYP1A4 and CYP1A5) and proteins (CYP1A) (Kubota et al., 2006; Nakayama et al., 2006). Similarly, in European herring gull embryo hepatocytes, cytochrome genes CYP1A4 and CYP1A5 were found to be upregulated following exposure to dioxins. (Hervé et al., 2010; Head and Kennedy, 2007).

Results from previous avian or mammalian studies suggest that biotransformation enzymes are catalyzed by CYP1A4, CYP1A5, and a wide range of other CYPs (Head and Kennedy, 2019; Routti et al., 2013). In particular, CYP1A4 is widely used as a biomarker for exposure of wildlife to substances that bind to the aryl hydrocarbon receptor (AhR), initiating the transcription of several enzymes involved in chemical biotransformation process (Xia et al., 2020; Crump et al., 2019). Ligands

to activate AhR include dioxins, PCBs, and many PAHs, such as benzo(a) pyrene (BaP). CYP1A expression is commonly used as a biomarker for toxicant exposure because it is directly activated via the AhR and has been validated to reflect the effects of pollutants on exposed biota (Strobel et al., 2018). In birds, bioassays measuring CYP1A4 or CYP1A5 induction from dioxin-like compounds and PAHs, at the level of mRNA expression, have been described in a number of avian species including chicken, herring gull, great cormorant, double-crested cormorant, European starling (*Sturnus vulgaris*), Japanese quail (*Coturnix japonica*), ring-necked pheasant (*Phasianus colchicus*), and jungle crow (*Corvus macrorhynchos*) (Head and Kennedy, 2019).

Biotransformation is interconnected to the organisms' overall changes in physiology. For instance, fasting or decreased food availability may indirectly induce the expression of biotransformation genes (Routti et al., 2013). This is particularly relevant in long-distance migrants, like most seabird species (Table S2). During migration or breeding, when birds use stored fat, lipophilic contaminants stored in adipose tissue relocate to other organs, such as the liver and brain, through the bloodstream (Routti et al., 2013; Hao et al., 2021). Therefore, contaminants can have a uniquely negative effect on fasting birds, of which little is known about. This phenomenon has also been studied in laboratory conditions, in relation to the levels of gene expression. For example, European herring gull chicks were administered fish liver oil containing halogenated organic pollutants (HOC), and the changes in their gene expression was compared to the control group receiving pure oil (Routti et al., 2013). Although concentrations of HOC metabolites were higher in the exposed chicks, the levels of CYP family gene expression did not differ between groups. Only after food intake was reduced in half, for the gulls in both groups, did the expression levels of genes involved in the cytochrome P450 pathway (CYP1A4, CYP1A5 and CYP3A) increase, and this increase was more pronounced in the pollution exposed group (Routti et al., 2013).

To assess whether and what effects pollutants have on biotransformation gene expression, studies must take into account the ecology of the studied species. Differences in contaminant concentrations may be caused by a myriad of ecological factors, like diet, feeding habitats, migration, life history, etc. For example, Arctic black-legged kittiwakes and northern fulmars showed different patterns in the levels of biotransformation enzyme (CYP1A4/CYP1A5, CYP2B, CYP3A, glutathione S-transferase (GST) and UDP-glucuronyltransferase (UDPGT)) activity, gene expression, and methylsulfone (MeSO₂-) PCB concentrations in liver (Helgason et al., 2010). These differences may be due to the species-specific biotransformation potential, but at the same time, other factors such as differences in migratory patterns, foraging behavior, and food specialization may also influence the concentration and metabolism of toxicants, as suggested and discussed by Helgason et al. (2010). The diet could be considered as a fundamental route of exposure for toxic substances demonstrating the qualitative linkage between dietary habits and contaminant concentrations not only between species but also within. Lucia et al. (2012b) investigated inter-individual variations in diets of godwits (*Limosa*) that could explain exposure to contaminants, and found that trace elements like Ag, As, Hg and Se concentrations were positively linked with the trophic position of birds. Godwits, like many other species, periodically switch diets in response to changes in habitat or prey availability, e.g. during migration and breeding periods.

Even within one gene family (e.g. CYP-genes), it can be challenging to select the appropriate genes for molecular ecotoxicological studies. To date, most studies have focused on the analyses of preselected genes that may not reveal affected metabolic pathways. For example, the experiment with herring gull chicks described above showed a different activity of the CYP protein (7-ethoxyresorufin-O-deethylation; EROD) involved in HOC metabolism between the experimental and control groups, but the expected difference in the expression of CYP mRNAs (CYP1A4, CYP1A5) between the two groups was not found (Routti et al., 2013). It is possible that CYP proteins were induced by other genes not

analyzed in this study, such as CYP2B (Routti et al., 2013). In addition, the timing of sample collection is important because the lifespan of mRNA is short. The level of mRNAs in genes may not always indicate the activity/level of proteins, as the protein that is the final product of the gene may also undergo post-translational modifications (Regoli et al., 2011).

In addition to the CYP pathway, the glutathione pathway is often targeted in seabird gene expression studies (Nakayama et al., 2008a; Helgason et al., 2010; Gibson et al., 2014). Glutathione is the most important intracellular antioxidant and takes part in contaminants' detoxification processes (Cooper and Hanigan, 2018). Indeed, on the level of biochemical analyses, glutathione and its associated enzymes have been suggested to be the most reliable biomarker of pollutant exposure in wild animals (Isaksson, 2010). Interestingly, there is an indication that links between pollutant exposure and glutathione pathway gene expression could be gender-specific, but potentially also linked to specific contaminants. For example, Gibson et al. (2014) investigated the association between blood Me-Hg levels and the expression of a set of pre-defined glutathione pathway genes in double-crested cormorants. The study found that Me-Hg concentrations were positively correlated with the expression of some glutathione pathway genes, glutathione S-transferase μ 3 (GSTM3) and glutathione peroxidase 3 (GPX3), but only in the blood of female cormorants. Another study in great cormorants showed sex-specific links between glutathione pathway-related gene expression and pollution exposure. Levels of hepatic perfluorinated compounds (PFC) correlated positively with expression of glutathione pathway-related genes, glutathione peroxidase 1 (GPX1) and glutathione S-transferase alpha 3 (GSTA3), but only in male cormorants (Nakayama et al., 2008a). Sex-specific relationships can be explained by the differences in oxygen consumption and energy requirements of males and females during reproduction (Gibson et al., 2014), but also by hormonal differences (e.g. endogenous estrogen may be more effective in females as a defense against peroxisome proliferators such as PFCs, Nakayama et al., 2008a). The results indicate that specific pollutants (in this case, Me-Hg and PFCs) could affect the gene expression of males and females differently. To elaborate further, sex-specific differences such as maternal depository of contaminants to eggs (especially to the first egg laid) is well-documented and even quantified (Ackerman et al., 2016). This idea has been repeatedly studied with mainly non-biotransferable and non-polar toxins such as methylmercury (Ou et al., 2015; Heinz et al., 2010). Additionally, a lesser-known effect found a positive correlation between contaminants (total Hg) in the males' blood and in the eggs (Ackerman et al., 2016) – a phenomenon which adds variability into the theory. Whether the differences between males and females seen in the above-mentioned studies in glutathione-pathway gene expression arise from sex-related uniqueness of physiology, or from the different effect to specific pollutants, remains to be studied.

1.4. Other physiological pathways

Even if pollutants can be removed from the organism or stored in less bioactive tissues (like fat), homeostasis of the organism can be affected, either directly by the toxicants, or by the pollutant metabolites produced in the process of xenobiotic removal (Stegeman and Lech, 1991). This is the field where it is more difficult to make predictions and use pre-selected genes, as we do not know what kind of cellular pathways might be damaged or upregulated to respond to damage. So far, the focus of pre-selected gene studies has been on the endocrine system (mainly thyroid function) and antioxidant enzymes, with ToxChip analyses also incorporating lipid metabolism, immune system, and genetic information processing related genes (Table 1). Common target genes include thyroid hormone-inducible hepatic protein (THRSP), deiodinase genes (D), delta-aminolevulinic synthase 1 (ALAS1), insulin-like growth factor 1 (IGF1), and liver basic fatty acid binding protein (LBFABP) – all of which could be considered as more

generalized/nonchemical-specific responders (Xia et al., 2020; Pagé-Larivière et al., 2018; Técher et al., 2018; François et al., 2016; Porter et al., 2013). As the main advantage of measuring gene expression over protein detection (which is more closely related to actual physiological effects than RNA levels) is the possibility to focus on a wider range of physiological processes. Two very good examples have been published so far. Nakayama et al. (2008a) studied the exposure to perfluorinated contaminants (PFC) and dioxins and related compounds (DRCs) in the liver of adult great cormorants, and found an elevated toxicity of the mixture of PFCs and DRCs, compared to the individual contaminants. The physiological pathways highlighted by non-targeted gene expression analysis were xenobiotic metabolism (both glutathione and CYP pathway), DNA stability protection (heat shock proteins) and energy production, among others. In the second example, the study by Jenko et al. (2012), used the suppression subtractive hybridization (SSH) method and highlighted changes in the expression of genes related to CYP pathway, but also immune response, thyroid hormones, cellular signaling, and iron transport and storage. The interplay of such a variety of responses to MeHg in laughing gull (*Larus atricilla*) serves as an important starting point for future investigations. Together, these endpoints provide some evidence of compensatory effects and suggest that several pathways may be important for overcoming toxicity effects in avian species like laughing gulls (Jenko et al., 2012).

Studies focusing on pre-selected genes have also targeted pathways related to cellular homeostasis. For example, modifications of the expression of cancer-related genomic pathways in relation to pollutant exposure have been described. In double-crested cormorants exposed to PAHs, genes involved in DNA repair and apoptosis (ribonucleotide reductase small subunit 2 (P53R2), pro-apoptotic regulator (BAX), growth arrest and DNA damage-inducible protein alpha (GADD45 α)) were upregulated in the liver and lung, while genes associated with the cancer-suppressing P53 gene pathway (ubiquitin protein gene (MDM2)) were downregulated in both tissues (Wallace et al., 2017). This suggests that pollutants are associated with both adaptive (cancer defense mechanisms, protection against oxidative stress) and harmful (e.g., cancer-promoting) changes in gene expression. Finally, different chemical contaminants have been described that affect genes involved in thyroid function in ring-billed gulls (Técher et al., 2016), European herring gulls and glaucous gulls (*Larus hyperboreus*, Ucán-Marín et al., 2009), lipid metabolism in herring gulls (Xia et al., 2020; Crump et al., 2016), and protection against metal damage in black-tailed gulls (*Larus crassirostris*, Kim et al., 2013).

2. Future directions and concluding remarks

Knowledge of the effects of pollution on seabird gene expression is still in its infancy. This research topic seems to be promising, as studying differences in gene expression is a cost-effective method for finding early signs of the negative effects of pollutants on wild population health (e.g. Zahaby et al., 2021; Helgason et al., 2010). In addition, seabirds are known, good model species to assess the state of marine ecosystems because of their long-life span, wide geographical distribution and top-predator status, which makes them highly susceptible to contaminants' bioaccumulation and biomagnification processes along the food chain (Burger and Gochfeld, 2004; Schreiber and Burger, 2002).

We suggest here that the age of the bird could be one of the most relevant factors determining the pollution burden, but also the activated genomic pathways in seabirds (Fig. 1). In hatchlings, the main exposure occurs through maternal deposit (Ackerman et al., 2016), and the organismal reaction should focus on damage prevention, as damage incurred in young age can have long-lasting fitness effects. Therefore, stressors experienced in young age can switch the organism (and its gene expression) on a set pathway where defense mechanisms are upregulated (Angelier, 2022). In adults, the most relevant exposure route is chronic exposure through food (Lucia et al., 2012b; Fossi et al., 1995), and xenobiotic metabolism should be the most active pathway

observable on the level of gene expression. In older birds, pollutants have accumulated in the tissues over their lifetime (Meitern et al., 2020), potentially leading to damage accumulation of the tissues, and we therefore suggest that older birds be targeted for a better understanding of pollutant damage effects, as these effects might be at least partially hidden in younger age classes, where damage is kept under control by upregulated defense mechanisms.

Currently, the expression of xenobiotic metabolism pathway genes (CYP) in response to habitat contamination has been the most studied effect in seabirds (Table 1). Adding more emphasis on homeostasis-related pathways would allow us to understand the health effects of pollutants and pollutant cocktails, but also help to reveal differences in vulnerabilities between species, as well as within species, between sexes and age groups. As studies have shown, even within the CYP gene family, results can depend on the selection of specific genes, bird species, gender, age, seasonal effects, or chemical contaminant (Routti et al., 2013; Lucia et al., 2012b; Helgason et al., 2010). Several studies in our review (e.g. Sharin et al., 2021; Pagé-Larivière et al., 2018; Porter et al., 2013) presented differential expression patterns in response to different chemicals. Chemical specificity of a biomarker is a valuable tool in regulatory assessment of contaminant effects, and more work is needed to find better specific and semi-specific biomarkers, that could be associated to a certain chemical or chemical group, respectively. To help meet the demand for toxicity evaluation, new methods, such as high-throughput toxicity screening, are currently being developed to permit rapid determination of toxic, molecular, and/or biochemical effects of a wide range of chemicals (Pagé-Larivière et al., 2018). Overall, we suggest focusing on methodologies allowing us to analyze a larger number of genes at a time, such as full transcriptome sequencing. Such methods would help to identify new genes affected by pollutants, and to quantify the relative expression profile of the changes. Another valuable direction would be to use proteomics to validate the observed changes (Banks et al., 2000).

We suggested that studies in ecological settings focus more on methods that explore sub-lethal health effects to allow for early intervention. However, it is important to discuss how the results of such studies could be linked with regulatory concerns. The aim of studying sub-lethal effects is to allow early intervention, as many health effects may only manifest in time periods that are longer than standard research project or monitoring protocols, including trans-generational effects. More studies on pollution-related gene expression and fitness effects are needed to include such research in monitoring programs – the latter is the direct link that is often needed by authorities for regulatory purposes. The fact that only a single seabird study (Iwata et al., 2010) has so far related changes in gene expression with changes in fitness, points more to the difficulties in assessing the causal links between fitness exposure to pollutant cocktails in the wild, and less to the possibility that these effects do not occur. As many studies so far analyze changes in gene expression, not in live seabirds, but in hepatocyte culture (Table 1), and/or use lethal sampling procedures, a future challenge in the field is to use more non-invasive sampling methods, to be able to include (long term) fitness measurements in environmental decision-making. Regulations regarding environmental pollution should stem from precautionary principles, and gene expression studies on wild animals could be the basis for new regulatory guidelines. As studies in poultry, in controlled conditions, have reliably linked gene expression changes in response to pollution with fitness effects (O'Brien et al., 2009; He et al., 2020), we can assume that similar effects can take place in ecological settings (for example, food restriction that is common in the wild magnifies pollutant effects; Routti et al., 2013). Looking at the studied physiological pathways, we suggest that changes in pathways other than xenobiotic metabolism, such as endocrine system, nervous system, or cell growth and death (Table 1), could be the basis of precautionary regulatory concerns. Such changes suggest strongly that the first line of defense (xenobiotic metabolism) has been overwhelmed and potential fitness effects are likely to occur and can therefore be used by

decision-makers.

As studies so far mostly focus on high latitude birds (Table 1), more studies of seabirds from warmer latitudes, especially from tropical regions, would give an opportunity to widen the range of physiological challenges under investigation: more and different types of contaminants, different ecological constraints, higher biodiversity, differences in 'pace of life' and basal metabolic rate etc. In particular, a fascinating understudied research area includes 'urban exploiter' species (Kark et al., 2007). Cities, acting as natural laboratories with a mix of anthropogenic stressors (Sepp et al., 2017), create a comparative experiment setting which could include heavily affected 'urban exploiter' populations' versus more 'natural' populations of the same species.

From a methodological point of view, to date, in most of the studies, birds have been euthanized and internal organs (liver, brain, lung, thyroid) sampled. This approach can be questioned in terms of ethics and can not be used with endangered species that urgently need to be studied to improve conservation practices. Thus, one of the future directions that needs to be developed is the comparison of gene expression levels between blood and internal organs in response to pollutant exposure, to develop less invasive methods: i.e. blood sampling, skin biopsies (see, for example, Rattiste et al., 2015 for a skin biopsy study of a common gull), or feather follicles (Lucia et al., 2012b; Zheng et al., 2020). Avian embryos (eggs) represent a useful biomonitoring tool for determining complex mixture effects, and the combination of a rapid extraction method, e.g. *in vitro* bioassay with chicken hepatocytes, and targeted endpoint evaluation (biochemical and transcriptomic) shows great promise as an environmental effects monitoring approach (Crump et al., 2019).

Finally, in addition to the genomic pathways studied so far, future studies of pollution-affected gene expression offer promising prospects to investigate more health consequences and target a wider range of physiological processes, to better understand the physiological trade-offs arising from the response to pollution (e.g., reduced investment in reproduction or parasite protection), and evolutionary potential of wild seabirds in a polluted world. As an outcome of pollution studies with long-lived animals, more effective early detection of sublethal effects can be used as a relevant tool for decision makers and conservationists that aim to take action and anticipate these effects to prevent future consequences at the population level.

Author statement

RK, LN and TS contributed to the conception and design of the review, and wrote the first draft of the manuscript. RM, JC, JF and MG wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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