Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2020:45

Persistent organic pollutants and effects on developmental and functional processes in mice

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Silje Modahl Johanson

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Oslo, 2020

Thesis number 2020:45
ISSN 1894-6402
ISBN 978-82-575-1710-6

Series of dissertations at the

Norwegian University of Life Sciences

Thesis number 2020:45

ISSN 1894-6402

ISBN 978-82-575-1710-6

Printed by Andvord Grafisk AS

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Acknowledgements

I would like to thank the previous and current Head of Department at the Department of Production Animal Clinical Sciences, Irma Oskam and Olav Reksen, for providing me with the opportunity to complete this PhD. I would also like to thank all animal caretakes at the section for Experimental Biomedicine, Christer W. Johnsen, Harry Hjelmseth, Arturas Kavaliauskis, Carina Berentsen, Andrea J. Eickstedt and all weekend staff (as well as Eirill Ager-Wick and Veslemøy S. Oma), for excellent support during the animal experiments, for providing a very nice lunch spot and for creating a good working environment.

Thank you Gudrun S. Boge, Ruth Halsne and Tina (Cathrine) Trangerud for conducting the animal experiment and initial analysis in Paper I. Furthermore, I would like to thank Kristine E. Aa. Hansen for incorporating me into the interpretation and writing process of Paper II.

This PhD would not have been completed without the guidance, motivation, support and inspiration from my excellent team of supervisors, Erik Ropstad, Jan Erik Paulsen, Karin E. Zimmer and Steven Verhaegen. I would also like to direct an additional thank you to my main supervisor Erik Ropstad for believing in me, for providing me with a great start on the PhD, for the quick replies and for always having a door open to answer whatever questions I might have. I'm also deeply grateful for the opportunities that Erik and Jan Erik have provided me and for including me in research projects other than my own. This has aroused my curiosity and increased my interest into several fields of research in which I'm hoping to continue to work with in the future. Thank you, Steven, for sharing your knowledge with me and for teaching me how to work with cells even though this ended up not to be included in my project. And thank you, Karin, for always helping me and answering whatever questions I might have.

I would like to thank Hanne F. Berntsen and Karin E. Zimmer for creating the POP mixture, Jan L. Lyche and Mette H. B. Müller for assisting in the chemical analysis and interpretation of results, Galia Zamaratskaia for conducting the CYP activity analysis, Özgün C. O. Umu for conducting the analysis and statistical tests on microbiota, Mona Aleksandersen for conducting histology and histopathological examinations and Gunn C. Østby for excellent help during sampling of mice, preparation of samples, initial analysis of mammary gland development and evaluation of ovarian follicles. I would also like to send a species thanks to Jonathan R. Swann and his research group at Imperial College London for allowing me to conduct metabolomics

analysis in an esteemed and very inspiring research environment. Without the knowledge and help for you, Jon, I would have been lost in the complexity of metabolomics. In addition, thanks to Eckbos Legat for founding my stay in London.

Lastly, I would like to thank my family and friends for all their love, encouragements and support, for listening, for showing an interest and for giving me advise. A special thanks to Kine Øren for triggered my interest into climbing and for continuing to be my friend even after you stopped working at NMBU. And thank you, Maria Christou, for being my good friend, for introducing me to Freddo Cappuccino and Frappé, for always listening and for always making me laugh. I'm also very happy that you introduced me to more PhD Candidates at the Veterinary School so that I got to know Helene Midttun, Mariella E. Guere Calderon, Giulia Malachin and 'the Italians'. This made the lunches, coffee breaks and everyday PhD life a lot more fun and it was great to know more people in the same PhD situation as myself. Thank you, Ajay Yadav, for inspiring me to work hard. And thank you, Mette H. B. Müller, for being the fantastic, energetic and enthusiastic person that you are and for inspiring me to pursue a carrier in academia. To my parents; Mette and Lennart, and to my aunt, Hanne, thank you for always showing an interest in my research and for the support and encouragement during this PhD. Thank you, Guri Odden, for being my very good friend and for always being there when I need someone to talk to. And most importantly, thank you to my husband, Morten, for being so understanding and patient, for always listening, for showing an interest and for being a wonderful boyfriend. Without you I would not have completed this PhD.

Abbreviations

ACF	Aberrant crypt foci
AHR	Aryl hydrocarbon receptor
AOM	Azoxymethane
APC	*Adenomatous polyposis coli*
ASV	Amplicon sequence variant
BFR	Brominated flame retardant
CAR	Constitutive androstane/activated receptor
CRC	Colorectal cancer
CYP	Cytochrome P450
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DMH	1,2-dimethylhydrazine
EDC	Endocrine disrupting compound
EDI	Estimated daily intake
FAP	Familial adenomatous polyposis
GEM	Genetically engineered mouse
HBB	Hexabromobiphenyl
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCBD	Hexachlorobutadiene
HCH	Hexachlorocyclohexane
H&E	Hematoxylin and eosin
LOD	Limit of detection
Min	Multiple intestinal neoplasia
MS	Mass spectrometry
NMBU	Norwegian University of Life Sciences
NMR	Nuclear magnetic resonance
OECD	Organization for Economic Co-operation and Development
OCP	Organochlorine pesticide

OPLS-DA	Orthogonal projections to latent structures discriminant analysis
OTU	Operational taxonomic units
PBDE	Polybrominated diphenyl ether
PCA	Principal components analysis
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCN	Polychlorinated naphthalene
PCoA	Principle coordinate analysis
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PeCB	Pentachlorobenzene
PFAS	Perfluoroalkylated substance
PFDA	Perfluorodecanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFUnDA	Perfluoroundecanoic acid
POP	Persistent organic pollutant
PXR	Pregnane X receptor
ROS	Reactive oxygen species
SCCP	Short-chain chlorinated paraffin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEB	Terminal end bud
WHO	World Health Organization
WT	Wild type

List of papers

Paper I:

S. M. Johanson, E. Ropstad, G. C. Østby, M. Aleksandersen, G. Zamaratskaia, G. S. Boge, C. Trangerud, R. Halsne, J. L. Lyche, H. F. Berntsen, K. E. Zimmer, S. Verhaegen

Perinatal exposure to a human relevant mixture of persistent organic pollutants: Effects on mammary gland development, ovarian folliculogenesis and liver in CD-1 mice.

Manuscript.

Paper II:

K. E. Aa. Hansen, S. M. Johanson, C. Steppeler, M. Sødring, G. C. Østby, H. F. Berntsen, K. E. Zimmer, M. Aleksandersen, J. E. Paulsen, E. Ropstad.

A mixture of persistent organic pollutants (POPs) and azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model.

Chemosphere. 2019. **214**:534-542. DOI: 10.3945/jn.117.251082

Paper III:

S. M. Johanson, J. R. Swann, Ö. C. O. Umu, M. Aleksandersen, M. H. B. Müller, H. F. Berntsen, K. E. Zimmer, G. C. Østby, J. E. Paulsen, E. Ropstad.

Maternal exposure to a human relevant mixture of persistent organic pollutants reduces colorectal carcinogenesis in A/J Min/+ mice.

Chemosphere. 2020. **252**:126484. DOI: 10.1016/j.chemosphere.2020.126484

Summary

Initial exposure to persistent organic pollutants (POPs) occurs by placental and lactational transfer during fetal and neonatal life and continues during the adult stage through ingestion, inhalation and dermal absorption. Many POPs have functional groups that resemble endogenous molecules rendering them with the potential for endocrine disruption during sensitive periods of organ development and function. Thus, adverse health effects may arise from exposure to POPs such as cancer and dysfunction of the endocrine, immune, reproductive, developmental or neurological systems.

Mice are extensively used in research related to human health and disease as they are small animals with short generation time and high genetic similarity to humans. In addition, transgenic and knockout technology has led to the development of murine models with high phenotypic similarity to human disease. However, several differences between humans and mice are present in the absorption, distribution, metabolism, excretion, sensitivity and susceptibility to POPs. Furthermore, attention has recently shifted from assessing the toxicity of single compounds or well-defined chemical mixtures to evaluating large and environmentally relevant mixtures of POPs.

The present study utilized two mouse models (CD-1 and A/J Min) and two exposure regimes (dietary or perinatal) to explore the effects of a human relevant mixture of POPs on female mammary gland development and ovarian folliculogenesis, liver morphology and function, and colorectal cancer development, intestinal microbiota and metabolome. The mixture composition was based on chemicals present in Scandinavian food products and the individual compound concentrations were adjusted to 0x (Control), 5 000x (Low) or 100 000x (High) human estimated daily intake levels for the general Scandinavian population.

As shown previously, the present study also demonstrated gestational and lactational transfer of POPs from mothers to offspring. Furthermore, the results showed absorption, distribution, accumulation and persistence of POPs in murine tissues. The Low dose resulted in human relevant concentrations (for some chemicals) and can be considered at least partly human relevant in both its composition and concentration.

Perinatal exposure to the mixture restricted mammary gland development and led to a premature arrest of gland growth in female CD-1 mice. In addition, it decreased ovarian follicle maturation and possibly also increased follicle atresia. Together this indicated potential endocrine disruption. Furthermore, the mixture caused persistent hepatocellular hypertrophy in CD-1 mice, but not in A/J Min mice. Thus, a strain-dependent difference in hepatic sensitivity was illustrated and, together with the induction of cytochrome P450 enzymes, indicated that the mixture may cause hepatotoxicity in sensitive strains.

The two exposure regimes, dietary and perinatal, resulted in contradictory effects on colorectal carcinogenesis in A/J Min mice. Dietary exposure moderately increased cancer development while perinatal exposure reduced carcinogenesis. The increase was synergistically enhanced when combined with one injection of azoxymethane. Interestingly, perinatal POP exposure modulated the biochemical and microbial environment of the intestine possibly to reduce colorectal carcinogenesis in the predetermined cancer model.

In conclusion, the human relevant mixture of POPs affected several developmental and functional processes in mice. The results of the present study can facilitate future mechanistic investigations into how human relevant chemical mixtures may affect biological development and function.

Sammendrag (summary in Norwegian)

Eksponering for persistente organiske forbindelser (POPs) starter i foster- og neonatalstadiet igjennom morkaken og brystmelk, og fortsetter videre igjennom matinntak, innånding og dermal absorpsjon. Mange POPs har funksjonelle grupper som etterligner endogene molekyler, noe som gir kjemikaliene et potensiale for endokrine forstyrrelser i løpet av sensitive perioder for organutvikling og funksjon. Alvorlige helseeffekter kan dermed oppstå som følge av eksponering, slik som kreft og dysfunksjon av ulike kroppsfunksjoner.

Mus er mye brukt i forskning på human helse og sykdommer fordi de er små dyr med kort generasjonstid og har høy genetisk likhet til mennesker. I tillegg har transgen- og knockout-teknologi ført til utvikling av musemodeller med høy fenotypisk likhet til humane sykdommer. Likevel finnes det flere ulikheter mellom mennesker og mus, blant annet når det gjelder absorpsjon, distribusjon, metabolisme og ekskresjon av POPs. Videre har oppmerksomheten skiftet fra å studere toksisiteten av enkeltstoffer eller veldefinerte mikser av kjemikalier til å undersøke store og miljørelevante sammensetninger av POPs.

I denne avhandlingen ble det brukt to musemodeller (CD-1 og A/J Min) og to eksponeringsregimer (diett og perinatal) til å undersøke effektene av en humanrelevant miks av POPs på hunnlig brystutvikling og utvikling av follikler i ovariene, levermorfologi og funksjon. Forekomst av tykktarmskreft, sammensetning av tarmmikrobiota og metabolom i tarminnhold og tarmvev ble også undersøkt. Miksen hadde en sammensetning av stoffer basert på kjemikalier som er tilstede i Skandinaviske matprodukter og konsentrasjonene av de individuelle stoffene ble justert til 0x (Kontroll), 5 000x (Lav) eller 100 000x (Høy) nivået av estimert humant daglig inntak for den generelle Skandinaviske populasjonen.

I likhet med tidligere studier viste også dette studiet at POPs overføres fra mor til barn igjennom graviditet og amming. Videre viste resultatene absorpsjon, distribusjon, akkumulasjon og persistens av POPs i vev fra mus. Lav eksponeringsdose resulterte i konsentrasjoner (for noen av kjemikaliene) som var humanrelevante og kan delvis vurderes til å være humanrelevant i både sin komposisjon og konsentrasjon.

Perinatal eksponering for miksen begrenset brystutviklingen og førte til en prematur stans i kjertelveksten i CD-1 hunnmus. Videre førte eksponeringen til en reduksjon i follikkelmodningen

og mulig også en økning av follikkelatresi i ovariene. Til sammen indikerte dette en mulig endokrin forstyrrelse. Miksen førte også til persistent hypertrofi av leverceller i CD-1 mus, men ikke i A/J Min mus. Dette tydet på forskjeller i leversensitivitet mellom musemodeller og, sammen med en induksjon av cytokrom P450 enzymer, indikerte dette at miksen kunne føre til levertoksisitet i høysensitive mus.

De to eksponeringsregimene, igjennom diett og perinatal overføring, førte til motstridende effekter på tarmkreft i A/J Min mus. Eksponering igjennom dietten førte til en moderat økning av kreft, mens perinatal eksponering reduserte karsinogenesen. Økningen ble synergistisk forsterket i kombinasjon med én injeksjon av azoxymetan. Perinatal POP-eksponering modulerte det biokjemiske og mikrobielle miljøet i tarmen, noe som mulig reduserte tarmkreftutviklingen i den forutbestemte kreftmodellen.

Vi konkluderer med at den humanrelevante miksen av POPs påvirket utviklingen og funksjonen til flere kroppsfunksjoner i mus. Disse resultatene gir et godt grunnlag for videre forskning knyttet til hvordan humanrelevante blandinger av POPs kan påvirke utvikling og helse hos dyr og mennesker.

1 Introduction

1.1 History

Synthetic chemicals for use in agriculture and industry were introduced as far back as in the 1920s and increasingly used during the 1940s and '50s. The insecticide dichlorodiphenyltrichloroethane (DDT) became famous during World War II for its ability to control vectors of malaria and typhus. However, guided by the book 'Silent Spring' by Rachel Carson (Carson 1962), questions were raised about the widespread use of DDT and other chemicals prior to investigation of their adverse effects on humans and wildlife. DDT was linked to drastic decreases in populations of predatory birds due to eggshell thinning and subsequent reproductive failure (Peakall 1970). Furthermore, evidence showed that chemicals such as DDT had the ability to accumulate to high concentrations in animals at the top of the food chain and cause unexpected adverse effects (Woodwell 1967). In 1972, the use of DDT in agriculture was put under strict regulations and a phase-out process of DDT and polychlorinated biphenyls (PCBs) was started. During the coming years, regulations were expanded to include more synthetic chemicals with toxic effects in wildlife and humans, and in 2004 the Stockholm Convention[1] on Persistent Organic Pollutants was endorsed by 172 countries to ban or heavily restrict the use of 12 ('the dirty dozen') pesticides, industrial chemicals and by-products (Table 1). By 2017, an additional 16 persistent organic pollutants (POPs) had been incorporated into the Stockholm Convention due to their persistent, accumulative and toxic nature, and the treaty was ratified by 181 countries (Secretariat of the Stockholm Convention 2017). The Stockholm Convention, together with other international agreements that also aim to protect human health and the environment from potential harm from synthetic chemicals and hazardous waste (e.g. the Basel[2] and Rotterdam[3] Conventions), has resulted in a drastic reduction in the production and use of POPs. However, despite being under heavy restrictions for almost 50 years, POPs are still ubiquitous in the environment and human exposure is, thus, inevitable.

[1] www.pops.int
[2] www.basel.int
[3] www.pic.int

Table 1. POPs included or under consideration to be included in the Stockholm Convention.

Initial 12 POPs	POPs included by 2017	Under consideration
Aldrin[1]	α-HCH[1,3]	Dechlorane Plus[2]
Chlordane[1]	β-HCH[1,3]	Dicofol[1]
DDT[1]	Chlordecone[1]	Methoxychlor[1]
Dieldrin[1]	DecaBDEs[2]	PFHxS[2]
Endrin[1]	Endosulfan[1]	PFOA[2]
Heptachlor[1]	HBB[2]	
HCB[1,2,3]	HBCD[2]	
Mirex[1]	Hexa- and heptaBDEs[2]	
Toxaphene[1]	HCBD[3]	
PCBs[2,3]	Lindane[1]	
PCDDs[3]	PeCB[1,2,3]	
PCDFs[3]	PCP[1,2]	
	PFOS[2]	
	PCN[2]	
	Tetra- and pentaBDEs[2]	
	SCCPs[2]	

[1]Pesticides; [2]Industrial chemicals; [3]By-products.
See abbreviations for full chemical names.

1.2 Persistent organic pollutants

1.2.1 Chemical properties, categorization and use

All POPs consist of a carbon backbone where one or more carbon atoms are bound to one or more halogen atoms, thus forming chlorinated, brominated or fluorinated compounds (Figure 1). Because of the strength of the covalent carbon-halogen bond, POPs have a high chemical stability, which causes them to resist degradation by biological systems and results in long elimination half-lives in animals, humans and the environment (O'Hagan 2008, Secretariat of the Stockholm Convention 2017). For instance, the half-life of PCB-153 has been reported to be 14.4 years in humans (Ritter *et al.* 2011).

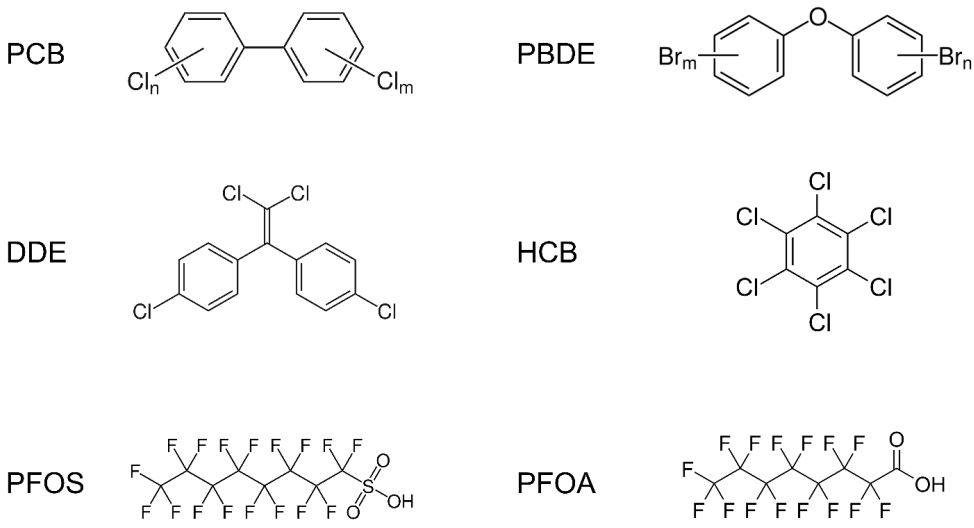

Figure 1. Chemical structures of selected POPs including polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB), perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA).

The halogen atoms have a low capacity to form hydrogen bonds (hydrophobic) and tend to be attracted to lipids (lipophilic), although there are exceptions such as for the fluorinated compounds. POPs with high lipophilicity can readily cross over cell membranes by passive diffusion and accumulate in adipose tissues of living organisms (bioaccumulation). Furthermore, many of these compounds have functional groups that resemble those of endogenous molecules (e.g. hormones). Thus, a variety of POPs can disrupt the hormonal system and homeostasis in the body (Diamanti-Kandarakis *et al.* 2009, Gore *et al.* 2015).

POPs used as pesticides are directly emitted into the atmosphere by spraying. Other POPs are semi-volatile and are released to air by volatilization. Thus, the atmosphere contributes greatly to the long-range transport of POPs (Bogdal *et al.* 2013). Through biological processes of air-water exchange such as snow or rain, POPs are conveyed to large bodies of water and can be globally distributed with ocean currents. In addition, POPs can be transported in polluted organisms through migration and by the export of e-waste (discarded electronic and electrical equipment) to developing areas (Breivik *et al.* 2016). Consequently, POPs have a widespread

distribution and are found in remote areas far away from industrial or agricultural cites with direct release of the chemicals (AMAP 1997, Bogdal *et al.* 2013).

As put forth by the Stockholm Convention, POPs can be categorized as pesticides, industrial chemicals or by-products. However, categorization by the type of halogen atom (chlorine, bromine or fluorine) is also possible.

In the chlorinated category we find the organochlorine pesticides (OCPs) such as DDT and hexachlorocyclohexanes (HCHs). These compounds were used worldwide for disease vector control and for controlling pests on agricultural crops in the 1940s. The Stockholm Convention still allows the use of DDT to control diseases such as malaria in regions that do not have other cost-efficient alternatives (Secretariat of the Stockholm Convention 2019a). Dichlorodiphenyldichloroethylene (DDE) is a metabolite of DDT and, because of its persistent nature, is considered a major environmental pollutant (Faroon and Harris 2002). Other examples of chlorinated POPs are hexachlorobenzene (HCB) and the class of PCBs. HCB is primarily a fungicide that was much used from 1945, but it is also a by-product from the production of other halogenated compounds (van Ommen and van Bladeren 1989). The class of PCBs consists of 209 isomers that were widely used from the 1930s in heat transfer fluids, in electric transformers and as additives in paint, paper and plastics (Safe 1993). Despite being under heavy restrictions and bans since the 1970s and -80s, HCB and PCBs are still detected in humans, animals and the environment worldwide.

Hexabromocyclododecane (HBCD) and the polybrominated diphenyl ethers (PBDEs) are among the brominated POPs. These are flame retardant chemicals that were highly used from the 1950s to reduce the flammability of plastics, textiles, electronics and synthetic building materials (Segev *et al.* 2009). As these brominated flame retardants (BFRs) are lipophilic and highly persistent, they accumulate in human adipose tissue and breast milk (Hites 2004). The commercial mixtures of octa- and pentaBDE (with hexa- and heptaBDE, and tetra- and pentaBDE as main components, respectively) were included in the Stockholm Convention and banned from use in 2004. DecaBDE was banned in 2009 and HBCD was put under heavy restrictions in 2013.

The only fluorinated POP listed in the Stockholm Convention by 2017 is perfluorooctane sulfonic acid (PFOS). However, perfluorooctanoic acid (PFOA) and perfluorohexane sulfonic acid (PFHxS) are under consideration for inclusion in the treaty due to their persistent and toxic nature.

These compounds are among the perfluoroalkylated sustances (PFASs) and have been much used in textile impregnation, wax, lubricants and firefighting foam from the 1950s due to their unique surface tension properties (Renner 2001). In addition to PFOA and PFHxS, hundreds of other PFASs are widely produced and used today including perfluorodecanoic acid (PFDA), perfluorononanoic acid (PFNA), perfluoroundecanoic acid (PFUnDA).

1.2.2 Human exposure

Humans are exposed to POPs through ingestion, inhalation and dermal contact (WHO 2010). For the general population, ingestion of contaminated food is the main route of exposure, especially for PCBs, OCPs and (partly) PFASs (Djien Liem *et al.* 2000, Trudel *et al.* 2008). Through long-range transport and due to the highly persistent nature of these compounds, POPs end up in low concentrations in fruits, vegetables and grains, and in higher concentrations in meat, fish and dairy products. Because of this increase in concentration with increasing trophic level (biomagnification), animals at the top of the food chains, including predatory animals and humans, accumulate high concentrations of POPs (WHO 2010). Chronic high-level POP exposure is well documented in indigenous human populations of the Arctic region with traditional diets consisting largely of local wildlife such as sea mammals (AMAP 2015a). Other routes of exposure, particularly to BFRs, are through inhalation of indoor dust and ambient air (Lorber 2008).

Occupational use of pesticides or industrial chemicals often lead to additional POP exposures. Furthermore, unusual chemical accidents or releases may lead to acute high-level exposure and potentially have devastating consequences on human health. For instance, the accidental exposures of Japanese (in 1968) and Taiwanese (in 1978 to -79) residents to rice oil contaminated with PCBs and polychlorinated dibenzofurans (PCDFs) were later associated with adverse effects on pregnancy and cognitive development of prenatally exposed children (Chen *et al.* 1992, Tsukimori *et al.* 2008). Another example is the spraying of herbicides such as Agent Orange by the United States military forces during the Vietnam War (1961-1971) with the aim to remove vegetation that could be used as cover by the North Vietnamese forces. This extensive use of herbicides was later linked to an increased risk of birth defects both in and outside of Vietnam (Ngo *et al.* 2006).

POPs residing in the blood of pregnant women are known to cross the placental barrier and, thus, initiate exposure prenatally (Vizcaino *et al.* 2014, Winkens *et al.* 2017). After birth, POPs stored in maternal lipid tissues are mobilized and redistributed to the lipid-rich breast milk, which continues to expose the infants during the breast-feeding period. This mobilization has also been considered a route for excretion of POPs in mothers (Schecter *et al.* 1996). DDT was the first pollutant to be reported in human breast milk as early as in 1951 (Laug *et al.* 1951). Recently, a large variety of POPs were reported in breast milk from Norwegian mothers (Iszatt *et al.* 2019, Lenters *et al.* 2019). In addition, inhalation of dust and air containing POPs and ingestion of other substances than breast milk (mostly due to the frequent hand-to-mouth behavior) are considered important additional exposure routes of POPs in infants and young children (Johnson-Restrepo and Kannan 2009).

Critical periods of development

Exposure to POPs starts *in utero* and continues throughout the postnatal periods from infancy to childhood and puberty, all of which are periods of rapid growth and development (WHO 2010). The rate of growth and development of bodily functions peaks at around 27-28 weeks of gestation followed by a steady decline throughout the first 3 years of life. Birth only has a minor effect on this decline, which reaches a plateau around 4 years of age (Hindmarsh 2012). During development, several periods can be critical as they include vital processes occurring at molecular, cellular, organ or organism level. As organ systems develop at different rates, the critical periods of development can differ in length and time. It is the difference in these critical periods (between themselves and compared to the general development) together with the duration and concentration of exposure that define the severity of the disturbance (WHO 2010). Thus, developing humans and animals are particularly sensitive to stimuli from harmful agents such as POPs during development. Multiple studies have shown both acute (such as fetal death, growth restriction and birth defects) and chronic effects (revealed later in life such as neurological disorders, cardiovascular and infectious diseases and cancer) to be associated with exposure to POPs during critical periods of development (as reviewed in Winans *et al.* (2011), Fenton and Birnbaum (2015), Gore *et al.* (2015) and Heyer and Meredith (2017)).

1.2.3 Mixed exposures

By monitoring chemicals in the environment, evidence has shown that humans and animals are exposed to a number of chemicals at different combinations via food, water, air and consumer products. Generally, the toxicity of chemicals including POPs have been evaluated in studies using single chemicals at relatively high doses. This is partly due to the theoretical and practical challenges with assessing the toxicity of mixtures as the chemicals may interact and cause effects not seen in single chemical studies. However, attention has recently shifted to evaluating the effects of more realistic exposure scenarios with multiple chemicals, also at relatively low doses (Kortenkamp 2014).

Chemical interaction may occur in different ways. The simplest way is in the form of no interaction where the chemicals act by dose addition or independent action to produce the effect. Dose addition is expected when the chemicals interact with the same biological site and by the same molecular mechanism (mechanism of action), while independent action is predicted when chemicals act by different mechanisms at different sites (IGHRC 2009, Reffstrup *et al.* 2010). According to dose addition, effects can be expected at chemical doses that individually are below those required to cause effects in single chemical studies (the threshold of effect). On the other hand, independent action does not anticipate an effect when the individual chemical doses are below the threshold of effect (Kortenkamp 2014).

However, if the effect of a mixture deviates from the effect predicted by dose addition or independent action, it may be caused by (IGHRC 2009):

- Direct chemical-chemical interaction that creates a chemical complex with a different effect compared to the original chemicals;
- Modification of the absorption, distribution, metabolism or elimination of the chemicals;
- Competition between chemicals that bind to the same biological site;
- Alterations in the responsiveness of the target (cell or tissue);
- Chemicals having functionally competing effects on the target.

Where chemical interaction arises, there is the possibility of potentiation, synergism, inhibition or antagonism on the overall effect. Potentiation occurs when one chemical enhances the toxicity of another chemical without changing its own toxicity, while synergism describes the situation

where the toxicities of both chemicals are enhanced. Oppositely, inhibition or antagonism occurs when one chemical reduces the toxicity of another chemical without itself being affected or by also reducing its own toxicity, respectively (IGHRC 2009, Reffstrup *et al.* 2010).

The nature of the chemical interaction may change with the dose and duration of exposure, as well as the discovery of more sensitive endpoints. Furthermore, the threshold of effect may be difficult to predict due to variance around the baseline (background noise) and the possibility for non-monotonic dose-response relationships (Vandenberg *et al.* 2012, Kortenkamp 2014). Thus, it is difficult to assess the overall effect of chemical mixtures from studies using single chemicals only.

1.2.4 Metabolism

After POPs have entered the body through ingestion, inhalation or dermal absorption, they are distributed to all tissues via the lymphatic and circulatory systems. The ability of tissues to take up POPs from the blood is dependent on the partition coefficient of the chemical between blood and lipid, the lipid content of the organ and the blood flow (Morgan and Roan 1971). The liver has a central role in the metabolism of xenobiotics and is considered a common target organ for POP exposure. The metabolism of xenobiotics including POPs can be divided into phase I and phase II reactions (Williams 1947).

The cytochrome P450 (CYP) mixed function oxidases are responsible for 70-80% of the phase I reactions and oxygenates the xenobiotic to a more polar compound with the purpose to increase the hydrophilic properties of the chemical. This more polar compound can further be metabolized by phase II enzymes, such as uridine diphosphate glucuronosyltransferase, glutathione S-transferase and sulphotransferase, which conjugate the newly formed compound with an endogenous molecule resulting in an even more hydrophilic compound. This polar conjugated xenobiotic can either be further metabolized (by phase III reactions) or directly excreted from the cell through active transport by membrane transporters (Nebert and Dalton 2006). However, metabolism by phase I enzymes such as CYPs can activate procarcinogens or generate reactive metabolites and by-products (such as reactive oxygen species (ROS)), which could be more toxic than the original xenobiotic (Bondy and Naderi 1994, Nebert and Dalton 2006).

The expression of CYP enzymes is regulated by nuclear receptors such as the aryl hydrocarbon receptor (AHR), the pregnane X receptor (PXR) and the constitutive androstane/activated

receptor (CAR) (Waxman 1999, Tolson and Wang 2010). Due to the ability of POPs to passively diffuse over cell membranes, exposure to these chemicals have been shown to activate nuclear receptors and subsequently increase the expression of CYPs (Sanders *et al.* 2005, Pacyniak *et al.* 2007, Szabo *et al.* 2008, van der Ven *et al.* 2008). Upregulation of hepatic CYP expression at early life stages has been stated as an early indicator of liver toxicity and carcinogenicity (Dunnick *et al.* 2018). In addition, reduced xenobiotic metabolism has been reported as justification for the relatively high susceptibility to POPs in fetuses and neonates (Milsap and Jusko 1994, Damstra 2002).

PFASs have a lower lipophilicity than the other POPs due to the high electronegativity of the fluorine atoms (O'Hagan 2008). Thus, these compounds bind to proteins and accumulate in protein-rich tissues such as the liver (Klevens and Ellenbogen 1954, Han *et al.* 2003, Qin *et al.* 2010). The metabolism of PFASs is not well known, however, substantial differences in metabolic and excretion rates have been reported between humans and other species (e.g. PFOA has an elimination half-life of 3.8 years in humans and 17-19 days in mice (Lau *et al.* 2007)). Despite the low lipophilicity of PFASs, these compounds can still be transferred from mothers to offspring through the placenta and breast milk (Fromme *et al.* 2010).

1.3 Health effects of POPs

In 1995, the increasing evidence of toxicity by POPs in humans and wildlife led the United Nations Environment Programme (UNEP) to start the discussions about implementing international agreements and regulations of these chemicals. Consequently, the Stockholm Convention on POPs was adopted in 2001 (and entered into force in 2004) with the aim to protect human health and the environment from persistent, globally distributed and accumulative chemicals (Secretariat of the Stockholm Convention 2019b).

The importance of using evidence on health effects in wildlife to forecast the risk of health effects in humans was emphasized by the World Health Organization (WHO) and indicated that POPs can cause endocrine, immune, reproductive, developmental or neurological dysfunction (Ross and Birnbaum 2001). Epidemiological studies have also found positive associations between POPs and various types of cancers such as colorectal (Howsam *et al.* 2004), breast (Høyer *et al.* 2000, Arrebola *et al.* 2015, Morgan *et al.* 2016), ovarian (Sharma *et al.* 2015) and testicular cancer (McGlynn *et al.* 2008, Purdue *et al.* 2009, Giannandrea *et al.* 2011).

In 2004, the WHO defined the term 'adverse effect' for use in chemical risk assessment as a:

'*change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.*' (page 10 in IPCS (2004)).

A weight-of-evidence (WOE) approach is usually taken to determine if a chemical can have an adverse effect on the environment, wildlife and humans. This approach is of great value when aiming to understand more about the relationship between the dose or time of exposure and the affected endpoint. Some endpoints are straight forward adverse as they fulfill one or more of the abovementioned criteria. However, the adversity of other endpoints may be more complicated to assess such as when the adverse effect (e.g. breast cancer) is caused by exposure during a critical period of development but does not manifest until adult life (Fenton 2009, Fenton and Birnbaum 2015).

1.3.1 Endocrine disruption

Physiological processes and homeostatic function are maintained and regulated by hormones that are produced by the endocrine glands (such as the thyroid, mammary, ovarian, testicular or adrenal glands; Figure 2) and distributed throughout the body via the circulatory system. Multiple of these processes are vital for development, health and disease. Endocrine disruption is the interruption of the ability of a cell or organ to communicate with other cells or organs through the use of endogenous molecules (mostly hormones). Endocrine disrupting compounds (EDCs) are either natural or synthetic compounds which alter the hormonal and homeostatic systems within an organism by interfering with the synthesis, secretion, transport, binding action, metabolism or elimination of hormones (Diamanti-Kandarakis *et al.* 2009). Many POPs are well-known EDCs (Gore *et al.* 2015). Endocrine disruption by POPs may occur through multiple mechanisms including interaction with nuclear hormone receptors (e.g. estrogen, androgen, progesterone and thyroid receptors), non-steroid receptors (e.g. neurotransmitter receptors) or orphan receptors (e.g. aryl hydrocarbon receptor), and pathways involving the biosynthesis and metabolism of hormones (Diamanti-Kandarakis *et al.* 2009). Depending on the timing of exposure and the dose of the EDC, endocrine disruption can leave permanent changes in the

physiology, morphology or behavior of the organism, which further may affect organ development and function, or increase the susceptibility to disease (Gore *et al.* 2015).

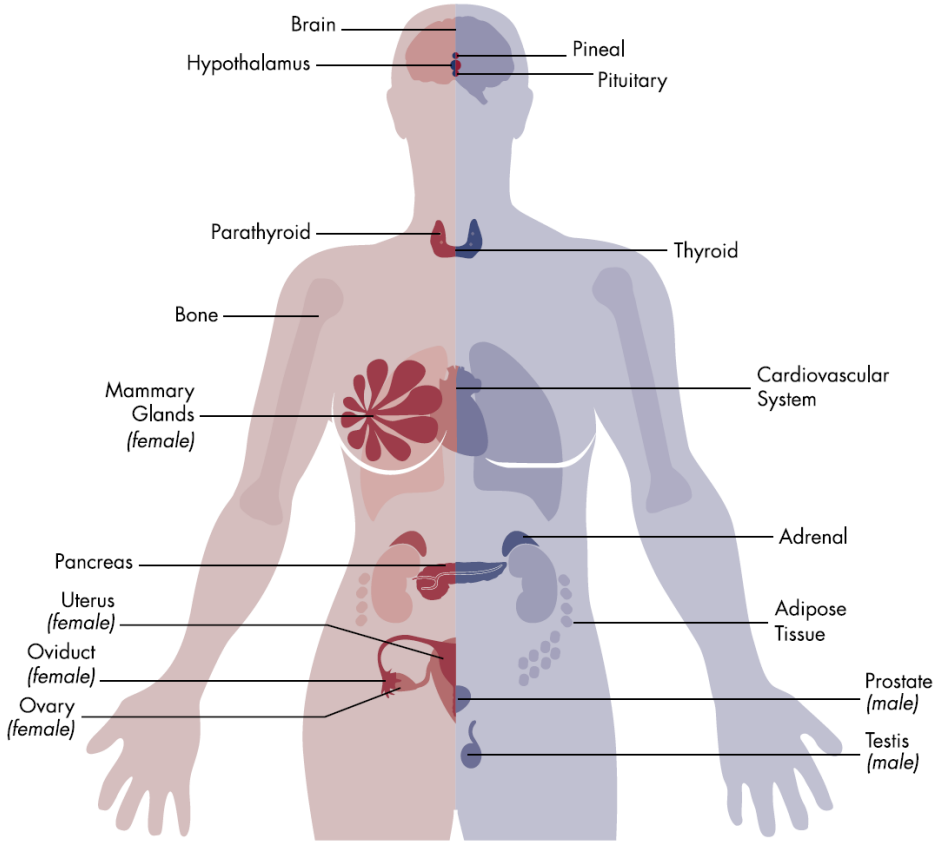

Figure 2. The female (left) and male (right) endocrine glands. These glands produce signaling molecules (hormones) that are distributed throughout the body by the circulatory system and regulate and maintain development, physiological processes and homeostatic functions. Reprinted from Gore *et al.* (2015) with permission from Oxford University Press (license number: 4853621324365).

1.3.2 Reproductive dysfunction

EDCs may influence the reproductive ability of animals and humans by affecting the development and function of the reproductive organs (Gore *et al.* 2015). Both the female and male reproductive systems require correct development of intricate structures, such as the ovary, testis and mammary gland, in order to reach optimal reproductive function. This development, as well as the overall function of the reproductive organs, are regulated by hormones. In females, studies have shown that exposure to EDCs affect ovarian and mammary gland development, structure and function (including impaired follicle formation, inhibited follicle growth, increased follicle atresia, increased sensitivity to carcinogens and lactational impairment). This may further cause adverse effects such as infertility, premature menopause and cancer (Macon and Fenton 2013, Gore *et al.* 2015). In males, exposure to EDCs has been linked to a range of reproductive disorders caused by disruption of hormonal systems during the development of the reproductive organs including cryptorchidism, hypospadias, poor semen quality and increased risk of developing testicular germ cell cancer (Gore *et al.* 2015).

Effects on the ovary

The primary function of the adult ovary is to differentiate an ovarian follicle and to release a mature oocyte for fertilization and reproduction. Furthermore, as an endocrine gland the ovary produces steroid hormones that support pregnancy and causes the development of secondary sexual characteristics. The number of primordial follicles (the ovarian follicle pool) that can develop into mature oocytes is fixed at the time around birth. Most of these primordial follicles are maintained in a resting state for months or years. However, some follicles are initially recruited and develop from primordial to primary, from primary to pre-antral and from pre-antral to antral follicles. Most antral follicles undergo atresia (degeneration and resorption). However, after puberty and under optimal hormonal stimulation (from follicle stimulating hormones), a subset of antral follicles escapes atresia and one dominant follicle continues the development into a mature oocyte, which is released during ovulation (Figure 3). The remaining theca and granulosa cells transform into the corpus luteum (McGee and Hsueh 2000).

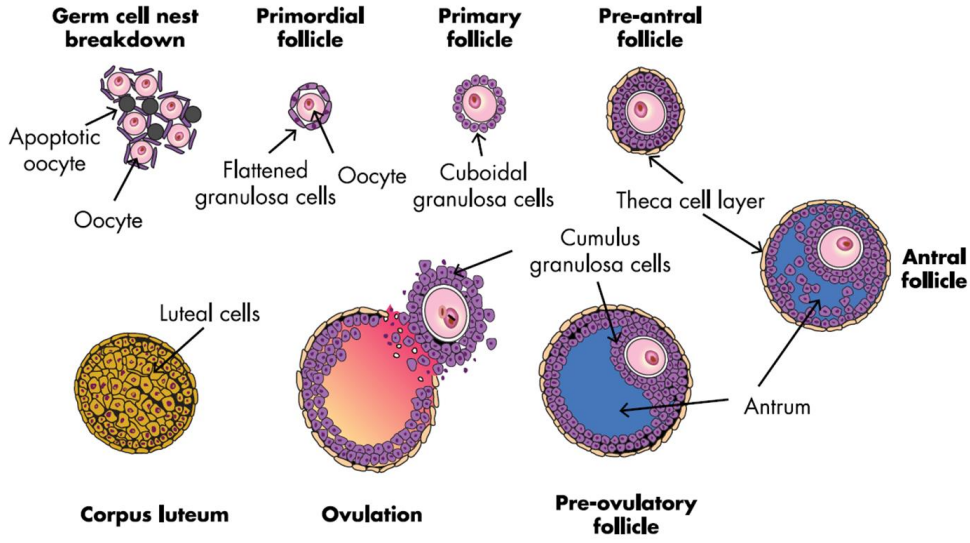

Figure 3. Schematic overview of ovarian follicle development. The germ cell nest breaks down around birth and results in the fixed pool of primordial follicles. The primordial follicles continue their growth to primary, pre-antral, antral and pre-ovulatory follicles. The oocyte within the pre-ovulatory follicle further matures and is released during ovulation. The remaining theca and granulosa cells form the corpus luteum. Adapted from Gore *et al.* (2015) with permission from Oxford University Press (license number: 4853621324365).

Follicle formation, maturation and, ultimately, the female reproductive ability is under strict regulation by the endocrine system (including local paracrine factors, steroid hormones and gonadotropins). POPs with endocrine disrupting properties may disturb this regulation by modulating hormone levels (e.g. by interacting with nuclear receptors) that are vital for folliculogenesis (Uzumcu and Zachow 2007). In addition, POPs may impact ovarian function through the production of ROS (which may initiate atresia) and alterations in DNA methylation patterns (Vabre *et al.* 2017). Studies have shown that exposures to POPs may have multiple detrimental effects on the ovary which could result in infertility, premature ovarian insufficiency or abnormal production of sex steroid hormones (reviewed in Vabre *et al.* (2017) and Patel *et al.* (2015)). Furthermore, exposure to EDCs during fetal and neonate development (when the primordial follicles are formed) may not only lead to adult health effects but may also cause alterations that can be transmitted to the next generation (Uzumcu and Zachow 2007).

Effects on the mammary gland

The mammary gland serves as the primary organ of energy transfer from mothers to infants and undergoes most of its development postnatally. The developmental process is regulated by hormones, growth factors and stromal factors, which does not yield a fully developed and differentiated gland until late in pregnancy. This developmental process is similar between humans and rodents and starts with the epithelial bud forming around the nipple (at 6-7 days before birth in rodents) (Fenton 2006, Rudel *et al.* 2011). After birth, the gland epithelium grows at the same pace as the body and extends towards the lymph node. In the mouse, the highly proliferative terminal end buds (TEBs) are formed after post-natal day 14 and leads the way for future epithelial branching until the entire mammary fat pad has been filled (Figure 4). After this, the TEBs differentiate into terminal structures (at around post-natal day 63 in the mouse) and are no longer present in the mature gland (Watson and Khaled 2008).

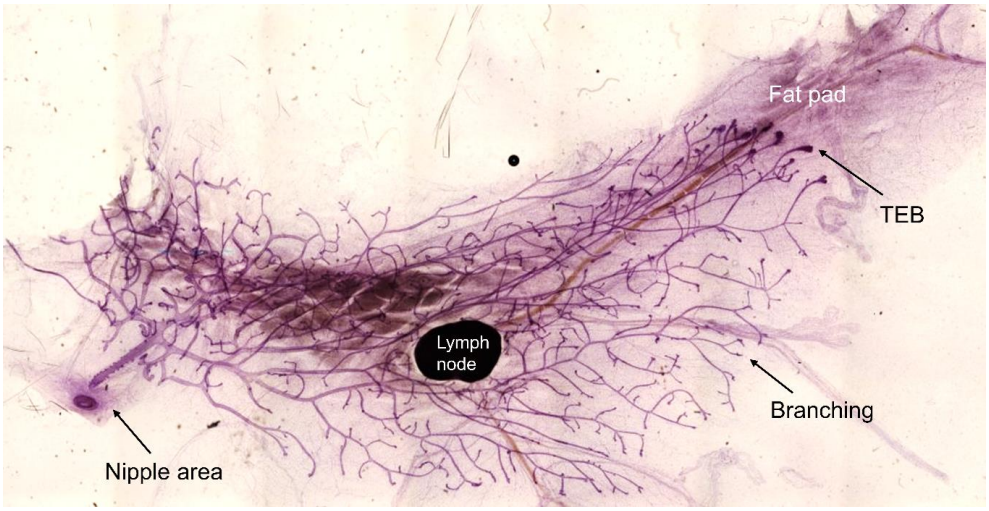

Figure 4. The fourth mammary gland in 6-week-old female CD-1 mice. Terminal end buds (TEBs) are present and lead the way for further branching of gland epithelium and extension throughout the fat pad away from the nipple area and lymph node.

Three phases have been suggested as critical for the development of a fully functioning mammary gland. These are when 1) the primary epithelial duct forms (coincident with the nipple) during prenatal development, 2) when the mammary gland grows exponentially (with the use of TEBs) around the time of puberty, and 3) when the gland prepares itself for functional lactation during

pregnancy. Interference by POPs with endocrine disrupting properties during these phases of development may alter the timing of developmental events, alter the sensitivity to toxic or carcinogenic compounds or cause lactational insufficiency during pregnancy (Fenton 2006). For instance, the highly proliferative and undifferentiated TEBs are considered particularly vulnerable to chemicals with carcinogenic properties and exposures that restrict TEB differentiation could drastically increase the risk of breast cancer (Russo and Russo 1996, Fenton 2006).

1.3.3 Cancer

Cancer is defined as abnormal cell growth and is one possible end-product of somatic cell evolution where the cell accumulates positive ('driver') mutations that makes it capable of avoiding regulation of cell proliferation, invading other tissues and spreading throughout the body (Martincorena *et al.* 2017). Cancer cells have several characteristic features (Figure 5) including the ability of unlimited proliferation, to avoid growth-suppressing signals and apoptosis, to sustain growth by angiogenesis (the creation of new blood vessels), to modify cellular metabolism and to evade recognition and destruction by the immune system (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011).

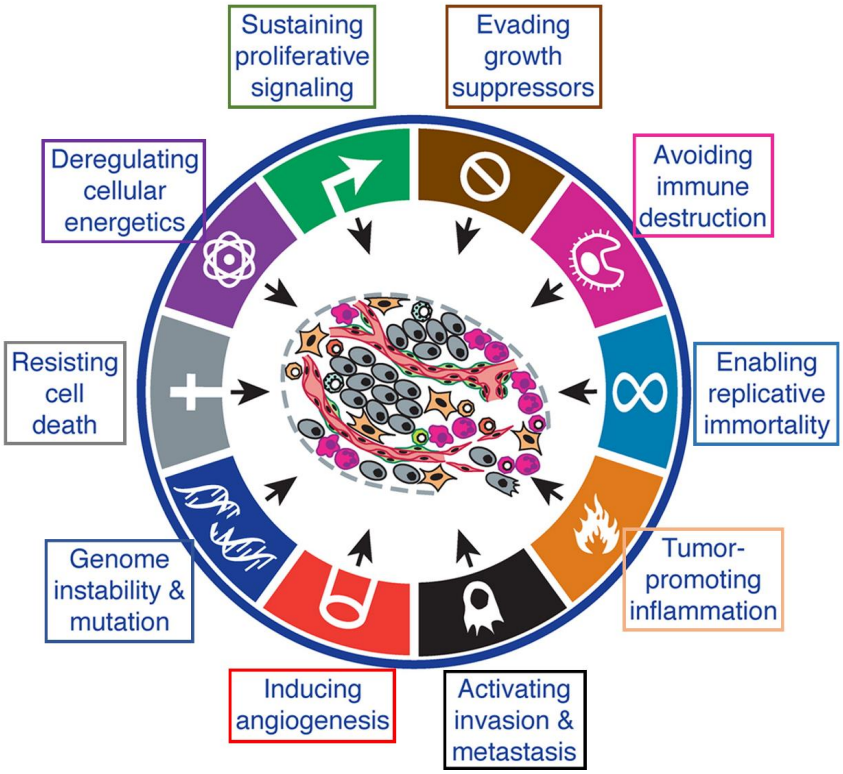

Figure 5. The hallmarks of cancer proposed by Hanahan and Weinberg (2011) which shows the characteristics of most (if not all) cancers. Sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion & metastasis, inducing angiogenesis and resisting cell death were proposed as hallmark functional capabilities for cancer development in 2000 (Hanahan and Weinberg 2000). An increasing body of research suggested to include an additional two hallmarks (deregulating cellular energetics and avoiding immune destruction) as well as two enabling characteristics (tumor-promoting inflammation and genome instability & mutation) in 2011. Adapted from Hanahan and Weinberg (2011) with permission from Elsevier (license number: 4853630237043).

The multistep theory of carcinogenesis was first developed by Armitage and Doll (1954) and can be divided into three stages: initiation, promotion and progression. During initiation, cells acquire irreversible alterations in DNA (mutations) (Barrett 1993). A single exposure to a mutagenic/genotoxic chemical may be enough for initiation if the mutations occur in a proto-oncogene or a tumor-suppressor gene. When activated by mutation, a proto-oncogene causes

proliferation of the cell. In contrast, a tumor-suppressor gene must be deactivated to result in uncontrolled cell growth. Multiple proto-oncogenes and tumor-suppressor genes are usually altered (mutationally or epigenetically) in common human cancers such as breast and colorectal (Boyd and Barrett 1990). During the second stage, promotion, the initiated cell clonally expands into a benign lesion. Here, the promotor may be a non-genotoxic chemical that provides a growth advantage for the lesion by inducing inflammation, oxidative stress or immune deficiency. Furthermore, promotion might be reversible up to a certain point (De Flora and Ferguson 2005). During the final stage, progression, the benign lesion develops into a malignant neoplasm that is self-sufficient in growth and can invade other tissues (Barrett 1993).

Generally, cancer susceptibility is governed by both genetic defects and environmental factors. Among the genetic defects are inherited mutations in tumor-suppressor genes such as *breast cancer gene 1* (*BRCA1*) and *adenomatous polyposis coli* (*APC*), which increases the risk of breast and colorectal cancer, respectively (Fearnhead *et al.* 2001, Campeau *et al.* 2008). Only about 5-10% of cancer cases are caused by genetic defects. Thus, environmental (or unknown) factors account for up to 95% of cases. Environmental factors include lifestyle choices such as diet, alcohol consumption and cigarette smoking, occupation, radiation (solar or ionization), the use of hormones and reproductive history (Anand *et al.* 2008). Exposures to POPs can be incorporated into multiple of these environmental factors for cancer susceptibility, and the WHO and the International Agency for Research on Cancer (IARC) have estimated that somewhere between 7 and 19% of all cancer cases are caused by toxic environmental exposures (Straif 2008, WHO 2009).

Most POPs are considered not to be directly genotoxic and may cause carcinogenesis indirectly through mechanisms such as the generation of ROS, the modulation of DNA repair pathways or by epigenetic modification (EFSA Panel on Contaminats in the Food Chain (CONTAM) 2011, Goodson *et al.* 2015, IARC 2017). In addition, some chemicals (e.g. the lower chlorinated PCBs) might be bioactivated to more reactive intermediate compound that can directly cause DNA mutations (Ludewig and Robertson 2013). Already in 1979, Ames (1979) suggested to test both natural and man-made chemicals in the human diet and environment for their ability to damage DNA. This led to a scientific and regulatory emphasis on the mutagenic ability of single or well-

defined mixtures of chemicals. On the other hand, the carcinogenic potential of complex and environmentally relevant chemical mixtures was not pursued.

The advances made in understanding cancer biology has revealed weaknesses in risk assessment practices that only investigate the carcinogenic potential of individual chemicals. Here, chemical interaction that causes an originally non-genotoxic chemical (when tested on its own) to become genotoxic, or that affects the responsiveness of the cell or tissue in ways that promote carcinogenesis, would not be considered. Furthermore, non-monotonic dose-response relationships could cause unanticipated effects at low doses. Thus, the carcinogenic risk of mixtures would potentially be underestimated (Vandenberg *et al.* 2012, Goodson *et al.* 2015).

Colorectal cancer

The colon is the lower part of the intestinal tract that extends from the caecum to the rectum. The surface of the colon is relatively flat compared to the finger-like structures (villi) covering the surface of the small intestine. Surrounding the villi in the small intestine and covering the surface of the colon are invaginations of the epithelial lining, called crypts of Lieberkühn. At the bottom of each crypt resides the stem cells. These stem cells produce transit-amplifying cells that migrate up the crypt (and further up the villi in the small intestine) towards the intestinal lumen and differentiates into distinct cell types (Figure 6). All intestinal epithelial cells are renewed every 3-5 days in the small intestine and every 5-7 days in the colon of mice (Barker 2014).

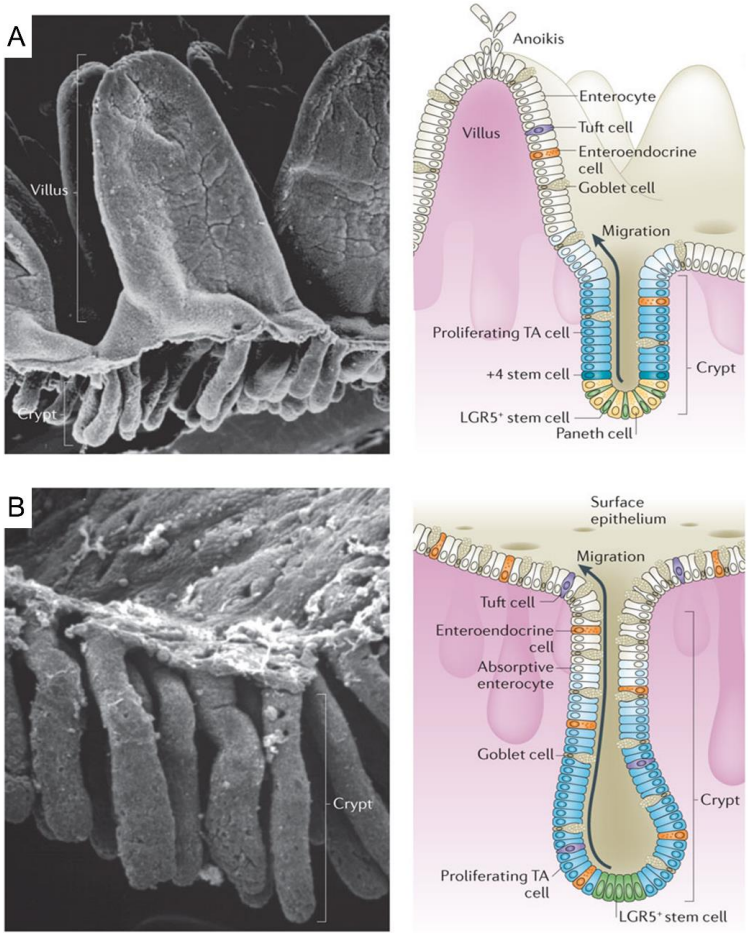

Figure 6. Structure of the small intestinal (A) and colonic (B) epithelium. Images on the left are taken by scanning electron microscopy (originally from Magney *et al.* (1986)). Stem cells reside on the bottom of each crypt and produce proliferating transit-amplifying (TA) cells, which migrate up the crypt and differentiate into distinct cell types (goblet cells, tuft cells, enteroendocrine cells and enterocytes) with various functions. Adapted from Barker (2014) with permission from Springer Nature (License Number: 4853631015848).

Colorectal cancer (CRC) is the third most common type of cancer in humans worldwide and accounted for 9.3 and 10.2% (males and females, respectively) of all cancer cases diagnosed in Norway in 2017 (Cancer Registry of Norway 2018). CRC can be divided into either sporadic or hereditary, where approximately 30% of cases are in patients with a family history of CRC.

Furthermore, approximately 5% of all CRC cases are caused by syndromes such as Lynch syndrome (also called hereditary nonpolyposis colorectal cancer) and familial adenomatous polyposis (FAP) syndrome, which have well-characterized mutations in germline DNA (Jasperson *et al.* 2010).

Colorectal carcinogenesis develops through the stages of initiation, promotion and progression in which intestinal epithelium is transformed from normal tissue, via benign lesions (adenomas), into malignant carcinomas (Figure 7) (Kinzler and Vogelstein 1996, Sancho *et al.* 2004). Initiation is achieved by mutation of the tumor-suppressor gene *APC*, which takes place in approximately 80% of sporadic CRC cases and is responsible for the FAP syndrome (Fearnhead *et al.* 2001, Fodde 2002). Promotion and progression is further accomplished by mutations in other tumor-suppressor genes, such as *TRP53* (*transformation related protein 53*) and *TGFBR2* (*transforming growth factor beta receptor 2*), and (proto-) oncogenic mutations of *RAS* (most commonly of *KRAS* (*Kirsten rat sarcoma viral oncogene homolog*)), *BRAF* (*v-Raf murine sarcoma viral oncogene homolog B*) and *PI3KCA* (*phosphatidylinositol-4,5-Bisphosphate 3-kinase catalytic subunit alpha*) (Markowitz and Bertagnolli 2009).

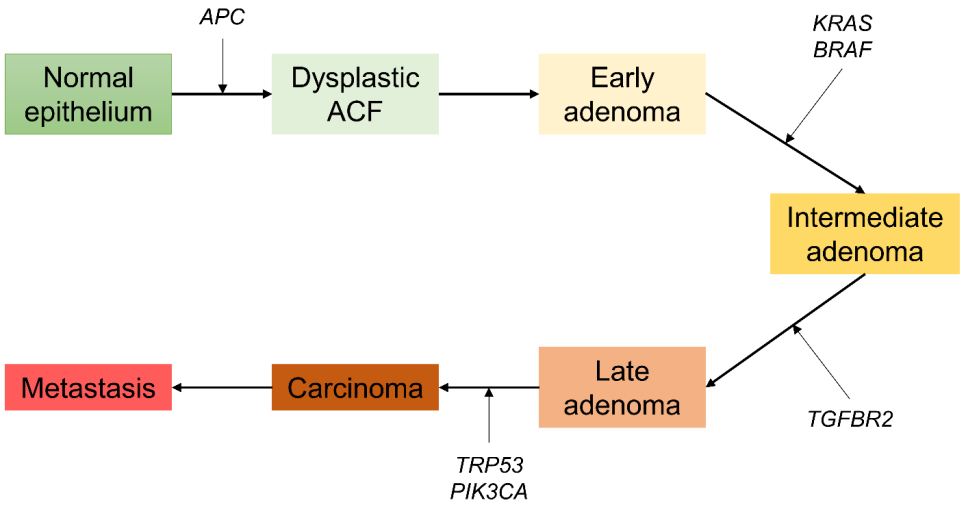

Figure 7. The progression of CRC from normal epithelium, via dysplastic aberrant crypt foci (ACF) and early, intermediate and late adenoma stages, to carcinoma and metastasis. Mutation in *APC* initiates carcinogenesis and promotion is a result from mutations in other genes such as *KRAS*, *BRAF*, *TGFBR2*, *TRP53* and *PIK3CA*.

In addition, colorectal carcinogenesis is influenced by other factors such as dietary components (including POPs) and bacteria (Zackular *et al.* 2013, Louis *et al.* 2014). The colon and small intestine contain a vast population of bacteria; the gut microbiome. These bacteria rely on host nutrient intake and energy and return effects that are beneficial for the host including improvement of immune function, protection against pathogens and maintenance of intestinal barrier integrity and physiology (Rooks and Garrett 2016). However, the gut microbiota has also been shown to negatively affect the host by modulating the immune system, activating pro-carcinogenic chemicals in the diet (such as POPs) or inducing oxidative stress and inflammation (Gagnière *et al.* 2016).

1.4 Methods for studying health effects of POPs

1.4.1 History of toxicity studies

The study of toxic effects of chemicals was initiated by Paracelsus (1493-1541) as early as in the start of the 1500s and expanded to show specific organ damage caused by toxins in the early 1800s (by Mathieu Orfila, 1787-1853). The use of animals to study the lethality of chemicals (finding a 50% lethal dose) started in 1920. Shortly after, a method for testing skin and eye irritation of chemicals and pharmaceuticals using rabbits was established. In the 1960s, long-term bioassays testing the chronic toxicity and carcinogenic potential of chemicals were developed for use on rats, mice and rabbits. At the same time, the devastating consequences of the chemical thalidomide (marketed as a cure for morning sickness in pregnant women) were revealed as the infant mortality rate and the number of babies born with birth defects skyrocketed. Thus, regulatory agencies introduced the need for risk assessment of all pharmaceuticals and chemicals already available or being introduced into the market. Formal risk assessment procedures were introduced by the Organization for Economic Co-operation and Development (OECD) at the start of the 1980s (Parasuraman 2011, OECD 2014). In 2001, a Mode of Action framework for chemical carcinogenesis was published by the International Programme on Chemical Safety (IPCS) with the purpose to increase the ability of translating tumor responses from bioassays into cancer risk in humans (Sonich-Mullin *et al.* 2001). This framework has later been extended (Boobis *et al.* 2006, Boobis *et al.* 2008, Meek *et al.* 2014) and the OECD guidelines for testing

the potential effects of chemicals on human health and the environment are continuously updated and expanded[4].

1.4.2 Animal models for studying health effects in humans

When chemical exposures (e.g. from accidents) are linked to documented health effects in humans, the cause-and-effect relationship and clarification of the mechanism and mode of action is derived from experimental studies using animal-based models. Furthermore, adverse health effects of pesticides, pharmaceuticals and industrial chemicals in humans is generally studied in animals. In order to predict the responses to exposure most accurately, animals with high similarity to humans are preferred (OECD 2014). However, these animals are only models and have strengths and weaknesses when translating the effects onto humans. For instances, thalidomide did not show any adverse effects in mice. However, later investigations revealed vital differences in the thalidomide metabolism and susceptibility between mice and humans. Thus, the toxicity studies conducted on mice failed to reveal the devastating consequences this chemical could have when administered to pregnant women (Rehman *et al.* 2011).

Several considerations must be made when selecting the correct animal to be used as a model for human effects. These include interspecies differences in absorption, distribution, metabolism, excretion, sensitivity, susceptibility, and critical periods of development (Patisaul *et al.* 2018). Furthermore, excellent animal care standards are needed to conduct high quality research on animal models (Workman *et al.* 2010). In order to accompany these standards, practical considerations including housing availability and cost arise. Thus, the use of larger and more demanding species (e.g. sheep, mini pig, dogs, ferrets, rabbits, hamsters, guinea pig and non-human primates) in research is rare. Other species such as zebrafish (*Danio rerio*), *Daphnia Magna*, *Caenorhabditis elegans* and *Drosophila melanogaster* have also been used to study the health effects of EDC exposures (mostly for chemical screening, genetic effects and in neuroscience). Although these organisms have led to significant discoveries, there are concerns about their translational ability of health effects onto humans (Patisaul *et al.* 2018).

Along with the scientific progress has come the development of *in vitro* and *ex vivo* methods and techniques for expanding the knowledge on mechanisms behind the observed health effects.

[4] www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

However, the complexity of interactions, feedback loops, endocrine systems and disease progression in the whole organism is not adequately replicated using such methods. Thus, animal models allow for the investigation of chemically induced effects at the phenotypic, physiological, behavioral and molecular levels that have a higher translational value compared to *in vitro* and *ex vivo* techniques (Patisaul *et al.* 2018).

Mice are extensively used in research on human health and disease. For instance, up to 95% of research on cancer development and response to treatment was conducted on mice in 2008 in the UK (Workman *et al.* 2010). The use of mice in human health research has several advantages including a 99% similarity in genes, a small size and short generation time compared to larger mammals, and a detailed understanding of the mouse biology and genetics acquired from their long-time use as research animals. Multiple strains of laboratory mice are available for purchase and the use of transgenic and knockout technology has led to the development of mouse models with characteristics that mimic a wide range of human diseases and health problems (e.g. diabetes and cancer). However, differences in metabolism, life expectancy, environment and genetic variability (and more) between mice and humans should not be forgotten (European Commision 2010).

Murine models for *Apc*-driven colorectal cancer

Genetically engineered mouse (GEM) models are mice with alterations in specific genes, such as oncogenes or tumor-suppressor genes, that mimic human disease. These mice have numerous benefits including testing of safe and effective chemicals for human application, studying mechanisms and pathways of disease and facilitating the development of targeted treatments. GEM models with activation or deactivation of oncogenes or tumor-suppressor genes, respectively, develop tumors in the setting of an intact immune system and have several similarities to their human disease counterparts. Thus, these models can be studied for their response in tumor formation after treatment with chemicals such as POPs (Hansen and Khanna 2004, Sharpless and DePinho 2006).

The *APC/Apc* tumor-suppressor gene has a homology of 86% at the nucleotide level, and 90% at the amino acid level after protein formation, between humans and mice (Su *et al.* 1992). This gene has a central role in colorectal carcinogenesis as it participates in multiple cellular functions such as proliferation, differentiation, apoptosis, adhesion and migration. For instance, *APC* is a

vital component in the β-catenin destruction complex that facilitated the phosphorylation of β-catenin and marks it for further degradation. β-catenin is an onco-protein and accumulation of β-catenin within the cell nucleus, as a result of *APC* dysfunction, leads to transcription (with the help of T cell and lymphoid enhancer factors) of *Wnt* (*wingless-type MMTV integration site family*) target genes (e.g. the oncogenes *c-myc* and *cyclin D1*) and increased cell proliferation (Reya and Clevers 2005). *APC* is further involved in the distribution of β-catenin and E-cadherin within the cell and, thus, has a central role in controlling cell adhesion. Furthermore, *APC* regulates cell polarity and migration through binding to actin filaments, it interacts directly with the microtubule cytoskeleton and it is involved in mitosis through spindle formation (Fearnhead *et al.* 2001, Fodde 2002).

Because of the high similarity in the *APC/Apc* gene between humans and mice, and the critical role of *APC* in CRC development, GEM models with mutations in this gene are of interest. Multiple mouse models with either germline or conditional mutations in *Apc* have been developed for studying *Apc* function in CRC and the susceptibility of colorectal lesions to environmental factors (Zeineldin and Neufeld 2013). The multiple intestinal neoplasia (Min) mouse model is a commonly used GEM model with a heterozygous nonsense mutation at codon 850 of the *Apc* gene, which leads to the truncation of the Apc protein (Su *et al.* 1992). This model has a great phenotypic and genetic similarity to humans with the FAP syndrome and was discovered by a coincidence after treating C57BL/6 mice with the mutagen ethylnitrosourea, which resulted in the development of multiple adenomas throughout the intestines (Moser *et al.* 1990). In both Min mice and patients with FAP, only one allele of *Apc* is originally mutated (Min/+), and the gene function is retained in the cell by the non-mutated allele (+). Mutation or silencing of the wild type (WT) allele, and consequently loss of heterozygosity (LOH), is required for cancer initiation. This mutation occurs spontaneously and becomes the 'second hit' in the two-hit hypothesis by Knudson (2001). Initiation results in the formation of multiple intestinal lesions, some of which will continue the progression to malignancy (Kinzler and Vogelstein 1996).

As *Apc* encodes a large multi-functional protein, other mouse models have been developed with a truncated Apc protein that is longer (e.g. Apc^{1638N}, Apc^{1309} and Apc^{1322T}) or shorter (e.g. $Apc^{\Delta 242}$, $Apc^{\Delta 474}$, $Apc^{\Delta 580}$, $Apc^{\Delta 14}$ and $Apc^{\Delta 716}$) than that of the Min mouse. Other murine models

have also been developed with complete deletion of the *Apc* gene ($Apc^{\Delta e1\text{-}15/}$), with alterations of the Apc protein (Apc^{mNLS} and $Apc^{\Delta SAMP}$) or with changed expression levels of *Apc* (Apc^{NeoF} and Apc^{NeoR}) (Zeineldin and Neufeld 2013). Collective traits for most of these models with germline mutations in *Apc* is a relatively short lifespan and lethality of the homozygous embryo. Thus, models with conditional *Apc* mutations have been developed with mutations manifesting only at specific developmental stages and/or in specific organs (Sansom 2009). These models use the CRE recombinase enzyme to catalyze site-specific recombination of DNA between two *loxP* sites, and subsequent excision of the in-between DNA. In conditional models, the *loxP* sites have been inserted into introns of the *Apc* gene, which causes a frameshift mutation and truncation of *Apc* in the presence of CRE (Zeineldin and Neufeld 2013).

Many of the models for *Apc*-driven colorectal carcinogenesis have different genetic backgrounds. Thus, different phenotypes of cancer development may occur. The traditional Min mouse model with the C57BL/6J strain background mostly develops lesions in the small intestine (Møllersen *et al.* 2004). Furthermore, the adenomas that develop only rarely progress into carcinomas (Rosenberg *et al.* 2009). In contrast, the A/J Min mouse model (with the A/J genetic background) develops numerous lesions in the colon and displays a continuous transition from early pre-neoplastic lesions (flat aberrant crypt foci (flat ACF)) to carcinomas (Figure 8). Thus, it has been argued that the A/J Min mouse model may reflect human colorectal carcinogenesis more closely than the traditional Min model as patients with CRC develop lesions mostly in the colon and rectum (Sødring *et al.* 2016).

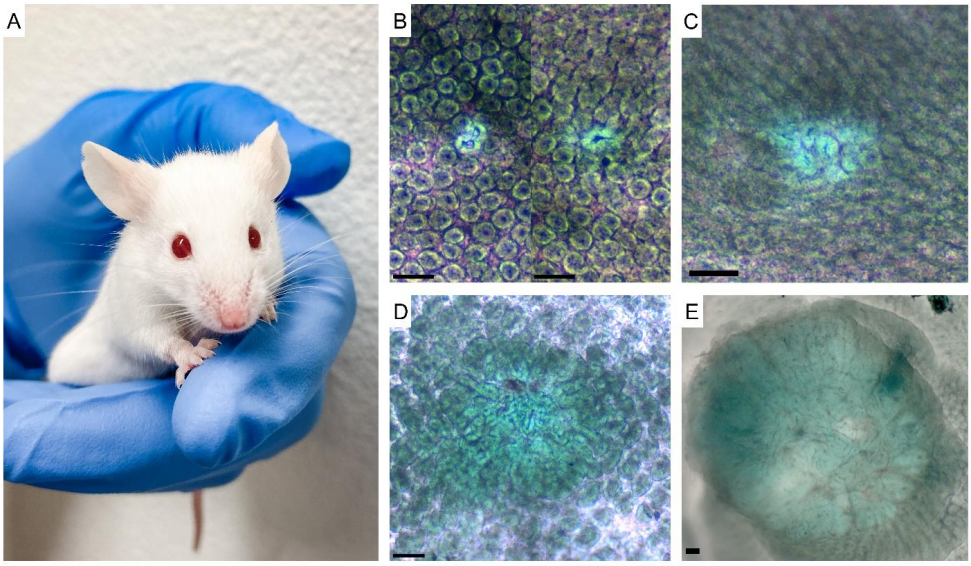

Figure 8. The A/J Min mouse (A: photo by A. J. Eickstedt) which develops intestinal lesions with a continuous transition from one or two dysplastic crypts (B), via aggregates of dysplastic crypts (C: medium and D: large flat ACF), to large adenomas and carcinomas (E). Pictures are taken at 4-fold magnification (scale bar = 100 μm) of colon stained with methylene blue from a 20-week-old A/J Min mouse.

Methods for inducing colorectal cancer

To accelerate carcinogenesis in GEM models and, thus, reduce the time for cancer to develop, treatment with known carcinogenic chemicals is common. Azoxymethane (AOM) is frequently used for inducing sporadic and hereditary CRC in rodent models such as the Min mouse. AOM is a potent carcinogen which initiates cancer by alkylating DNA and facilitating base mispairings. Initially, the precursor compound 1,2-dimethylhydrazine (DMH) was used. However, the downstream metabolite AOM has proved to be a more potent carcinogen and has a higher stability in solutions, thus, replacing DMH in cancer studies (Neufert *et al.* 2007).

After injection (usually administered intraperitoneally or subcutaneously), AOM needs a stepwise activation to exert its final potential as a carcinogen. Firstly, AOM is hydroxylated by CYP enzymes (mostly CYP2E1) in the liver. This creates the metabolite methylazoxymethanol

(MAM) (Sohn *et al.* 2001), which is excreted in the bile and further metabolized by microbiota (or microbial components) before it interacts with the DNA of the intestinal epithelial cells (Papanikolaou *et al.* 1998). AOM mainly acts as an initiating agent in CRC formation, however, some tumor-promoting activity has been recorded (Bissahoyo *et al.* 2005). In addition, laboratory mice with different genetic backgrounds have shown distinct differences in their susceptibility to AOM-induced CRC. For instance, the A/J mouse is highly sensitive, while the C57BL/6 mouse is generally more resistant, to tumor initiation by AOM. However, differences in AOM susceptibility has also been reported between different sublines of the C57BL/6 strain (Bissahoyo *et al.* 2005, Rosenberg *et al.* 2009).

1.4.3 Histological techniques in effect evaluation

Histological techniques are frequently utilized to visualize microscopic features of tissues or cells and to recognize specific changes associated with disease. These techniques all include fixation, sectioning, staining and examination under a microscope and most of them were first described in the 19th century (Slaoui and Fiette 2011). Fixation, commonly using neutral buffered formalin, is conducted to preserve the natural structure of the tissue and to avoid cell degradation. However, it has been found to destroy DNA, RNA and proteins. Various methods of processing may be conducted before embedding the tissue in paraffin wax and sectioning it into thin sections (usually 3-10 µm thick). Furthermore, staining is performed to highlight important features and to enhance the contrast of the tissue (Alturkistani *et al.* 2015). The Hematoxylin and Eosin (H&E) stains have been regularly used in the study of diseased tissue (histopathology). Hematoxylin highlights the nuclear details in the cells while the counterpart eosin distinguishes between the nuclei and cytoplasm. Before the development of H&E staining, the Carmine stain was much used to study tissue structures (Titford 2009).

Although the histological techniques are relatively old, they are still much used in evaluating how exposure or disease alters cells or tissues by comparing treated groups with non-treated controls. For instance, the carmine stain is regularly used when studying developmental alterations in the rodent mammary gland after exposure to EDCs (Fenton *et al.* 2002, Foster *et al.* 2008, Kodavanti *et al.* 2010, White *et al.* 2011, Tucker *et al.* 2015, Tucker *et al.* 2018). Furthermore, several advanced stains and techniques have significantly improved the field of histopathology, including immunohistochemistry, antibody binding and electron microscopy (Titford 2009).

1.4.4 Omics technologies in effect evaluation

The omics technologies study the complete system at different layers of biomolecular organization including DNA (genomics), gene expression (transcriptomics), protein expression (proteomics) and metabolite expression (metabolomics/metabonomics) (Figure 9). Genomics was the first of the omics to be developed and it is now understood as the determination of the entire DNA of an organism including a detailed genetic mapping (Gubb and Matthiesen 2010). With the complete sequencing of the human genome by the Human Genome Project (first published in 2001 (McPherson *et al.* 2001, Sachidanandam *et al.* 2001, Venter *et al.* 2001)) a new era of hypothesis-driven whole system approach to research was initiated using high-throughput tools for the analysis of information at many levels of biomolecular organization (Hood and Galas 2003). This project was initiated in 1990 as an international collaborative research program with the aim to map and completely sequence the human genome (Gubb and Matthiesen 2010). The whole system approach allows for the study of responses to genetic modification or environmental factors. Such environmental factors may come from xenobiotics, dietary components, gut microbiota or lifestyle choices, which all have effects on the organism and require intricate interactions between internal processes to maintain the physiological homeostasis (Claus and Swann 2013).

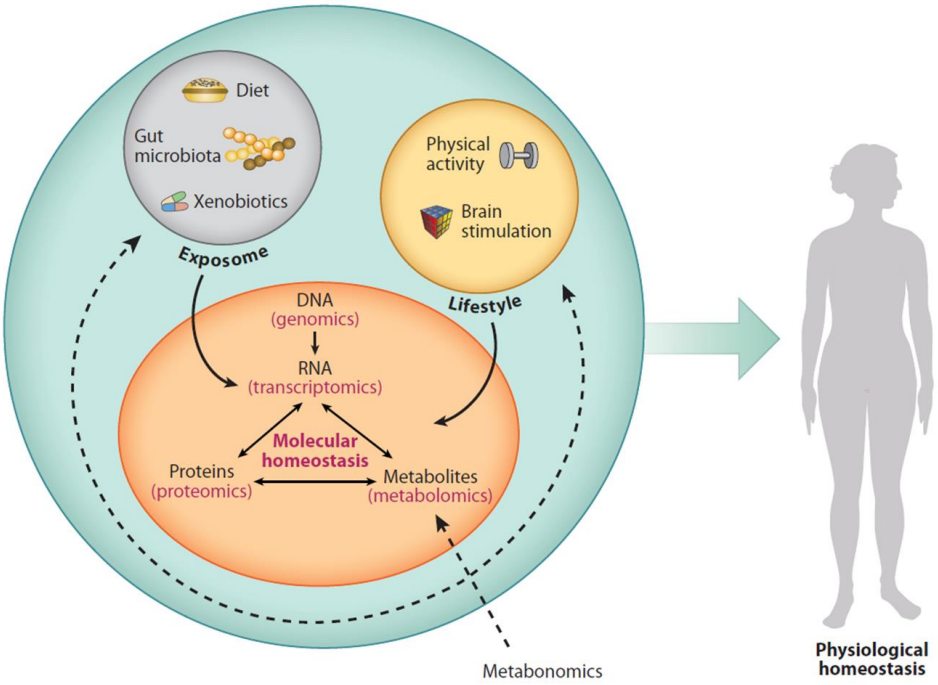

Figure 9. The biological system is under constant influence by external (exposome) or lifestyle factors and an intricate interaction between RNA, proteins and metabolites are required to maintain physiological homeostasis. The external factors include diet, activity of the gut microbiota and xenobiotics (including pharmaceuticals and pollutants), while lifestyle includes factors such as physical activity, brain stimulation and stress. The genomics, transcriptomics, proteomics and metabolomics technologies are useful when studying responses to genetic stimuli or environmental disturbance. Reprinted from Claus and Swann (2013) with permission from Annual Review, Inc (license ID: 1043279-1).

The Human Microbiome Project was launched as an extension of the Human Genome Project to understand the microbiome in relation to human genetic and physiological diversity (Turnbaugh *et al.* 2007). Within this project, the genomics technology was utilized to identifying and classifying microorganisms in tissues and organs (e.g. the gut microbiota). The composition and homeostasis of bacterial communities are important for human health and the imbalance of microbes (dysbiosis) in the gut has been linked to various conditions such as inflammatory bowel diseases, Parkinson's disease, obesity and an increased risk of cancer (Mulak and Bonaz 2015, Wlodarska *et al.* 2015, Gérard 2016, Sun and Kato 2016). The profiling of microbial communities

is generally conducted by sequencing the small ribosomal subunit of RNA organization (16S rRNA). This 16S region is found in all prokaryotes and consists of highly variable regions flanked by highly conserved regions that can be used for RNA amplification with polymerase chain reaction (PCR) (Wang and Qian 2009). The variable regions serve as identifiers of distinct microbes and the results are compared to known genetic sequences in a database (Kim and Chun 2014).

However, not all genes are transcribed simultaneously in an organism, tissue or cell. Thus, functional genomics aims to understand normal and abnormal gene regulation and expression patterns. Incorporated into this field is the transcriptomic technology, which studies the expression levels of mRNA in the organism, tissue or cell in question (Gubb and Matthiesen 2010). One key method in transcriptomics is the use of microarrays and the resulting gene expression profiles can be compared to find differences in transcription regulation under different conditions (e.g. chemical exposures) or developmental periods (Gibson 2003).

Moving one layer up in biomolecular organization, the proteomic technology studies the protein structure, function and composition in an organism or tissue. Proteomics is highly complex as the protein composition is constantly changing and varies with different developmental periods, tissues, cell types and environmental factors. Furthermore, the protein concentration and composition are affected by regulation or modification of transcription, translation, post-translation, transport or activity. Although complex, the development and improvement of mass spectrometry (MS), tandem MS and protein microarray techniques has ensured that the proteomic technology is highly successful (Gubb and Matthiesen 2010).

Small alterations in the proteome of an organism can often result in much more dramatic changes in the small molecule metabolites present (the metabolome). Furthermore, the product of the gene-protein-metabolite cascade can be more accurately assessed by measuring the metabolites and, thus, the biological state of the organism (Claus and Swann 2013). Alterations in metabolite composition and concentration reflect the diet, lifestyle and environmental factors affecting the organism and is useful for identifying biomarkers of disease (Gubb and Matthiesen 2010). Metabolomics aims to quantify and characterize all small molecule metabolites (< 1500 Da) in a specific tissue or organism (Wishart *et al.* 2007). When studying the metabolic response of an organism to stimuli or genetic manipulation, the term ‘metabonomics’ is much used. However,

both metabolomics and metabonomics have identical analytical and modelling procedures and the terms are often used interchangeably (Nicholson and Lindon 2008). Metabonomics/metabolomics is useful for determining the final metabolic alterations of a chemical exposure, for broadening the understanding of chemical toxicity and for connecting a chemical exposure to an observed health effect (Bouhifd *et al.* 2013).

The most commonly used analytical techniques for metabolomics are MS and nuclear magnetic resonance (NMR) spectroscopy. To simultaneously measure multiple metabolites with the MS technique, metabolite separation by liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) is required prior to spectrometry. When separation has been conducted, MS allows for the detection of metabolites with a high sensitivity and precision (Bouhifd *et al.* 2013). On the other hand, the NMR technique has the advantage of a simple and rapid sample preparation, reproducibility and a high robustness (Claus and Swann 2013).

For all omics technologies, statistical tools and advanced analyses are applied to interpret and visualize the complex data sets obtained. For metabolomics, different chemometric and pattern-recognition techniques are generally used. Depending on the hypotheses to be tested or questions to be answered, different statistical analyses are available which can identify biomarkers of disease or classify discrete differences between treatment groups (Nicholson and Lindon 2008, Claus and Swann 2013).

2 Aims of the study

The assessment of chemical mixtures for regulatory purposes is only partly covered by current guidelines as these mostly include the evaluation of individual chemicals or defined chemical mixtures. Thus, research on the health effects of large and human relevant mixtures of POPs is highly needed. Furthermore, the developmental processes of various organs are controlled by complex signaling pathways and are dependent on stimuli from endogenous molecules at specific time intervals to achieve their optimal function. Hence, disturbance by chemicals with endocrine disrupting properties, particularly during critical periods of development, may disrupt vital processes and possibly lead to adverse health effects.

The overall aim of the PhD study was to expand the current knowledge on effects of human relevant mixtures of POPs on several developmental and functional processes in mice. The study included the use of a mixture of POPs relevant for the Scandinavian population as its composition reflected the overall composition of POPs in human food and its concentration was based on estimated daily intake (EDI) levels for the human population. To further accompany the wish for human relevancy, the mixture was administered through the diet to mice and mouse dams during gestation and lactation anticipating maternal transfer of POPs.

The overall aim was achieved by using two mouse models, one outbred strain regularly used in toxicity assessment (the CD-1 mouse) and one GEM model for CRC development (the A/J Min mouse). The exposure regimes and mouse models allowed for the evaluation of effects of the POP mixture on several biological processes and led to the identification of three sub-aims:

1. Assess effects on female mammary gland development and ovarian folliculogenesis (Paper I).
2. Describe effects on liver morphology and function (Papers I, II and III).
3. Investigate effects on CRC development, intestinal microbiota and metabolome (Papers II and III).

3 Methodological considerations

This section encompasses a short description of the methods used in Papers I, II and III. For more details see materials and methods in the individual papers.

3.1 The POP mixture

The POP mixture (Table 2) was designed and created by Berntsen *et al.* (2017) and is described briefly in Papers I, II and III. The mixture contained 29 different chlorinated, brominated and fluorinated POPs and aimed to model an environmental mixture relevant for human consumption. Compounds reported in studies published between 2004 and 2012 to occur at the highest levels in food products ingested by the Scandinavian population were included. Estimated daily intake (EDI) levels of the 29 compounds were identified for humans and adapted to a 25 g mouse consuming 3 g of feed/day. However, due to differences in xenobiotic metabolism between humans and mice (Walton *et al.* 2001, Martignoni *et al.* 2006) and possible background exposure of POPs in regular mouse feed, the EDI levels were upwards adjusted 5 000-fold (creating the Low dose) to achieve exposure levels more relevant to humans. This resulted in POP concentrations similar to the maximum allowed levels by feed companies. For the High dose, a 20-fold higher level of all compounds was chosen, resulting in 100 000xEDI concentrations. Interestingly, the individual level of each POP was mostly lower than the no observed adverse effect level (NOAEL), where these levels were available. Furthermore, Berntsen *et al.* (2017) estimated the levels of POPs in the Low dose to be mostly higher than the tolerable daily intake (TDI) concentrations used by regulatory agencies at the time of mixture preparation (ATSDR 2005, EFSA 2005, Health-Canada 2007, EFSA 2008).

Table 2. Composition and concentrations of PCBs, OCPs, BFRs and PFASs in the POP mixture. Human average EDI levels (ng/day) for a 70 kg human were based on a literature review of Scandinavian EDI values and adapted to a 25 g mouse (pg/day). The mixture was incorporated into AIN-93G mouse feed, estimating a consumption of 3 g feed/day, at one Low (5 000xEDI) and one High (100 000xEDI) dose. Nominal and measured concentrations are reported as ng/g in feed. Adapted from Berntsen *et al.* (2017) with permission from Taylor & Francis.

Compounds	Human average EDI	Mouse adapted EDI	Nominal Low	Measured Low	Nominal High	Measured High
PCBs						
PCB-28	10	3.5	5.8	3.1	117	46
PCB-52	23	8.3	13.8	15.0	275	182
PCB-101	39	14.0	23.3	25.4	467	377
PCB-118	68	24.3	40.4	37.2	808	612
PCB-138	97	34.5	57.5	53.8	1150	957
PCB-153	97	34.5	57.5	61.4	1150	981
PCB-180	26	9.3	15.4	17.4	308	263
Σ7PCBs	360	128.4	213.7	213.3	4275	3418
OCPs						
HCB	84	30.0	50.0	37.4	1000	588
α-chlordane	63	22.5	37.5	45.0	750	723
Oxychlordane	21	7.5	12.5	9.8	250	297
Trans-nonachlor	21	7.5	12.5	14.9	250	264
α-HCH	36	13.0	21.7	21.2	433	421
β-HCH	29	10.5	17.5	22.3	350	398
γ-HCH (Lindane)	40	14.3	23.8	31.4	475	435
Σ7OCPs	294	105.3	175.5	182.0	3508	3126
p,p'-DDE	201	71.8	119.6	136.0	2392	2390
Dieldrin	126	45.0	75.0	70.4	1500	1470
BFRs						
BDE-47	68	24.3	40.4	39.7	808	642
BDE-99	13	4.8	7.9	8.6	158	126
BDE-100	11	3.8	6.3	5.6	125	91
BDE-153	2	0.8	1.3	1.5	25	22
BDE-154	4	1.5	2.5	2.8	50	38
ΣBDE-47-154	98	35.2	58.4	58.2	1166	919
BDE-209	105	37.5	62.5	64.8	1250	1141
HBCD	21	7.5	12.5	9.9	250	203
PFASs						
PFHxS	1.2	0.4	4.9	1.7	98	42
PFOS	18	6.5	10.8	3.2	217	74
PFOA	31	11.0	18.3	6.0	367	121
PFNA	9.5	3.5	5.8	2.1	117	42
PFDA	13	4.8	7.9	3.1	158	57
PFUnDA	6.7	2.4	4.0	1.6	80	28
ΣPFASs	79.4	28.6	51.7	17.7	1037	364

The POPs were dissolved in appropriate solvent (acetone for all BFRs except BDE-209, cyclohexane for all PCBs, OCPs (except HCB and β-HCH) and PFASs, or chloroform for HCB and β-HCH) and added to corn oil. BDE-209 did not dissolve in any solvents used and was added directly to the oil. Corn oil was chosen to replace the more generally used soybean oil, as corn oil would be more relevant for human consumption. The solvents were evaporated under N_2 flow at room temperature and the oil containing POPs was incorporated into AIN-93G mouse feed at the Low or High doses. A control dose was also made where the corn oil only contained the solvents remaining after evaporation. Furthermore, a reference diet was created using AIN-93G mouse feed and corn oil.

All chlorinated and brominated compounds in the mixture are included in the Stockholm Convention. PFOS and PFOA were included in the treaty in 2009 and 2019, respectively. Furthermore, PFHxS was acknowledged for its persistence, global distribution and potential risk to humans and the environment by the Persistent Organic Pollutants Review Committee in 2018 and the committee recommended to list this chemical in Annex A of the Convention during their fifteenth meeting in October 2019[5]. However, at the time of this PhD, this chemical has still not been fully incorporated into the treaty. The remaining PFASs (PFNA, PFDA and PFUnDA) are not included or proposed for inclusion in the treaty.

3.2 Ethical considerations

All animal experiments were approved by the Institutional Animal Care and Use Committee at NMBU and the Norwegian Food Safety Authority (Paper I: application ID FOTS 7722, Paper II: application ID FOTS 8127, Paper III: application ID FOTS 11549). They were conducted in accordance with The Norwegian Regulation on Animal Experimentation at the section for Experimental Biomedicine, NMBU, in Oslo, Norway. The section is licensed by the Norwegian Food Safety Authority[6]. All mice were kept under specific pathogen free (SPF) conditions and followed a health-monitoring program recommended by the Federation of European Laboratory Animal Science Association (FELASA)[7].

[5] chm.pops.int/TheConvention/POPsReviewCommittee/Meetings/POPRC15/Overview/tabid/8052/Default.aspx
[6] www.mattilsynet.no
[7] www.felasa.eu

3.3 Murine models

The outbred CD-1 mouse strain was chosen as a model for effects of the POP mixture on mammary gland development, ovarian folliculogenesis and liver (Paper I), as this model produces large litters and has an excellent maternal behavior (Patisaul *et al.* 2018). Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Wilmington, USA) and gave rise to the CD-1 dietary exposed females, as well as the males used for breeding with the dietary exposed females.

To study the effect of the mixture on CRC (Papers II and III), the A/J Min mouse model was chosen as it spontaneously develops intestinal lesions. Embryos homozygous for the Min trait (Min/Min) are not viable (Moser *et al.* 1995). Thus, the Min mouse model used in Papers II and III is heterozygous for the Min trait (Min/+). These mice were bred in-house at the section for Experimental Biomedicine by mating A/J Min/+ males with A/J WT (+/+) females. Allele-specific PCR on DNA extracted from ear punch samples (with the product visualized by gel electrophoresis) was necessary for determination of offspring with the Min/+ genotype (Dietrich *et al.* 1993). The A/J Min mouse model was created at the National Institute of Public Health by transferring the Min trait from the C57BL/6L Min mouse to the A/J mouse strain and backcrossing for > 12 generations to secure the status as inbred (Moen *et al.* 2016).

3.4 Exposure regimes

The animal experiment in Paper I was conducted by G. S. Boge, C. Trangerud and R. Halsne in 2015. The F1 females born from the timed-pregnant dams were administered the mixture of POPs through the diet from weaning (at 3 weeks of age). A total of 75 mice of the CD-1 strain were randomly assigned to either the Control, Low or High dose of POPs. Mice continued to receive POPs through the diet during one round of mating, pregnancy and lactation, which resulted in offspring exposed to the mixture of POPs *in utero* and through lactation. After weaning, the perinatally exposed offspring were given the reference diet to ensure an identical nutrient and oil composition as in the POPs diets. Furthermore, female offspring were sacrificed at 3 (weaning), 6 (pubertal) and 9 (adult) weeks of age, while male offspring were sacrifices at 9 and 30 weeks of age. Dietary exposed dams were sacrificed during pregnancy (on gestation day 17) or at 21 days post-partum.

The animal experiment in Paper II was conducted by K. E. Aa. Hansen in 2016. A total of 87 A/J Min mice were used, divided between two experimental sections. In the first section, 66 mice were randomly assigned to either the Control, Low or High dose of the mixture at weaning. After completion, mice were incorporated into the second experimental section and received one subcutaneous injection of AOM (8.5 mg/kg) during the second week after birth. After AOM treatment, a total of 21 A/J Min mice were randomly assigned to one of the three doses. Mice in both experimental sections were sacrificed at 13 weeks of age after 10 weeks of dietary exposure to the POP mixture. The relatively low number of mice in the second section of the experiment was caused by a high mortality of pups after AOM injection. Thus, breeding for this experimental section was terminated prior to completion due to animal welfare reasons.

The animal experiment in Paper III was conducted by the PhD Candidate in 2017-2018. Female A/J WT mice (54 in total) were randomly assigned to a Control or Low dose of the mixture at weaning and exposed throughout 3 consecutive rounds of mating, pregnancy and lactation with A/J Min/+ males. The resulting Min/+ offspring were given the reference diet from weaning until euthanasia at 20 weeks of age.

In all animal experiments, feed and water were available *ad libitum* and mice were housed in groups in either open type III cages or closed type III individually ventilated cages (IVC) with standard aspen bedding, red polycarbonate houses and cellulose nesting material.

3.5 Chemical analysis

The concentrations of PCBs, OCPs, BFRs and PFASs were analyzed in pooled liver samples from dietary exposed CD-1 dams and their perinatally exposed female offspring in Paper I, and in individual liver (only PFASs) or adipose (PCBs, OCPs and BFRs) samples from perinatally exposed A/J Min mice in Paper III. The analysis was conducted by laboratory technicians at the Laboratory of Environmental Toxicology at NMBU, which is accredited by the Norwegian Accreditation for testing POPs in biological material according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). This accreditation requires strict routines and quality control measures including repeatability, reproducibility, procedural blanks and local, interlaboratory and international reference material and tests. The analytical method for PCB, OCP and BFR quantification is based on Brevik (1978) and Polder *et al.* (2014). The analysis of PFASs,

described by Grønnestad *et al.* (2017), is not included in this accreditation but is validated according to the same quality control measures and procedures.

The resulting concentrations of the lipid-soluble POPs can either be presented as wet weight (i.e. compound weight/volume of sample) or as lipid weight (i.e. compound weight/lipid concentration). However, presentation as lipid weight is recommended as this describes a standardized body burden estimation and allows for the comparison of compound concentrations between different tissues and with levels reported in other studies (Bernert *et al.* 2007). PFASs have a lower lipophilicity than other POPs and, thus, lipid adjustment is not recommended for these compounds.

For POP concentrations detected in more than 60% of liver and adipose tissues in Paper III, levels below the limit of detection (LOD) were replaced with the LOD values for the respective compound and included in the statistical tests.

3.6 Histological techniques

Whole mounts are useful when evaluating alterations in the developing rodent mammary gland as they allow for the evaluation of epithelial extension throughout the fat pad and the presence or absence of TEBs (Rudel *et al.* 2011). This histological technique does not involve sectioning and the carmine stain is generally used. Furthermore, image analysis using imaging software (e.g. ImageJ) can be utilized to quantitatively assess morphological features in the glands. Paper I included the evaluation of mammary gland development in perinatally exposed female CD-1 offspring using whole mounts and the modified Sholl analysis for quantification of epithelial branching density (Stanko *et al.* 2015, Stanko and Fenton 2017). Imaging software was also utilized on fixed, sectioned and H&E stained mammary glands to measure the amount of glandular tissue occupying the fat pad.

Furthermore, every tenth H&E stained section from one randomly chosen ovary of perinatally exposed female CD-1 offspring in Paper I was used for counting the numbers of healthy follicles including primordial, primary, pre-antral and antral follicles. In addition, histopathological examination was conducted on H&E stained liver sections from dams, female and male CD-1 offspring in Paper I, and from A/J Min offspring in Paper III using standardized nomenclature guidelines (Thoolen *et al.* 2010).

Swiss rolls were made of the intestines from A/J Min mice in Papers II and III according to the procedure originally described by Moolenbeek and Ruitenberg (1981) and modified by Sødring *et al.* (2016). H&E stained sections at three depths (top, middle and bottom) were evaluated for pre-neoplastic lesions (with hyperplastic and/or dysplastic cells), adenomas (cancerous lesions restricted to the mucosa) and carcinomas (cancerous lesions with infiltrative growth through mucosa into the submucosa).

All histopathological examinations, as well as the analysis of mammary glandular tissue, were conducted blinded to dose by a board-certified pathologist (M. Aleksandersen). The overall developmental evaluation of the mammary glands, including the number of TEBs, was conducted by G. S. Boge, R. Halsne and G. C. Østby. The ovarian follicle numbers were counted by G. C. Østby. The PhD Candidate conducted the Sholl analysis on mammary gland whole mounts and prepared histological sections of liver and intestines (in Papers I and III).

3.7 CYP activity

The activities of CYP1A1, CYP1A, CYP2B10, CYP3A11, CYP2E1 and CYP2A5 were analyzed in liver samples from perinatally exposed female CD-1 offspring in Paper I. The analysis was conducted at the Department of Molecular Sciences, Swedish University of Agricultural Sciences, in Uppsala, Sweden by G. Zamaratskaia. The method of hepatic microsome preparation has been described by Rasmussen *et al.* (2011) and the enzymatic activity quantifications were conducted as explained by Zamaratskaia *et al.* (2010) (for CYP1A1, CYP1A and CYP2B10), Pilipenko *et al.* (2017) (for CYP3A11) and Zamaratskaia *et al.* (2009) (for CYP2E1 and CYP2A5). Protein concentrations for adjusting the final enzymatic activities was measured using a commercially available kit and bovine serum albumin as a standard.

3.8 Surface microscopy

The effect of dietary (Paper II) or maternal (Paper III) exposure to the mixture of POPs on CRC in the A/J Min mouse model was analyzed by surface microscopy on intestines stained with methylene blue. The method has previously been described by Sødring *et al.* (2016). The methylene blue stain enables the visualization of flat ACF in the colon. These lesions were first identified by Paulsen *et al.* (2000) in C57BL/6J Min mice and, in contrast to the original ACF (first described by Bird (1987)), flat ACF lies flat against the surrounding epithelium (Paulsen *et*

al. 2000, Paulsen *et al.* 2005, Paulsen *et al.* 2006). Furthermore, they appear bright blue-green when stained and have increased or deformed crypts with elongated luminal openings (see Figure 8 B, C and D). Importantly, unlike classical ACF that only show signs of mild dysplasia, the flat ACF have more severe dysplastic features already from the mono-crypt stage. This provides evidence of a direct relationship between the small flat ACFs and the larger adenomas and carcinomas (Paulsen *et al.* 2001, Sødring *et al.* 2016).

Transillumination was used to count and measure the diameter of the lesions in the colon and small intestine of A/J Min mice. Lesion load was identified as the total intestinal area covered by lesions. In the colon, lesions were identified as either flat ACF or tumors, where tumors have a crypt multiplicity of more than 30 aberrant crypts (covering more than approximately 0.4 mm^2). One crypt has previously been measured to cover an area of approximately 0.002 mm^2 and the smallest lesions include only 1-4 crypts. Lesion size classes have also been defined previously for the A/J Min mouse model using a logarithmic scale starting with the smallest flat ACFs at 0.002-0.008 mm^2 (Sødring *et al.* 2016). The small intestine does not contain flat ACF and, thus, all aberrant crypt aggregations were defined as lesions.

The identification of intestinal lesions was conducted by K. E. Aa. Hansen for Paper II and by the PhD Candidate for Paper III.

3.9 Omics techniques

3.9.1 Metabolomics

Metabolomic analysis was conducted by the PhD Candidate on cecal tissue and content from dietary (Paper II) and perinatally (Paper III) exposed A/J Min mice at Imperial College London, UK. ^{1}H NMR spectroscopy was done on extracts of tissue and content according to the protocol by Beckonert *et al.* (2007) and the description in Paper III. Some data treatment was necessary to prepare the ^{1}H NMR metabolic spectra for statistical analysis and metabolite identification. This included digitalization into spectral regions, removal of internal standard, water and outliers, alignment (using the recursive segment-wise peak alignment algorithm (Veselkov *et al.* 2009)) and normalization (using a probabilistic quotient-based approach) of spectra. Metabolites were identified using Chenomx NMR Suite[8] with the Profiler module and a database developed at

[8] www.chenomx.com/products/

Imperial College. Metabolic pathways were identified based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Kanehisa and Goto 2000)[9].

3.9.2 16S rRNA sequencing

The analysis of microbiota was conducted on feces from A/J Min mice perinatally exposed to the mixture of POPs in Paper III. Following DNA extraction by Ö. C. O. Umu at the Department of Food Safety and Infection Biology, NMBU in Oslo, sequencing of 16S rRNA was conducted by Eurofins Genomics using amplification of the V3-V4 region with the primers 347F (5'-TACGGGAGGCAGCAG-3') and 800R (5'-CCAGGGTATCTAATCC-3') creating two 300 base pair paired-end reads. These paired-end reads were processed, analyzed and joined using Qiime2 (Bolyen *et al.* 2019) with the VSEARCH plug-in (Rognes *et al.* 2016), and the remaining sequences were denoised using Deblur (Amir *et al.* 2017). The reads were then clustered into amplicon sequence variants (ASV) and ASVs that were present in 3 or more samples were aligned using MAFFT program (Katoh *et al.* 2002) to create a phylogenetic tree (using FastTree (Price *et al.* 2010)). The differences between the identified ASVs were visualized by generating an unweighted UniFrac distance metric (Lozupone and Knight 2005). The bacterial diversity (alpha diversity) and composition (beta diversity) was computed and taxonomic classification was made using q2-feature-classifier plug-in (Bokulich *et al.* 2018) and Greengenes 16S rRNA gene database (McDonald *et al.* 2012).

3.10 Statistical methods

In Papers I and II, the statistical analyses were conducted by E. Ropstad in JMP Pro®. Plots were created (by the PhD Candidate for Paper I and by C. Steppeler and K. E. Aa. Hansen for Paper II) in R studio (with the packages 'ggplot2' and 'ggpubr') and in Microsoft Excel®. A p-value $\leq$ 0.05 was considered statistically significant for all tests.

The weight measures (body, liver and combined ovaries and uterus) and intestinal length were normally distributed and with a satisfactory homogeneity in variances between doses. Thus, these data were analyzed using multivariate linear regression by standard least square personality, which generated least square mean values that were further analyzed using analysis of variance (ANOVA).

[9] www.genome.jp/kegg/pathway.html

In Paper I, the regression models were computed within each sampling time and included the explanatory variables:

- dose (Control, Low or High),
- weight at weaning (only for dam body weight),
- number of offspring per litter (only for offspring body weight) or
- body weight (only for liver and ovaries + uterus weight).

In Paper II, the overall model was as follows:

$$Y = \mu + G_i + E_j + e$$

Where:

Y = observation of body or relative liver weight, relative colon or small intestine length.

μ = overall mean body or relative liver weight, relative colon or small intestine length.

G_i = effect of sex, where i = male or female.

E_j = effect of dose, where j = Control, Low or High.

e = error.

Furthermore, in Paper I the numbers of TEBs in female CD-1 offspring at 3 and 6 weeks of age, as well as the mammary gland branching density (for 3, 6 and 9 weeks), were normally distributed and with homogenic variances. Thus, these data were also analyzed by multivariate linear regression within each sampling time and with dose and body weight as explanatory variables.

In Paper I, a contingency table analysis was conducted on the ratio between male and female CD-1 offspring, and the severity scores of hepatic histopathology within each sampling time and using the Steel-Dwass method to compare doses. An ANOVA followed by Dunnett's or Tukey HSD (honestly significant difference) test was used to investigate differences between doses within each sampling time in the number of live fetuses and offspring, and the number of ovarian follicles. The numbers of TEBs (9 weeks only) and mammary epithelial intersections, the epithelial area and length, the percentage of the fat pad covered by glandular tissue, and the hepatic CYP activities were not normally distributed (even after log-transformation) and were non-parametrically analyzed for differences between doses within each sampling time using the Steel or Steel-Dwass methods.

In Paper II, lesion numbers (from surface microscopy and histopathology), size and load were not normally distributed. Log-transformation granted some improvement, which allowed for the

evaluation of the effect of gender. Only small differences were noted between the genders and, thus, this variable was excluded, and the final analysis was conducted as a non-parametric test investigating the differences between doses using the Steel method.

In Paper III, all statistical analyses (except for 16S rRNA sequencing data) were conducted by the PhD Candidate using R (with the package 'ggplot2') or MATLAB (with scripts developed at Imperial College London). Due to randomness, only 3 A/J Min male offspring were obtained in the Control group and genders were pooled for further analysis. The threshold for significance was set to 0.05.

The concentrations of POPs were analyzed for differences between doses (Control and Low) using Welch two-sample t-test for compounds with a normal distribution (after log-transformation) and Mann-Whitney U test for those not achieving normality. The liver and cecal weights, and the colon or small intestine length were analyzed relative to the body weight. One outlier was removed from the cecal weight data (due to a large tumor in the cecum). After this, all weight and length data were normally distributed and with a satisfactory homogeneity in variances and analyzed for differences between doses using Welch test. Only 3 variables in the data from lesion identification by surface microscopy (flat ACF number and size, and lesion load in the small intestine) became normally distributed after log-transformation and were analyzed by Welch test. The remaining variables, including lesion numbers from the histopathological examination, were investigated for differences between doses by the non-parametric Mann-Whitney U test.

Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was performed on mean centered ^{1}H NMR spectral data obtained from perinatally exposed A/J Min mice with spectra as the feature matrix and dose (Control or Low) as the predictor matrix. The OPLS-DA is a supervised method that allows for sample classification (Claus and Swann 2013). On the other hand, principal components analysis (PCA) is an unsupervised method that requires no *a priori* knowledge of the sample class (or dose). However, this method can be used to investigate similarities between groups and to detect outliers in the data, as was done for the ^{1}H NMR spectral data in Paper III. In addition, OPLS regression models were built (separately for each dose) to investigate possible correlations between spectra and intestinal lesion or bacterial data. For OPLS-DA and OPLS regression, a model predictive performance (Q^2Y) was determined by 7-

fold internal cross-validation and the validity and robustness was assessed through permutation testing (1000 permutations) (Westerhuis *et al.* 2008). Furthermore, coefficient plots were created from the models and used to identify distinct metabolites that differed between the doses.

Statistical analyses of 16S rRNA sequencing data were conducted by Ö. C. O. Umu. The distance metric created between the identified ASVs from the 16S rRNA sequencing of fecal bacteria was visualized by principle coordinate analysis (PCoA) and analyzed by permutational multivariate analysis of variance (PERMANOVA) to investigate the difference in bacterial composition between the Control and Low doses (Paper III). Differences in microbial diversities were explored using the Alpha group significance method in Qiime2. Furthermore, differences in bacterial relative abundances were checked using analysis of composition of microbiomes (ANCOM) at different taxonomic levels (Mandal *et al.* 2015).

4 Results: summary of papers

Paper I

Perinatal exposure to a human relevant mixture of persistent organic pollutants: Effects on mammary gland development, ovarian folliculogenesis and liver in CD-1 mice.

S. M. Johanson, E. Ropstad, G. C. Østby, M. Aleksandersen, G. Zamaratskaia, G. S. Boge, C. Trangerud, R. Halsne, J. L. Lyche, H. F. Berntsen, K. E. Zimmer, S. Verhaegen.

Manuscript.

The study aimed to investigate the effects of perinatal exposure to the mixture of POPs on mammary gland development, ovarian follicle maturation and liver morphology and function in CD-1 mice. Dams were exposed from weaning, throughout mating, gestation and lactation to the mixture at Control, Low or High doses. Chemical analysis revealed efficient uptake of POPs from the diet and distribution to the hepatic tissue in dams. Furthermore, all compounds in the mixture (except PCB-52 and HBCD) were transferred to the offspring during gestation and lactation. After end of lactational exposure, the compounds decreased in concentration with increasing age of offspring.

Overall, perinatal exposure to the mixture of POPs modulated mammary gland development in female CD-1 offspring. Weaning offspring (3 weeks of age) showed restricted gland development (only High dose) compared to control. Furthermore, pubertal (6 weeks) and adult (9 weeks) offspring exhibited a premature arrest of gland growth by reducing branching density, epithelial area and length. Ovarian folliculogenesis was also affected by perinatal exposure to the mixture of POPs, as evident by a reduced number of primary and antral follicles in weaning offspring indicating a delayed rate of follicle maturation and possibly an increased rate of follicle atresia.

Dietary exposure to the High dose increased the liver weight of dams and pubertal female offspring. Furthermore, a persistently increased severity of hepatocellular hypertrophy (centrilobular) was seen in both dams and offspring, in addition to a dose-dependent induction of hepatic CYPs (in female offspring). Thus, the mixture of POPs may potentially induce oxidative stress and hepatotoxicity in CD-1 mice.

Paper II

A mixture of persistent organic pollutants (POPs) and azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model.

K. E. Aa. Hansen, S. M. Johanson, C. Steppeler, M. Sødring, G. C. Østby, H. F. Berntsen, K. E. Zimmer, M. Aleksandersen, J. E. Paulsen, E. Ropstad.

Chemosphere (2018).

The study aimed to investigate whether dietary exposure to the mixture of POPs could affect colorectal carcinogenesis alone or in combination with a single treatment with the carcinogenic chemical AOM. The mixture of POPs was given to A/J Min mice at Control, Low or High doses through the diet for 10 weeks and mice in the second section of the study were administered one subcutaneous injection of AOM during the second week after birth.

The High dose had an adverse effect on the mice independent of AOM treatment by decreasing the body weight and increasing the liver weight. Dietary POP exposure caused moderate and dose-dependent increases in colorectal carcinogenesis as the High dose initiated the formation of new lesions while the Low dose promoted the growth of already existing lesions. AOM alone showed a promoting effect on colorectal carcinogenesis by stimulating lesion growth without initiating lesion formation. On the other hand, when combining AOM treatment with dietary POP exposure, a synergistic increase in lesion formation was seen in the colon, and to a smaller extent in the small intestine. Furthermore, combined treatment also promoted carcinogenesis and resulted in a severe lesion burden for mice given AOM and the High dose. Histopathological evaluations further demonstrated this synergistic effect by showing increased numbers of preneoplastic lesions, adenomas and carcinomas in the colon. These results emphasize the importance of anticipating interacting effects when assessing the carcinogenic potential of chemical mixtures.

Paper III:

Maternal exposure to a human relevant mixture of persistent organic pollutants reduces colorectal carcinogenesis in A/J Min/+ mice.

S. M. Johanson, J. R. Swann, Ö. C. O. Umu, M. Aleksandersen, M. H. B. Müller, H. F. Berntsen, K. E. Zimmer, G. C. Østby, J. E. Paulsen, E. Ropstad.

Chemosphere (2020).

The study aimed to investigate the effect of perinatal exposure to the mixture of POPs on CRC, intestinal metabolite composition and microbiota in the A/J Min mouse model. Mice were exposed to the mixture *in utero* and through lactation at the Control or Low doses and sacrificed at 20 weeks of age. Chemical analysis revealed maternal transfer, accumulation and persistence of POPs in adipose and liver tissues of mice. Furthermore, the lipid-adjusted concentrations were 2-35x higher than the average plasma concentrations reported in the Scandinavian population, thus, proving that the Low dose could be considered relevant to humans.

Weight measures or hepatic morphology were not affected by perinatal exposure. Although the exposure increased the size of flat ACF, it reduced the formation and growth of colonic tumors, indicating a restriction of colorectal carcinogenesis by perinatal exposure to the mixture of POPs. In addition, histopathological evaluation showed more carcinomas and adenomas in the small intestine and colon, respectively, of control mice. Furthermore, alterations caused by perinatal exposure were seen in the composition of metabolites associated with the metabolism of amino acids, lipids, glycerophospholipids and energy in intestinal (cecal) tissue. Indications were also present of an exposure-modulated renal xenobiotic excretion and hepatic metabolism (by alcohol dehydrogenase). The microbial composition in feces was altered by perinatal POP exposure, which manifested as a change in the core microbiome and the relative abundance of one *Unclassified Sutterella* species (phylum *Proteobacteria*). However, no changes were seen in the bacterial diversity. These results highlighted that early-life exposure to the mixture of POPs could reduce colorectal carcinogenesis in A/J Min mice by modulating the intestinal microbial and biochemical environment.

5 Discussion

The result presented herein aid in the clarification of chemical mixture effects on several developmental and functional processes in mice. The female reproductive organs, liver and intestinal biochemical environment proved sensitive to developmental exposure. In addition, dietary exposure to chemical mixtures may increase the risk of CRC, particularly when combined with other cancer-promoting agents. This discussion will address the relevance of the obtained results in a broader context followed by an elaboration of some important methodological issues.

5.1 Confirmation of exposure

The levels of POPs measured in mice confirmed that the chemicals were taken up from the diet, distributed to the hepatic tissue and transferred from mothers to offspring. Many of the chemicals were also persistent in hepatic and adipose tissues and, thus, the effects seen in mammary glands, ovaries, livers and gut could have been caused by a combination of early-life exposure and a chronic disturbance from the most persistent POPs.

The lipid-adjusted POP concentrations measured in livers of dams dietary exposed to the Low dose (Paper I) were much higher for most compounds than the lipid-adjusted blood levels reported for humans by Berntsen *et al.* (2017) (up to 757 and 2450x for PCB-138 and BDR-209, respectively), with the exception of PFHxS, PFOS and γ-HCH (which were only 3, 3 and 7x higher in mice, respectively). When comparing the concentrations of chlorinated and brominated POPs in CD-1 dams to blood concentrations in women from the Arctic regions (highly exposed human populations (AMAP 2015a)), *p,p'*-DDE, *trans*-nonachlor and BDE-153 had only 2-8x higher levels in mice compared to humans (AMAP 2015b, Long *et al.* 2015, Bravo *et al.* 2019). Three studies reporting the POP concentrations in blood from pregnant Norwegian or Danish women showed 18-25x lower levels of *p,p'*-DDE in humans compared to pregnant mice (Veyhe *et al.* 2015, Caspersen *et al.* 2016, Bjerregaard-Olesen *et al.* 2017). Furthermore, *p,p'*-DDE was only 3-fold lower in blood of Norwegian women with Type 2 diabetes compared to dietary exposed CD-1 mice (Rylander *et al.* 2015). For the fluorinated POPs, the wet weight concentrations of PFHxS and PFOS were only 5-6x lower in blood of Swedish mothers (3 weeks post-partum) compared to pregnant mice (Gyllenhammar *et al.* 2018). Thus, dietary exposure to the Low dose of POPs resulted in at least partly human relevant concentrations in CD-1 mice (Paper I).

The PCBs and OCPs seemed to be more efficiently transferred to offspring during late gestation and lactation, compared to BFRs (particularly BDE-209) and PFASs, as these compounds had 2-3x higher concentrations in weaning CD-1 offspring compared to post-pregnant dams (Paper I). This may have been caused by a high rate of lactational transfer and/or a low metabolic capacity in fetuses and neonates (Milsap and Jusko 1994).

The levels of γ-HCH, PFHxS and PFOS in weaning mice were similar (with an 1-2-fold difference) to the human blood levels reported by Berntsen *et al.* (2017). However, all other compounds were up to 789x higher in mice than in humans. Furthermore, weaning mice also had higher levels than those reported in blood from 3-year-old Norwegian children (Caspersen *et al.* 2016). Interestingly, the levels of *p,p'*-DDE decreased fairly rapidly in CD-1 offspring, which resulted in only 3x higher levels in pubertal offspring compared to 6-11-year-old Danish and 3-year-old Norwegian children. In addition, adult mice (9 weeks of age) had a 4-5-fold lower *p,p'*-DDE concentration than Danish and Norwegian children (Mørck *et al.* 2014, Caspersen *et al.* 2016, Knudsen *et al.* 2017). *p,p'*-DDE was chosen to represent DDT and its metabolites in the mixture of POPs as this compound had the greatest contribution to the sum of DDTs in human food, blood and breast milk (Berntsen *et al.* 2017). The concentration of *p,p'*-DDE did not differ between 20-week-old A/J Min mice perinatally exposed to the Control or Low doses (Paper III), which together with the results from Paper I demonstrated a possible metabolism and excretion of this compound in mice. To the authors knowledge, metabolism of *p,p'*-DDE in mice has not been reported previously.

The chemical analysis in Paper I was conducted on only one pooled sample of liver tissue from each sampling time and dose. Thus, individual variation could not be identified, and statistical tests were not conducted. Furthermore, all compounds were measured in liver tissue even though the more lipophilic chemicals accumulate to a higher extent in lipid-rich tissues.

In Paper III, analysis was conducted on individual samples from perinatally exposed A/J Min mice, and the lipid-soluble PCBs, OCPs and BFRs were measured in adipose tissue while the PFASs were measured in liver tissue. Here, all compounds (except for PCB-52, α-chlordane and HBCD) were detected and 20 (of the 29) POPs in the mixture had significantly higher concentrations (up to 136x) in 20-week-old A/J Min mice perinatally exposed to the Low dose compared to controls. This confirmed the persistent nature and accumulative potential of most

chemicals and showed indications of possible metabolism and/or excretion of the lower chlorinated PCBs (PCB-28, -52 and -101), *p,p'*-DDE, α-chlordane, α- and γ-HCH, BDE-47 and HBCD (de Wit 2002, Grimm *et al.* 2015, Wacławek *et al.* 2019).

Of the PCBs, OCPs and BFRs with significantly different concentrations between the Low and Control doses (Paper III) the lipid-adjusted levels in mice were 2-35x higher than the human blood levels reported in Berntsen *et al.* (2017). Furthermore, PCB-118 and HCB had only 3 and 5x higher levels in A/J Min mice compared to 3-year-old Norwegian children, respectively (Caspersen *et al.* 2016), the concentration of β-HCH was 8x higher in mice compared to 6-11-year-old Danish children (Mørck *et al.* 2014) and BDE-99 had 4x higher levels in mice compared to Danish children (Knudsen *et al.* 2017). Of the PFASs, the concentrations of PFOS and PFOA were only 2-fold higher in A/J Min mice than in human blood and PFHxS had 3x higher levels in humans than in mice (Berntsen *et al.* 2017). Furthermore, the level of PFOA in mice was equivalent to the level reported in blood from 2-4 months old Swedish infants, and the concentrations of PFHxS was 2-fold higher in infants compared to A/J Min mice (Gyllenhammar *et al.* 2018). As the fluorinated POPs have a relatively low lipophilicity (O'Hagan 2008), lipid adjustment was not conducted. However, this prevented accurate comparison with other tissues and studies.

Notably, all detected POPs had higher concentrations in mice dietary or perinatally exposed to the High dose compared to mice given the Low dose (Paper I). Furthermore, many chemicals were detected in the Control dosed CD-1 and A/J Min mice. However, concentrations in the controls were 44-573x and 11-452x lower than in Low for CD-1 dams and weaning offspring, respectively. Berntsen *et al.* (2017) only detected HCB in the Control and reference feed. Thus, there seemed to be a low level of background contamination possibly originating from the laboratory animal environment.

Overall, the detection of POPs in mice confirmed that the mixture was absorbed, distributed, transferred and accumulated. The Low dose resulted in human relevant concentrations in murine tissues for a few compounds and, thus, it can be argued that this dose may be relevant to humans in both its concentration and composition. The High dose resulted in considerably higher tissue concentrations and can only be considered relevant to humans in its composition. Lastly, the

present study showed evidence of murine metabolism and/or excretion of *p,p'*-DDE and contamination with POPs in the environment of laboratory animals.

5.2 Mammary gland development and ovarian folliculogenesis

The sensitivity of the developing mammary gland, particularly during late-gestational exposure to chemicals, has been established previously (Fenton *et al.* 2002, White *et al.* 2007, White *et al.* 2009). Qualitative assessment of mammary gland development in female CD-1 offspring did not reveal any effects of perinatal exposure to the mixture of POPs (Paper I). However, 3-week-old mice exposed to the High dose had fewer TEBs compared to controls. The number of TEBs vary with age and are only apparent when the glandular tissue expands throughout the fat pad (Watson and Khaled 2008). Thus, alterations in the number of TEBs is coincident with an alteration in gland differentiation. In addition, as TEBs have a great number of proliferating cells, any modification in the presence of TEBs changes the susceptibility to carcinogenic stimuli and may increase the risk of cancer development (Fenton 2006, Osborne *et al.* 2015). In mice, TEBs are developed at 2 weeks of age (Watson and Khaled 2008) and weaning mice should, therefore, have TEBs present in their developing mammary glands. Hence, a reduced number of TEBs in exposed compared to control mice indicate a decreased rate of proliferation and a restriction of gland development.

The number of TEBs present is also coincident with the number of epithelial ducts in the mammary gland. A gland with a high branching density will, thus, have a higher amount of TEBs (Rudel *et al.* 2011). Although the number of TEBs was not affected by perinatal exposure at 6 and 9 weeks, the High dose increased the branching density of mammary gland epithelium significantly in pubertal and non-significantly in adult mice. This suggested a retention of TEBs causing more epithelial branching and a continuation of the developmental restriction observed in weaning mice. Furthermore, perinatal exposure to the Low dose decreased the epithelial expansion throughout the fat pad resulting in a shorter and smaller gland in pubertal and adult mice. This indicated that the restricted gland development observed in mice perinatally exposed to the High dose manifested as a premature arrest of gland development in mice perinatally exposed to the Low dose. During lactation, this restricted gland development could reduce milk production and, thus, delay the development and maturation of the next generation (White *et al.* 2011).

Multiple studies have previously reported restriction of mammary gland growth in female CD-1 mice due to developmental PFOA exposure (White *et al.* 2007, White *et al.* 2009, Macon *et al.* 2011, White *et al.* 2011, Tucker *et al.* 2015). Furthermore, perinatal exposure to DE-71 (a mixture of pentaBDEs) or *in utero* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), atrazine metabolites or bisphenol A (BPA) also delayed gland development in both mice and rats (Markey *et al.* 2001, Fenton *et al.* 2002, Enoch *et al.* 2007, Kodavanti *et al.* 2010, Filgo *et al.* 2016). On the other hand, no alterations have been reported after perinatal exposure to dieldrin in female BALB/c mice (Foster *et al.* 2008), gestational exposure to BPA analogues accelerated mammary gland development and caused inflammation, non-neoplastic mammary lesions and spontaneous adenocarcinoma formation in CD-1 mice (Tucker *et al.* 2018), and HCB exposure increased the formation of hyperplastic mammary ducts and modulated gland development in an AHR-dependent manner in C57BL/6 mice (Miret *et al.* 2017).

The effects of chemical mixtures on mammary gland development have not been thoroughly investigated. Only a few studies have been conducted and report no modulations of gland development in Sprague-Dawley rats following neonate or *in utero* exposure to mixtures of chemicals based on POP exposures documented in the Canadian population (Desaulniers *et al.* 2001, Foster *et al.* 2004) or a mixture of PCBs, PCDDs and PCDFs (Desaulniers *et al.* 2004). In addition, differences between murine strains in gland sensitivity and response have been demonstrated (Yang *et al.* 2009, Tucker *et al.* 2015), which emphasizes that caution should be taken when assessing the effects of environmental pollutants using a single strain.

Moreover, histological assessment revealed a reduction in the number of primary ovarian follicles caused by perinatal exposure to the Low dose in weaning CD-1 mice (Paper I). In addition, the numbers of antral follicles were reduced by perinatal exposure to both doses in weaning mice and non-significantly in adult mice. This suggested a delayed rate of follicle maturation, from primordial to primary and from pre-antral to antral follicles, caused by perinatal exposure to the mixture of POPs.

Previous evidence has suggested that POPs can disrupt ovarian folliculogenesis by directly affecting the ovary or by modulating endocrine pathways such as signaling along the hypothalamic-pituitary-ovarian axis (Lilienthal *et al.* 2006, Talsness *et al.* 2008). After primordial follicle assembly, the initial recruitment is triggered by stimulation from intraovarian

and/or unknown factors which promotes primordial follicles growth into primary follicles (McGee and Hsueh 2000). Once triggered, the follicle continues to develop until it either undergoes atresia or reaches the preovulatory stage. The later stages of initial follicle recruitment are completely dependent on gonadotropins (e.g. luteinizing hormone and follicle-stimulating hormone) secreted by the anterior pituitary in response to stimulation by gonadotropin releasing hormone (GnRH) from the hypothalamus. In addition, the gonadotropins stimulate the production of ovarian steroid hormones, which regulates GnRH secretion and creates a negative feedback loop tightly regulating follicle maturation (Yamaji *et al.* 1972, McGee and Hsueh 2000). Due to the endocrine disrupting properties of many POPs (Gore *et al.* 2015), it is likely that perinatal exposure to the mixture disturbed the hormonal regulation of ovarian folliculogenesis (Uzumcu and Zachow 2007). However, hormone levels were not measured in the present study and, thus, the effects of the mixture directly on the endogenous signals could not be determined.

The reduced number of antral follicles could also suggest an increased rate of follicle atresia. However, the number of atretic follicles were not quantified in the present study. An increased number of atretic follicles has previously been reported following perinatal exposure to a mixture of PCB-101 and -118 in CD-1 mice (Pocar *et al.* 2011) and *in utero* exposure to PCB-126 in Sprague-Dawley rats (Muto *et al.* 2003). Increased follicle atresia can result in an acceleration of ovarian reserve depletion, induce premature ovarian failure and early onset of menopause, shortening the reproductive lifespan (Vabre *et al.* 2017, Ge *et al.* 2019). In humans, multiple OCPs, PCBs and BFRs have been associated with premature menopause (Cooper *et al.* 2002, Akkina *et al.* 2004, Knox *et al.* 2011, Taylor *et al.* 2014, Grindler *et al.* 2015). In addition, a disruption of the ovarian reserve formation, resulting in a deficient ovarian follicle pool and a reduced number of primordial follicles, can also reduce the reproductive lifespan.

Exposure to γ-HCH during the period of ovarian reserve formation reduced the number of oocytes in CD-1 fetuses (La Sala *et al.* 2009) and gestational exposure to BDE-99 or perinatal exposure to HBCD reduced the number of primordial follicles in adult rats (Lilienthal *et al.* 2006, Ema *et al.* 2008). Several studies exposing rats to single chemical (including TCDD, PCB-126, BDE-99, BDE-47, PFOA and PFOS) during early development have reported delays in ovarian follicle maturation (Heimler *et al.* 1998, Muto *et al.* 2003, Lilienthal *et al.* 2006, Shirota *et al.* 2006, Talsness *et al.* 2008, Du *et al.* 2019). Furthermore, *in utero* exposure to a mixture of PCBs

(Aroclor 1016) decreased the number of preantral and antral follicles in weaning Long-Evan rats (Baldridge *et al.* 2003). On the other hand, gestational exposure to another mixture of PCBs (Aroclor 1254) increased the number of antral follicles in adult Long-Evans rats, suggesting a prolongation of this phase by postponement of atresia (Lilienthal *et al.* 2006). Increased numbers of pre-antral and antral follicles were also seen in pregnant Sprague-Dawley rats exposed to a mixture of BFRs (including PBDEs and HCBD) (Lefevre *et al.* 2016).

Altogether, the present study demonstrated possible endocrine disruption by the mixture of POPs manifesting as a restriction of mammary gland development and an alteration of ovarian folliculogenesis and possibly also follicle degeneration. Premature arrest of mammary gland growth can lead to belated or limited milk production and reduced development in offspring. Modulations of gland development may also increase the risk of cancer development. Furthermore, alterations of ovarian follicle maturation and degeneration can reduce the reproductive lifespan by inducing premature ovarian failure and menopause.

5.3 Liver morphology and function

Enhanced liver weights were seen after dietary exposure to the High dose of the POP mixture both in CD-1 (Paper I) and A/J Min mice (Paper II). However, perinatal exposure only increased the liver weights significantly in 6-week-old female offspring (Paper I, High dose only). Dietary or perinatal exposure to the Low dose did not affect the liver weights in mice (Papers I, II and III).

Histopathological assessment was conducted on liver tissue from CD-1 and A/J Min mice in Papers I and III. No pre-neoplastic or neoplastic lesions were found in either mouse strains. However, both dietary and perinatal exposure to the mixture caused a significant increase in the severity of centrilobular hepatocellular hypertrophy in CD-1 mice (Paper I). Interestingly, this pathological condition was not seen in A/J Min mice following perinatal exposure (Paper III). However, as the samples in Paper III were taken at 20 weeks of age (17 weeks after weaning and end of lactational exposure) hypertrophy of the liver cells may have been present at an earlier age but reversed prior to sampling. Normally, hepatocellular effects such as hypertrophy are resolved when the chemical causing the effect has been metabolized and excreted from the system (Maronpot *et al.* 2010). However, chemical analysis in A/J Min mice showed persistence of most POPs. A/J Min mice were only exposed to the Control and Low doses of the mixture, which may

have had too low chemical concentrations to cause hypertrophy in this strain. Interestingly, hepatocellular hypertrophy was still present in 30-week-old male CD-1 mice (27 weeks after weaning) perinatally exposed to the Low dose. Thus, the present study demonstrated strain differences in the sensitivity to perinatal POP exposure.

The ability of A/J Min mice to metabolize xenobiotics has not been characterized and, to the authors knowledge, no studies have yet been published on the difference in hepatic metabolism of POPs between WT and Min mice. However, the contribution of the *Wnt*/β-catenin pathway to the hepatic expression of CYP isoforms including CYP1A2, CYP2A5 and CYP2E1 has been shown (Gerbal-Chaloin *et al.* 2014) and transgenic mice knockout for the *Ctnnb1* gene (encoding β-catenin) exhibit loss of or lower basal expression of CYPs (Sekine *et al.* 2006, Tan *et al.* 2006, Braeuning *et al.* 2009). Thus, it is reasonable to assume that the capacity of A/J Min mice to metabolize POPs varied from that of the CD-1 mice, which may have contributed to the strain-dependent differences observed.

Furthermore, dose-dependent increases in the activities of hepatic CYP enzymes were seen after perinatal exposure to the mixture of POPs in female CD-1 mice (Paper I). Of the enzymes analyzed, the enhanced activities of CYP1A1, CYP3A11 and CYP2B10 indirectly indicate activation of nuclear receptors (particularly AHR, PXR and CAR) in liver tissue. The dose-dependent inductions were less prominent with time corresponding to the temporal decline in measured hepatic POP concentrations. Interestingly, the activities of CYP2E1 and CYP2A5 did not follow the same pattern of increase and were (at some points) lower in perinatally exposed mice compared to controls. This may have been caused by a reduced capacity for xenobiotic metabolism after exposure during early development, which could predispose animals to hepatotoxicity and other adverse effects at times where CYP2E1 and CYP2A5 are required for detoxification. In addition, CYP2E1 is known to generate toxic metabolites and by-products during its metabolism of xenobiotics with low molecular weights (Ioannides and Lewis 2004, Bieche *et al.* 2007, Porubsky *et al.* 2008). Thus, a reduced enzymatic capacity and metabolism of POPs by CYP2E1 may have contributed to the observed hepatocellular hypertrophy. The physiological consequences of reduced xenobiotic capacities by CYP2E1 and CYP2A5 should be further investigated.

Previously, dose-dependent induction of CYP enzymes, proteins and transcripts have been shown in rats after perinatal exposure to a mixture of PBDEs (DE-71) (Zhou *et al.* 2002, Szabo *et al.* 2008, Dunnick *et al.* 2018), a mixture of PCBs (Bonfanti *et al.* 2014), a mixture of POPs and methylmercury simulating blood levels in the Canadian Arctic population (Chu *et al.* 2008) or a mixture of POPs based on blood levels in women from the Great Lakes/St. Lawrence region (Chu *et al.* 2005). Some of these mixtures also induced hepatocellular hypertrophy (Chu *et al.* 2005, Chu *et al.* 2008, Dunnick *et al.* 2018).

Enhanced xenobiotic metabolism by CYP enzymes can generate metabolites or by-products (e.g. ROS) that are toxic to the hepatic tissue and, thus, can induce hepatotoxicity (Bondy and Naderi 1994, Ioannides and Lewis 2004, Zangar *et al.* 2004). Furthermore, hepatotoxicity may occur when liver enlargement due to hepatocellular hypertrophy and/or hyperplasia exceeds the adaptive response of enzymes (Maronpot *et al.* 2010). The hepatic effects observed after perinatal exposure to the mixture of POPs (in CD-1 mice) have the characteristics of an adaptive liver response as defined by Maronpot *et al.* (2010) including dose-dependency and reversibility. However, a complete reversibility of effects was not seen in the present study and, thus, hepatotoxicity may occur at both the High and Low doses.

Hepatic carcinogenesis is another possible endpoint of perinatal exposure to the mixture as prolonged hypertrophy can favor the development of cancer. However, hypertrophy should not be considered the only predictor of cancer and, as proven by Allen *et al.* (2004), only 45% of rats exhibiting chemically induced liver hypertrophy developed cancer during a period of two years. Thus, increased enzymatic activities and hepatocellular hyperplasia are used as additional predictors of liver carcinogenesis (Allen *et al.* 2004, Maronpot *et al.* 2010). The activity of hepatic CYP enzymes was only measured in female offspring up to 9 weeks of age and no measurements of enzymatic induction was conducted on 30-week-old male offspring. Furthermore, hepatocellular hyperplasia was not seen in any of the CD-1 or A/J Min mice. Thus, it is debatable if perinatal exposure to the mixture of POPs can induce hepatic carcinogenesis.

Notably, the centrilobular location of the hypertrophy seen in CD-1 mice can be explained by the zonation of CYP enzymes in liver tissue, which is highest in hepatocytes located close to the perivenous region (central vein) (Oinonen and Lindros 1998, Maronpot *et al.* 2010). Thus, this further indicated that the hypertrophy was induced by CYP metabolism of POPs.

Overall, CD-1 mice showed a higher hepatic sensitivity to the mixture of POPs compared to A/J Min mice. Furthermore, dietary exposure caused hepatocellular hypertrophy and liver enlargement while perinatal exposure caused induction of CYP enzymes and persistent hepatocellular hypertrophy leading to a concern for hepatotoxicity and possible carcinogenesis.

5.4 Colorectal cancer, intestinal microbiota and metabolome

Dietary exposure to the High dose of the POP mixture increased the number of flat ACFs in the colon of A/J Min mice (Paper II) indicating an initiation for colorectal carcinogenesis. On the other hand, the Low dose increased the number of colonic tumors suggesting a growth stimulation of already existing lesions (promotion).

The mechanisms by which POPs can initiate and/or promote CRC are many including the induction of DNA mutations (directly or indirectly), the modulation of DNA repair pathways and the induction of oxidative stress (Ludewig and Robertson 2013, IARC 2017). Song *et al.* (2014) showed CRC promotion by *p,p'*-DDT through the production of ROS, by inhibiting detoxifying enzymes and by reducing antioxidants levels in intestinal cells. The resulting oxidative stress prevented degradation of β-catenin and, thus, increased cell proliferation and CRC growth. Hong *et al.* (2017) also concluded that POPs can cause CRC through increasing cell proliferation and reducing antioxidant levels contributing to accumulation of ROS, oxidative stress and inflammation. However, no investigation was conducted to directly study the mechanisms of CRC initiation and promotion following dietary exposure to the mixture of POPs in the present study. Thus, further studies into the mechanistic basis of colorectal carcinogenesis by chemical mixtures are needed.

Mixtures of PCBs have been shown to induce liver and lung carcinogenesis in mice and rats alone (Ito *et al.* 1973, Mayes *et al.* 1998) or after lesion initiation by NDMA (N-nitrosodimethylamine) or 1-NP (1-Nitropropane) (Anderson *et al.* 1986, Anderson *et al.* 1994, Nakanishi *et al.* 2001). However, the effects of chemical mixtures on CRC in rodents have been very scarcely investigated and only one study demonstrated an increased formation of pre-neoplastic intestinal lesions (ACFs) in Sprague-Dawley rats (Hong *et al.* 2017). Furthermore, *p,p'*-DDT exposure promoted colorectal carcinogenesis in BALB/c mice injected with a suspension of the human colorectal adenocarcinoma cell line DLD1 (Song *et al.* 2014). On the other hand, no CRC initiation or promotion was shown after gestational exposure to PFOS or PFOA in C57BL/6J

Min mice (Ngo *et al.* 2014). In addition, PFOS reduced intestinal tumor burden and growth in C57BL/6J Min mice and in a xenograft mouse model implanted with human colorectal lesions (Wimsatt *et al.* 2016, Wimsatt *et al.* 2018). In humans, some OCPs and PCBs have been positively correlated with CRC risk (Howsam *et al.* 2004, Lee *et al.* 2018, Abolhassani *et al.* 2019). However, no association between POP concentration and CRC was found in Egyptian patients (Abdallah *et al.* 2017).

Due to the promoting effect of the Low dose seen in Paper II, as well as the more humanly relevant POP concentrations in this dose compared to the High dose, the effect of the Low dose on CRC was investigated after perinatal exposure using A/J Min mice (Paper III). Perinatal exposure reduced the number, size and total intestinal area covered by colonic tumors indicating a decreased colorectal carcinogenesis. In addition, the exposure reduced the formation of adenomas in the colon and carcinomas in the small intestine. To the authors knowledge, this is the first study to show reduced CRC due to perinatal exposure to a mixture of POPs.

The multistep process of colorectal carcinogenesis is affected by various external factors including microbiota and intestinal metabolites. Furthermore, acquiring a diverse and balanced microbial community early in life is crucial for the development of a healthy immune system (Gagnière *et al.* 2016). With the advances made in omics technology, growing evidence suggest that dysbiosis of intestinal microbiota can promote chronic inflammatory conditions and produce possible carcinogenic metabolites (Sun and Kato 2016). Furthermore, the physiological changes that occur during CRC (such as changes in the intestinal barrier function and rupture of the intestinal epithelium) alter the local microbial and biochemical environment (Hirayama *et al.* 2009, Tjalsma *et al.* 2012). The presence of gut microbiota has been shown to play a vital role in the formation of CRC in Min mice (Li *et al.* 2012). In addition, exposures to various chemicals have been linked to microbial dysbiosis and the perturbation of multiple metabolic pathways increasing the risk of cancer development (Zackular *et al.* 2013, Jin *et al.* 2017, Zhang *et al.* 2017). However, there is limited understanding about the long-term effects of alterations in microbiota and metabolite composition in relation to CRC development following early-life exposure to large chemical mixtures. Thus, omics technologies were utilized in Paper III to eluate the role of microbiota and intestinal metabolites in CRC development after perinatal exposure to the mixture of POPs.

Although the bacterial diversity was not affected by the POP mixture, perinatal exposure altered the composition of microbiota and metabolites in the intestine of 20-week-old A/J Min mice. The altered bacterial composition could be displayed as a significant difference in the core microbiome between control and perinatally exposed mice, as well as a difference in the relative abundance of one *Unclassified Sutterella* species. The phylum *Proteobacteria* (including the *Unclassified Sutterella* species) had a higher abundance in human adenomas and rats with DMH-induced CRC (Shen *et al.* 2010, Zhu *et al.* 2014). This species was found at a higher abundance in controls compared to perinatally exposed mice and may have been partly responsible for the higher tumor burden seen in control mice.

Furthermore, the difference in core microbiome between controls and perinatally exposed mice was visualized as a difference in ASVs between doses. The exposed mice had one ASV affiliated to the *Bilophila* genus (phylum *Proteobacteria*), one ASV affiliated to each of the *Peptococcaceae* and *Mogibacteriaceae* families, and one ASV affiliated to the *Adlercreutzia* genus that were not present in the controls. Unfortunately, identification at lower classification levels was not possible due to poor resolution of the 16S rRNA data. The *Bilophila* genus contains the bacterial species *Bilophila wadsworthia*, which may contribute to cancer initiation and promotion as it produces hydrogen sulfide that damages the intestinal barrier and causes oxidative stress (Attene-Ramos *et al.* 2006, Louis *et al.* 2014, Dahmus *et al.* 2018). However, as mice perinatally exposed to the mixture of POPs showed reduced CRC promotion, it may be possible this ASV belonged to another bacterial species within the *Bilophila* genus.

The *Peptococcaceae* and *Mogibacteriaceae* families are classified in the order *Clostridiales*, which has been shown to have a negative association with intestinal lesion burden in humans (Baxter *et al.* 2014). This association may partly be caused by the production of butyrate, the preferred source of energy by intestinal epithelial cells, by bacteria in this order. Butyrate-producing species are considered indicators of a diverse and healthy microbiota and humans with advanced CRC have less butyrate-producing bacteria compared to healthy controls (Louis *et al.* 2014, O'Keefe 2016). Thus, the ASVs affiliated to the *Clostridiales* order may have contributed to the lower colonic tumor burden observed in perinatally exposed mice.

However, butyrate may also stimulate aberrant cell proliferation under certain conditions (Roediger 1982, O'Keefe 2016). One ASV affiliated to the *Adlercreutzia* genus was seen in

perinatally exposed mice, but not in controls. This genus belongs to the subclass *Coriobacteridae*, which was shown to have a higher relative abundance in human CRC tissue (Marchesi *et al.* 2011). Furthermore, mice with a higher relative abundance of this ASV had higher levels of butyrate and trimethylamine (TMA), which both have been linked to increased CRC development (Xu *et al.* 2015, O'Keefe 2016, Thomas *et al.* 2019). Thus, the ASV affiliated to the *Adlercreutzia* genus may have contributed to the only increase in colorectal carcinogenesis observed in perinatally exposed mice, namely a larger average size of flat ACFs.

To the authors knowledge, no studies have yet been published showing long-term effects of perinatal exposure to large mixtures of POPs on gut microbiota. However, perinatal exposure to a mixture of 12 PCBs induced dysbiosis and altered the bacterial composition in 4-week-old Sv129:C57BL/6 mice (Rude *et al.* 2019). In addition, alterations of bacterial composition and function in 1-month-old human infants was associated with several POPs in breast milk (e.g. PCB-167, BDE-28, PFOS and PFOA) (Iszatt *et al.* 2019). Various mixtures of PCBs modulated microbial diversity and/or community composition in mice following direct exposure (Choi *et al.* 2013, Cheng *et al.* 2018). Furthermore, multiple studies using single POPs (including PCB-126, PCB-153, BDE-47, BDE-99, *p,p'*-DDE, β-HCH or PFOS) have found changes in gut microbiota that can be linked to inflammation and altered intestinal metabolism in mice (Li *et al.* 2017, Liu *et al.* 2017, Xu *et al.* 2017, Chi *et al.* 2018a, Chi *et al.* 2018b, Lai *et al.* 2018, Li *et al.* 2018, Petriello *et al.* 2018).

Notably, as gut microbiota is a complex and diverse community, it is unlikely that single bacteria are responsible for driving carcinogenesis or that a defined bacterial composition would be found in all CRC patients. On the other hand, as suggested by Zackular *et al.* (2013), an effect on the overall community involving alterations in bacterial populations and their general metabolic function may play a critical role in CRC development. Thus, modulation of whole microbial communities can dramatically alter lesion burden.

The change in intestinal metabolite composition in A/J Min mice perinatally exposed to the Low dose of POPs was visualized as an increase or decrease in specific metabolites relative to the controls. By pathway identification, the altered metabolites were connected to modulations in the metabolism of amino acids, lipids, glycerophospholipids and energy in the intestinal tissue. Alterations in the same metabolic pathways have previously been found in humans with CRC

(Zhang *et al.* 2017). Furthermore, the metabolic profiles revealed changes in essential cell membrane components and a possible perturbation of hepatic metabolism by alcohol dehydrogenase and renal xenobiotic excretion in perinatally exposed mice. Previous studies in humans and rodents have associated exposure to POPs with effects on cell membranes (O'Kane *et al.* 2013, Carrizo *et al.* 2017, Pikkarainen *et al.* 2019). The modulation of xenobiotic metabolism and excretion observed in the present study may indicate that early-life POP exposure can predispose animals to deleterious effects from chemicals that require alcohol dehydrogenase and optimal renal function.

However, it should be noted that the predictive performance of the OPLS-DA model revealing a difference in the metabolic profiles of cecal tissue between control and perinatally exposed mice should be considered relatively low ($Q^2Y = 0.1127$). This can be explained by only a small difference between doses. Consequently, if a larger difference had been found, a higher predictive performance of the model would have been obtained. However, permutation testing revealed a significant p-value ($p = 0.028$) and the difference in metabolite composition was accepted as valid.

Dietary exposure to a mixture of PCBs (Aroclor 1254) affected cell membranes, altered fatty acid metabolism and caused mitochondrial dysfunction in rats (Lu *et al.* 2010, O'Kane *et al.* 2013). In addition, combined PCB and phthalate (DEHP) exposure perturbed lipid, tryptophan and phenylalanine metabolism in Kunming mice (Zhang *et al.* 2012). Sprague-Dawley rats exhibited long-lasting and dose-dependent alterations in the metabolism of glycerophospholipids, amino acids and carnitines following perinatal PCB-180 exposure (Pikkarainen *et al.* 2019). Furthermore, neonate or adult exposure to BDE-209 modulated the metabolism of amino acids, carbohydrates, lipids and energy in mice and rats (Yang *et al.* 2014, Eguchi *et al.* 2016, Jung *et al.* 2016). In addition, the metabolism of energy, lipids and amino acids was altered in adult or 10-days-old C57BL/6 mice following exposure to HBCD (Wang *et al.* 2016, Szabo *et al.* 2017). Thus, previous studies have shown effects on multiple metabolic pathways due to POP exposure.

Interestingly, metabolomics was conducted on identical samples from dietary exposed A/J Min mice in Paper II and did not show any difference between the metabolic profiles of the Control and Low doses. Thus, this proved the higher sensitivity of early developmental periods, compared to exposure from weaning until adulthood, to disturbance by chemical mixtures.

After cancer induction by AOM in the second experimental section of Paper II, the initiation and promotion of colonic lesions (both flat ACF and tumors) was dramatically increased by dietary exposure to the mixture of POPs. Histopathological examinations also revealed more pre-neoplastic lesions, adenomas and carcinomas in the colon and cancer initiation in the small intestine. This initiation and promotion were particularly severe for mice receiving the High dose, however, a numerical increase (non-significant) from controls was seen in the Low dosed mice.

Compared to the relatively moderate effect on colorectal carcinogenesis by POPs alone, the dramatic increase in initiation and promotion indicated a synergistic interaction between AOM and the mixture. Both AOM and POPs are metabolized by CYPs (mainly in the liver), which could have resulted in an enhancement of the carcinogenic ability of both components due to alterations in the balance between detoxification and activation (Hernández *et al.* 2017). After metabolization of AOM in the liver and excretion in the bile, MAM interacts with microbiota in the intestine (Papanikolaou *et al.* 1998, Sohn *et al.* 2001). As the mixture of POPs was administered through the diet, it could also have interacted with microbiota and the intestinal biochemical environment (Jin *et al.* 2017), which could further disrupt homeostasis and cause favorable conditions for cancer initiation and promotion. However, neither CYP induction or microbial alterations were investigated in Paper II and further studies should explore the possible interactions between individual POPs and between POPs and carcinogenic compounds in the human diet and environment.

The results presented in Paper II emphasize that adverse health effects can arise from synergistic interaction between carcinogenic chemicals and POPs present in Scandinavian food products. Interestingly, AOM treatment alone showed only a promoting effect on CRC and did not initiate the formation of new lesions. A strain-dependent tumor promoting ability of AOM has been shown previously (Bissahoyo *et al.* 2005). Furthermore, the mice receiving AOM treatment and the High dose of the POP mixture had a severe lesion burden at 13 weeks of age, which may have reduced the absorption of nutrients over the intestinal wall and caused the reduction in body weight observed.

Altogether, dietary exposure to the human relevant mixture of POPs showed a modest ability to initiate and promote CRC in A/J Min mice. On the other hand, perinatal exposure to the mixture reduced colonic lesion burden possibly due to modulations of the microbial community and

intestinal metabolite composition. When combining a single treatment of AOM with the mixture of POPs, a synergistic increase in colorectal carcinogenesis was seen highlighting the possibility of adverse effects from chemical mixtures. Furthermore, early developmental periods were sensitive to disturbances from POPs, however, this may not always lead to adverse health effects later in life.

5.5 Methodological issues

5.5.1 The POP mixture

The selection of POPs for inclusion in the mixture has been described and discussed in detail by Berntsen *et al.* (2017). Importantly, POPs were chosen based on which chemicals had the highest concentrations in human food products ingested by the general Scandinavian population. Some chlorinated chemicals (including polychlorinated dibenzo-*p*-dioxins (PCDD), PCDFs and the most dioxin-like PCBs (except PCB-118)) were deliberately excluded from the mixture because of their relatively high toxicity at low doses, which would have reduced the utilization of the mixture especially at the High dose. Other compounds (such as toxaphenes and endosulfan) were excluded due to limited studies on the levels of these compounds in the Scandinavian population.

The mixture aimed to include compound ratios that reflected those found in Scandinavian food products and was not based on the mode of action (MOA) of the individual chemicals. If the study population had been another than the Scandinavian population, a different composition of POPs would have been achieved as populations differentiate in their exposure to POPs by having different diets and regulations of past and present agricultural use. Furthermore, the EDI levels varied between the reported studies due to differences in sampling time (year, season etc.), diet, inclusion of food items, collection methods, questionnaires and analytical procedures. Thus, the inclusion of more studies in the concentration estimation (if available) would probably have resulted in different levels of the POPs. In addition, the strictly regulated and banned POPs have shown declining concentrations in human blood and breast milk during the last decades (Polder *et al.* 2008, Darnerud *et al.* 2015, Gebbink *et al.* 2015, Bjerregaard-Olesen *et al.* 2016, Bjerregaard-Olesen *et al.* 2017). Consequently, several EDI levels may be lower at present time compared to the time of mixture creation.

Differences in xenobiotic metabolism between humans and mice may confound extrapolation of mixture effects onto human health (Walton *et al.* 2001, Martignoni *et al.* 2006, European Commision 2010). Furthermore, Berntsen *et al.* (2017) discovered that feed companies allow for the use of relatively high POP concentrations in rodent feed. Thus, the EDI levels were increased with 5 000x for the Low dose. This dose was again increased 20-fold to create a High dose that would distinguish from the Low dose in murine tissues. Financial limitations restricted the creation of more doses. Importantly, other studies using similar POP mixtures also encounter the same problems when choosing compounds and concentrations. Thus, no estimated human relevant mixture will ever completely portray the complex exposure scenarios and continuously varying mixture concentrations that humans encounter. Berntsen *et al.* (2017) chose the compounds and concentrations to the best of their abilities. In addition, the mixture of POPs included a relatively large number of compounds, making it more humanly relevant compared to mixtures with less POPs. Hence, without forgetting the weakness of mixture estimation, it should be considered relevant to humans.

Furthermore, Berntsen *et al.* (2017) analyzed the chemical concentrations in the Control, Low and High doses to verify the presence or absence, as well as the levels of POPs, in feed. The measured concentrations varied from the desired nominal concentrations due to loss of chemicals during preparation or analytical difficulty in chemical analysis. In addition, HCB was detected in the Control dose and the reference feed possibly due to contamination of corn oil (Berntsen *et al.* 2017). Lastly, the POP mixture was incorporated into mouse feed formulated by the American Institute of Nutrition (AIN) in 1993 (AIN-93G) for gestating and growing rodents (Reeves *et al.* 1993). Thus, the nutrient and energy composition of feed was ideal for studies involving perinatal or developmental exposures.

5.5.2 Murine models

As previously mentioned, mice are commonly used when studying a large variety of chemically induced health effects and multiple outbred and inbred strains with different characteristics are available. However, when the goal is to extrapolate the effects onto humans, it is important to consider the dissimilarities in metabolism, sensitivity, mechanism of action, latency period and environment between mice and humans (European Commision 2010, Patisaul *et al.* 2018).

Furthermore, the value of results should be weighed against the use of laboratory animals and excellent care standards are needed to optimize results (Workman *et al.* 2010).

The CD-1 mouse

As an outbred strain, the CD-1 mouse yield results with a relatively large variability. However, this strain produces large litters and has an excellent maternal behavior enabling the survival of most, if not all, pups (Patisaul *et al.* 2018). Furthermore, it has been much used to study the effects of EDCs on mammary gland development (White *et al.* 2007, White *et al.* 2009, Macon *et al.* 2011, White *et al.* 2011, Tucker *et al.* 2015, Tucker *et al.* 2018) and the similarities and dissimilarities between murine and human mammary gland development have been established previously (IBCERCC 2013). The CD-1 mouse strain has also been used to study the effects on reproductive and developmental health of early-life exposure to single chemicals or mixtures of POPs (Lau *et al.* 2003, Thibodeaux *et al.* 2003, Lau *et al.* 2006, Wolf *et al.* 2007, Pocar *et al.* 2011). Moreover, this strain was relatively sensitive to developmental PFOA exposure compared to the inbred C57BL/6 strain (Tucker *et al.* 2015) and, thus, it was chosen to explore the effects of the mixture of POPs on mammary gland development, ovarian folliculogenesis and liver in Paper I.

The A/J Min mouse

GEM models with germline mutations in the *Apc* gene are of interest when studying the risk and susceptibility of CRC to environmental factors (Zeineldin and Neufeld 2013) and several models have been developed (including the Min mouse model (Moser *et al.* 1990, Sødring *et al.* 2016)). The germline *Apc* mutation in the Min mouse is particularly relevant for humans with FAP. However, this GEM model can also be used to study sporadic CRC as mutations in *Apc* is apparent in approximately 80% of cases (Fearnhead *et al.* 2001, Fodde 2002). The A/J strain with a germline mutation in *Apc* develops intestinal lesions with a phenotype similar to humans. In addition, the A/J Min mouse has a longer life expectancy, compared to the traditional C57BL/6 Min mouse model, and carcinomas develop in 100% of animals (Sødring *et al.* 2016). Both the A/J and the C57BL/6 strains are inbred and the use of inbred laboratory mice in cancer research is favorable due to a high homozygosity of gene loci (a minimum of 98.6% at 20 generations of inbreed), which decreases variability and increases reproducibility of results (Beck *et al.* 2000). The A/J Min mouse model has previously been used to study the effects of dietary fibers, various

meats and hemin (with or without nitrite) on colorectal carcinogenesis (Sødring *et al.* 2015, Moen *et al.* 2016, Steppeler *et al.* 2017). However, no studies have yet investigated the effects of POPs on CRC in this model.

5.5.3 Exposure regimes

Administration of POPs through diet *ad libitum* is a non-invasive method of exposure that causes little stress to the animals. In addition, it is a realistic exposure scenario for humans as the main route of POP exposure occurs through ingestion (Djien Liem *et al.* 2000, Trudel *et al.* 2008). Administration of chemicals by gavage would have secured equal dosing of animals, however, gavage induces stress that can negatively affect sensitive strains. The A/J Min mouse appeared sensitive to stress during breeding, which resulted in bad maternal behavior and cannibalism of pups. Thus, administration through diet was favorable compared to more invasive methods of exposure. However, as no determination of feed intake was conducted, individual mice may have consumed unequal doses of the POP mixture, which may further have resulted in a high variability of results. Thus, large group sizes were required to detect differences between doses and minor effects may have been overlooked.

POPs are known to be transferred from mothers to offspring, thus, enabling the possibility of a combined *in utero* and lactational exposure scenario. Oral gavage of neonate mice with the mixture of POPs could have been conducted in Papers I and III to ensure identical exposure levels. However, perinatal exposure was considered a more realistic scenario in which exposure would occur during multiple critical periods of development. Notably, the competition between offspring for milk resulted in an earlier start of solid food intake for some pups and, thus, POP exposure may have occurred partly by nibbling to feed prior to weaning.

Furthermore, it is important to note that the inclusion of more than one round of mating, gestation and lactation on the dietary exposed A/J WT dams in Paper III may have contributed to a higher exposure concentration of POPs with long half-lives for offspring produced by the second and third rounds, compared to offspring born from the first mating. However, due to unknown factors, the first round of mating produced very few offspring with the Min/+ genotype and the decision was made to include additional rounds of mating, pregnancy and lactation on the dietary exposed A/J WT dams to increase the number of replicates. In Paper I, only one round of mating was included on the CD-1 dams. However, the dose of POPs transferred from mothers to offspring is

dependent on both maternal concentration and litter size. A standardization of the litter size could have been conducted shortly after birth to reduce variance in individual POP exposure through lactation. However, this would have reduced the overall number of replicates within each dose and was, therefore, not conducted.

As the High dose of the mixture alone increased the liver weight and decreased the body weight of A/J Min mice in Paper II, and because the Low dose represented a more realistic human exposure scenario compared to the High dose, only the Low and Control doses were used in Paper III. However, this limited the confidence in results and, thus, it can be argued that the High dose should also have been included in Paper III. Moreover, only a limited number of perinatally exposed offspring was obtained in Paper III, which by randomness did not show an even distribution between genders within the Min/+ and +/+ traits. The decision to pool genders was made to provide a larger number of replicates within each dose and because previous studies have shown no differences between genders in the formation or progression of CRC in the A/J Min mouse model (Sødring *et al.* 2016, Steppeler *et al.* 2017). However, the colonic tumor load was higher in males compared to female A/J Min mice in the study by Moen *et al.* (2016) and, thus, the pooling of genders within doses could have concealed gender-specific differences in CRC in Papers II and III. Furthermore, the response in intestinal metabolite composition to POP exposure has been shown to vary between genders (Merhi *et al.* 2010, Zhang *et al.* 2012, Pikkarainen *et al.* 2019). Hence, future studies should include more replicates as to enable the investigation of effects in males and females separately.

Moreover, the development of a microbial community is dependent on maternal effects and may vary between cages and litters (Goodrich *et al.* 2014). Thus, randomization of pups should have been conducted shortly after birth to reduce confounding effects in microbiota (of particular interest in Paper III). However, as some murine strains are easily stressed during nursing, randomization could also have resulted in bad maternal behavior and reduced the number of replicates.

In Paper II, the exposure regime included a non-random division of mice between the two experimental sections. This prevented statistical analysis between sections and future studies should completely randomize recruitment into untreated and AOM-treated groups. In addition, all groups not treated with AOM should receive an injection of saline solution as to inflict the

same amount of stress onto all mice. AOM was used to decrease the latency period of CRC development in A/J Min mice of the second experimental section. A high mortality was observed shortly after AOM injection and 1-2 weeks prior to starting on the POPs diet. Thus, mortality was not caused by the mixture. Previous studies have used two consecutive injections of AOM at doses up to 10 mg/kg in young A/J Min mice (Paulsen *et al.* 2006, Moen *et al.* 2016). As only one injection of 8.5 mg AOM/kg body weight was included in Paper II, the cause of the high lethality is unknown. A higher number of replicates in the second experimental section would have been preferred and would possibly have resulted in a significant difference in CRC between the Control and Low doses.

5.5.4 Histological techniques

Evaluation of mammary glands

Mammary gland development is typically evaluated through basic measurements including counting of structures (e.g. TEBs) and observations of morphological characteristics (scoring of development). These measurements vary with the experience and interpretation of the evaluator and are not properly blinded to treatment as a baseline for gland development needs to be established prior to evaluation. In Paper I, mammary gland development was initially conducted by two evaluators, each repeating the evaluation three times for each whole mount. However, as the evaluators were not initially blinded to the Control dose and had little experience in mammary gland assessment the results should be interpreted with caution.

Stanko *et al.* (2015) emphasized that standardized methods for assessing rodent mammary gland development were needed in order to accurately compare results from different laboratories and research groups, and a method for quantifying branching density on 2D images of mammary gland whole mounts from rodents was established (Stanko *et al.* 2015, Stanko and Fenton 2017). This method is based on the Sholl method originally developed for assessing the branching patterns in dendritic cells (Sholl 1953). However, as mammary glands display the same tree-like structure as neurons, Stanko *et al.* (2015) proposed that this method could also be used for measuring mammary epithelial branching. The Sholl analysis uses skeletonized images in ImageJ (Schindelin *et al.* 2012) and a plugin that counts the number of intersections occuring in a series of concentric rings streching out from a predefined center (the mammary gland base). Epithelial

branching density is calculated by dividing the number of intersections by the epitheial area (not including the lymph node).

The Sholl analysis is a suitable method for quantitatively assessing the mammary epithelial branching in rodents in response to EDC exposure and origianlly described for the peripubertal rat mammary gland (Stanko *et al.* 2015). However, the method has also been conducted on mammary glands from CD-1 mice (Tucker *et al.* 2018) and does not require specialized microscopy equipment or expensive imaging software. In Paper I, a Sholl analysis was used on mammary gland whole mounts blinded to dose. It was important that the entire gland epithelium was present and equally stretched onto the charged microscope slide. Thus, torn or damaged whole mounts were not included in the analysis and an inexperience in whole mount preparation may have obscured the results. Additional quality assurance aspects described in Stanko *et al.* (2015) were followed and the method of whole mount preparation was conducted in accordance with Davis and Fenton (2013). As murine mammary glands do not grow as dense as rat glands, Stanko *et al.* (2015) proposed that the method could be used on whole mounts from mice at all ages. However, Tucker *et al.* (2018) found it very difficult to perform the method on mammary glands from CD-1 mice of > 3 months of age due to an extensive growth and density of the epithelium. The whole mounts from 9-week-old mice in the present study exhibited complex and sometimes very dense epithelial structures. Thus, more time had to be invested in the image processing, but it was possible to conduct the analysis.

To the authors knowledge, other standardized methods for qualitatively assessing mammary gland development without the use of commercial software and/or specialized microscopy equipment are lacking. Thus, the Sholl analysis was chosen due to its low cost and the availability of a detailed description (Stanko *et al.* 2015, Stanko and Fenton 2017). However, this method was conducted by a different evaluator than those assessing mammary gland development and counting the number of TEBs. Thus, differences in results could have been achieved due to personal differences in evaluation.

Evaluation of ovarian follicles

When investigating alterations in ovarian folliculogenesis, it was important to count the number of follicles blinded to dose. Furthermore, the final follicle numbers were relative to the counting procedure and did not represent the total number of follicles in the ovaries of female CD-1 mice

in Paper I. Thus, caution should be taken when comparing the follicle counts in the present study to those reported in other studies using different procedures. It can be argued that the fractionator technique (Gundersen *et al.* 1988) should have been applied to estimate a total number of follicles per examined ovary (as conducted in Pocar *et al.* (2011) and Tomic *et al.* (2002)). However, as this technique would involve multiplying all counts with the same number, the overall difference between doses would be the same as by not applying the technique.

In the present study, the ovarian follicles were categorized as described by Flaws *et al.* (2001). Other methods of follicle categorization are possible, such as by determining the follicle category based on granulosa cell numbers (Pedersen and Peters 1968) rather than follicle appearance, or by classifying the pre-antral and antral follicles according to size (Heimler *et al.* 1998, Baldridge *et al.* 2003). In addition, pre-antral and antral follicles have been defined as secondary and tertiary follicles, respectively (Lilienthal *et al.* 2006, Du *et al.* 2019). Furthermore, atretic follicles or corpus luteum were not counted in the present study, and no measures were taken to promote estrous cycle homogeneity in offspring. Thus, future studies should include an assessment of atretic follicles and corpus luteum, vaginal cytology and necropsy of mice in estrus, and the evaluation of both ovaries from all mice.

5.5.5 CYP activity

The analysis of CYPs can be conducted on mRNA, protein or activity corresponding to the transcriptional, translational or catalytical levels, respectively. As mRNA is the source of protein content and enzymatic activity, similarities in response to xenobiotic exposure could be expected. However, discrepancies between mRNA and CYP activity levels have been shown previously (Li *et al.* 2017) and can be caused by post-transcriptional modification that inhibits mRNA translation into protein. The catalytic activity using model substrates is commonly used to determine modulation in CYP activity caused by xenobiotic exposure. However, this method is vulnerable to overlapping substrates, which makes it difficult to select a substrate that is exclusively metabolized by only one CYP enzyme. For instance, pentoxyresorufin can be used as a substrate for CYP2B10 in mice and CYP2B6 in humans, and methoxyresorufin is catalyzed by both CYP1A1 and CYP1A2 in mice (reviewed in Hrycay and Bandiera (2009)). Furthermore, differences in CYP expression patterns, regulation and activity has been documented between mouse strains (Hrycay and Bandiera 2009). In the present study, substrates were chosen to

investigate differences between doses for CYP enzymes known to be involved in murine xenobiotic metabolism and commonly used as biomarkers for AHR, CAR and PXR activation (Whitlock 1990, Hrycay and Bandiera 2009, Tolson and Wang 2010). However, comparisons between results at different biochemical levels and between different strains and species should be conducted with caution.

5.5.6 Omics techniques

Metabolomics

Metabolomics/metabonomics provides a 'top-down' approach to the biochemical environment of a complex organism and monitors the global outcome of all influences on the system (Nicholson and Lindon 2008). As previously described, NMR spectroscopy and MS are the analytical techniques most commonly used in metabolomics. These techniques can be considered complimentary, rather than alternatives, with the ability to extract large amounts of information from a small volume of sample (Claus and Swann 2013). ^{1}H NMR spectroscopy measures all molecules containing hydrogen in a sample and yields NMR spectra containing a vast amount of information. This can be done without any prior knowledge of metabolite composition or concentration, and with only minor treatment of the sample prior to analysis. On the other hand, MS is generally more sensitive than NMR. However, some metabolites may give varying responses between experiments, which can be misleading. Furthermore, the metabolite separation in MS disrupts the molecular structures and interactions between molecules in the sample cannot be identified (Nicholson and Lindon 2008). Thus, the NMR technique was chosen for the present study. However, MS could possibly have been conducted as an additional measurement to confirm metabolites identified by NMR.

The NMR analysis utilized in the present study was able to detect relative differences in metabolite composition between perinatally exposed and control mice. Unfortunately, this method is relatively insensitive for metabolites in the ranges just about the detection limit and large degrees of spectral overlap could occur (Claus and Swann 2013). Thus, several metabolites may not have been identified as their presence was masked by baseline noise or other spectral peaks. Furthermore, NMR spectroscopy was conducted on cecal tissue and content, which may have had a different metabolite composition compared to the colon and small intestine. However, as the other parts of the intestine were fixated in formalin and used for surface microscopy,

metabolomics was only possible on the remaining intestinal tissue; the cecum. Thus, the metabolic differences seen in cecal tissue should be further confirmed in samples from colon and small intestine.

Gut microbiota

In addition to 16S rRNA gene sequencing, 'shotgut' metagenomic sequencing can be conducted to study the collective microbial genome. Metagenomics sequences the whole genome in all microorganisms without selecting for a particular gene (Scholz *et al.* 2016). This method yields a more detailed overview of the target environment compared to 16S rRNA sequencing. However, it requires a high read count and a reference genome for classification, it may incorrectly identify reads from the host, and it is relatively expensive (Breitwieser *et al.* 2019, Wang *et al.* 2019). On the other hand, 16S rRNA gene sequencing is a fast and cost-effective method that identifies numerous bacteria. Even though this method has some amplification bias, which may prevent identification at low taxonomic levels, it is desirable due to rRNA databases (such as Greengenes) that contain genes from millions of bacterial species (Breitwieser *et al.* 2019). Thus, 16S rRNA gene sequencing was used in the present study.

Following 16S rRNA sequencing, the reads were clustered into ASVs. The traditional method of 16S rRNA clustering is by categorization in operational taxonomic units (OTUs). The OTU method selects a radius of typically 3% variation in sequences that is assumed to be caused by noise (error) or variability within the taxonomic group. All sequences within this 3% radius are clustered as the same OTU (Caruso *et al.* 2019). On the other hand, clustering into ASVs does not make any assumptions about the variability of sequences. Instead, the method attempts to model the error and to cluster reads consistently with the error model. Thus, ASVs can improve the sensitivity and specificity of 16S rRNA gene sequences and can cluster sequences into units that vary with only one base pair (Callahan *et al.* 2017, Caruso *et al.* 2019).

5.5.7 Statistical methods

As several of the replicates in the present study cannot be considered completely independent (mainly due to litter effects), statistical methods should have taken this possible clustering effect into account. Furthermore, correlations between variables may have been overlooked and, thus, regression models (also including random effects) could potentially have been conducted on more

of the data. In addition, it was important to choose methods that corrected for the overall experiment wise error rate (type I error).

During the evaluation of effects on CRC, it was important to choose an experimental period that avoided type II errors during statistical analyses. A prolongation of the period may have led to larger differences between groups. However, this would have raised ethical concerns for animal welfare. Furthermore, as previously mentioned, the replicate numbers should have been increased to account for the high variability observed and to increase statistical power.

In Papers I and III, chemical concentrations below LOD were replaced with LOD for inclusion in the sums and statistical analysis. This may have resulted in an overestimation of the chemicals present, which further could have led to an overestimation of the descriptive statistics (mean, median, etc.). Other approaches for replacement of LOD values includes replacement with a distribution, ½ LOD or 0, however, the latter of these methods will lead to underestimation of the chemical concentrations (Baccarelli *et al.* 2005, Donders *et al.* 2006). Importantly, non-detectable levels are connected to a high degree of uncertainty and the method of replacement should be evaluated and described for each data set.

Multivariate methods for metabolomics

Metabolomics captures an extensive amount of molecular information from one sample and, thus, multivariate statistical methods are needed to extract latent metabolic information (Eriksson *et al.* 2004, Claus and Swann 2013). These techniques fall into two groups: the unsupervised clustering methods and the supervised methods. The unsupervised methods do not need any information about the data prior to analysis and includes PCA, which attempts to find a small set of variables that explains most of the variation in the data set. PCA further summarizes the multidimensional data into a few principal components that can be visualized to identify trends, groupings, outliers and metabolites (Trygg *et al.* 2007).

On the other hand, the supervised methods use information about the group membership of each sample to make predictions about the data set. One commonly used supervised technique is the partial least squares (PLS) analysis. This is a regression extension of PCA that includes two matrices (X and Y) in which it aims to estimate individually and to quantify the relationship between. In addition, PLS can be used in discriminant analysis (DA) where the Y matrix contains information about treatment, group or gender, depending on the question to be answered. Thus,

PLS-DA can be used to separate the multidimensional data into classes by focusing on maximum separation ('discrimination') rather than maximum variation (Eriksson *et al.* 2004, Trygg *et al.* 2007). However, the PLS model is affected by systematic variation in X that is not related to the Y matrix, which may make the interpretation of metabolites from model plots problematic. Thus, the OPLS-DA method was developed as a modification of the PLS technique where it separates the systematic variation in X into variation that is either linearly related or unrelated (orthogonal) to Y. Only the linearly related variation is then used for modelling Y. Hence, the OPLD-DA model can separate variation between classes from the variation within classes, something PLS-DA is not able to do (Trygg *et al.* 2007).

PCA is recommended as a starting point for multivariate analysis as it rapidly provides an overview of the data set and, thus, it was applied in the present study. Furthermore, as the metabolomics data could be classified into exposed and control samples, OPLS-DA was used to simplify interpretation and to find differentially abundant metabolites. As this method is not sensitive to systematic variation, it was the recommended technique in the present two-class problem.

However, all these multivariate techniques must be able to cope with noise, correlations and missing data as well as biological and experimental variation. Variability in NMR peak shifts may cause problems for the statistical analysis. Thus, peak alignment was conducted (Trygg *et al.* 2007). Other methods for pre-processing data prior to statistical analysis are also available and include centering, scaling, transforming, expanding and correcting signals. Furthermore, prior to any data treatment, the experiment should be designed in the most optimal way to extract the information needed to answer the questions asked (Eriksson *et al.* 2004).

Methods for analyzing microbial diversity

Microbial diversity can be estimated within (i.e. alpha diversity) and between samples (i.e. beta diversity). The beta diversity is considered relatively robust to issues of low sequence counts and noise and may reveal differences in microbial communities where alpha diversity does not show a difference (Goodrich *et al.* 2014). In the present study, the beta diversity was assessed using the qualitative unweighted UniFrac method which was purely based on the calculated distances between samples in their ASV composition. The distance metric generated by the UniFrac method can be further subjected to statistical testing. In addition, PCoA is commonly used on

microbial data sets and summarizes the beta diversity relationships (community compositional differences) between samples (Goodrich *et al.* 2014). Lastly, statistical tests can be conducted on the relative abundances of bacterial species, genera, families, orders, classes and phyla. However, as the abundances sum up to 1, standard statistical tests (e.g. t-test and ANOVA) are not appropriate and an ANCOM test should be used instead (Mandal *et al.* 2015).

5.6 Mouse-to-human translation

When determining human causality between a presumed cause and an observed effect, human data is considered the most valid metric (Guzelian *et al.* 2005, James *et al.* 2015). Only when human data is insufficient to answer the specific question, regulatory risk assessment could derive their health-protective guidelines from animal studies. However, when human causality is only based on non-human data, an unknown degree of uncertainty is introduced into the hazard and risk estimations. Thus, animal data should only be judged cautiously and not as the tipping point when reaching a conclusion on human causation (James *et al.* 2015).

The question of whether results acquired from animal-based models are predictive of adverse effects in humans is very important. As put forth in the evidence-based toxicology framework, animal data should be used as supporting information when identifying the mechanisms linking cause and effect (the Hill criteria of plausibility (Hill 1965)) and to facilitate future epidemiological and mechanistic studies (James *et al.* 2015). Thus, the results presented herein should be used to inspire future research on chemical mixture effects, preferably at low and human relevant concentrations, and to facilitate mechanistic investigations into the complex process of colorectal carcinogenesis.

During animal-to-human translation, the translational success rate can be determined by the construct, internal and external validity. The construct validity refers to the ability of the test to measure what it claims to be measuring, which is dependent on the disease in question, the animal model and the animal welfare during the experiment (Bailoo *et al.* 2014). In the present study, murine models were chosen based on their high sensitivity to endocrine disturbances during mammary gland development and their high similarity in CRC development to humans. However, the intrinsic differences in physiology, genetics, epigenetics and molecular biology between humans and mice should not be forgotten.

Internal validity focuses on optimizing the study design, conduct, analysis and reporting to eliminate the possibility of bias (Bailoo *et al.* 2014). Thus, the translation success rate of the present study was affected by the suboptimal experimental designs discussed previously. Lastly, external validity extends beyond the experimental setting and wish to generalize the results to other environmental conditions and other species of animals (including humans). The present study did not include a systematic variation in murine characteristics and environmental conditions and was, thus, not designed to optimize the external validity (Bailoo *et al.* 2014, Pound and Ritskes-Hoitinga 2018). Instead, the present study wished to follow the golden standard of experimental design by keeping all factors constant except for the independent variable under investigation. The murine models chosen were generally uniform (at the same age and weight) and other environmental conditions were kept as constant as possible. However, this homogenization compromised the external validity and consequently also the translational success rate. Furthermore, questions should be raised regarding the reproducibility of results as highly standardized and homogenized conditions may reveal conclusions that are only valid under the specific conditions and laboratory environment used (Richter *et al.* 2009, Bailoo *et al.* 2014). Thus, the mouse-to-human translational ability of the present study is debatable and further studies are needed to increase the external validity and reproducibility of results.

6 General conclusions

This thesis expands current knowledge on effects of POP mixtures relevant for human consumption. After absorption, distribution, maternal transfer and accumulation, the human relevant mixture of POPs restricted female mammary gland development, decreased ovarian follicle maturation and possibly increased ovarian follicle atresia in CD-1 mice. Strain-dependent differences in hepatic response to exposure was seen between CD-1 and A/J Min mice and sensitive murine strains may be inclined to develop hepatotoxicity due to prolongation of cellular hypertrophy and enzymatic induction. Dietary exposure moderately increased colorectal carcinogenesis in the A/J Min mouse model. Interestingly, contradictory effects were seen after perinatal exposure where the mixture reduced carcinogenesis possibly by modifying the intestinal biochemical and microbial environment. Thus, POP exposure during early development reduced the adversity of the predetermined cancer condition. Lastly, dietary exposure after AOM treatment resulted in a synergistic interaction between AOM and POPs which drastically increased cancer development. Overall, the results of the present study can facilitate future mechanistic investigations into how human relevant chemical mixtures may affect biological development and function.

7 Future perspectives

The present study provides a basis for future studies into the mechanistic basis of colorectal carcinogenesis and developmental effects caused by exposure to chemical mixtures. However, the fact that mixture composition and chemical concentrations is highly complex in the context of human exposure presents an extensive challenge when defining what is human relevant and to find animal models with sufficiently high validity and translational value.

There is a need for the use of *in vitro* models to elucidate the mechanistical aspects of colorectal carcinogenesis in relation to chemical exposure. These models may include organoids derived from murine or human intestinal stem cells which generate self-organizing 3D structures that mimic the architecture, function and genetic signature of the original tissue (Sato *et al.* 2009, Sato *et al.* 2011, Dutta *et al.* 2017). Colorectal carcinogenesis can be modelled in organoids using gene manipulation techniques, such as CRISPR/Cas9-mediated genome editing that introduce mutations into *APC*, *KRAS*, *TRP53* or *PIK3CA*, or by generating organoids from cancerous tissue (Sachs and Clevers 2014, Matano *et al.* 2015). Thus, the effects of POPs directly on the cancer process can be examined in intestinal organoids. Furthermore, direct chemical interaction (possibly leading to synergism) between individual POPs or between POPs and other carcinogenic agents can be studied directly on the target tissue.

To further study the possible beneficial effects of microbiota and metabolome on the intestinal health, specific bacteria or bacteria-derived metabolites can be introduced into the *in vitro* organoid environment. However, studying the effects of anaerobic microbes on organoids is challenging as organoids growth under oxygenated conditions. In addition, organoids do not encompass the total environment of the *in vivo* intestine including a complex microbial community and interactions with stroma, muscle, blood and immune cells (Dutta *et al.* 2017). Thus, specific bacteria can also be introduced into the intestinal environment of germfree mice. Both these *in vitro* and *in vivo* models can further be treated with human relevant chemical mixtures during microbial modulation to investigate the interaction between microbiota and POPs on intestinal health.

Lastly, it would be of interest to study how exposures to POPs during early life may have physiological consequences for xenobiotic metabolism and chemical sensitivity, and to characterize the basal enzymatic expression patterns of A/J Min compared to WT mice.

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Appendix: Papers I-III

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Perinatal exposure to a human relevant mixture of persistent organic pollutants: Effects on mammary gland development, ovarian folliculogenesis and liver in CD-1 mice.

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Abstract

The ability of persistent organic pollutants (POPs) with endocrine disrupting properties to interfere with the developing reproductive system is of increasing concern. POPs are transferred from dams to offspring and the high sensitivity of neonates to endocrine disturbances may be caused by underdeveloped systems of metabolism and excretion. The present study aimed to characterize the effect of *in utero* and lactational exposure to a human relevant mixture of POPs on the female mammary gland, ovarian folliculogenesis and liver morphology and function in CD-1 offspring. Dams were exposed to the mixture through the diet at Control, Low or High doses (representing 0x, 5 000x and 100 000x human estimated daily intake levels, respectively) from weaning and throughout mating, gestation and lactation. Perinatally exposed female offspring exhibited altered mammary gland development, resulting in a premature arrest of gland growth, and a suppressed ovarian follicle maturation. Increased hepatic cytochrome P450 enzymatic activities indirectly indicated activation of nuclear receptors and potential generation of reactive products. This could have contributed to hepatocellular hypertrophy, which was observed from weaning until 30 weeks of age, and potentially lead to hepatotoxicity. Further studies should address if human relevant mixtures of POPs can cause mammary or hepatic carcinogenesis, lactational impairment or infertility.

Keywords

Persistent organic pollutants; Mammary gland development; Ovarian folliculogenesis; Hepatocellular hypertrophy; Hepatic cytochrome P450 activity

Abbreviations

BFR, brominated flame retardant; CYP, cytochrome P450; EDC, endocrine disrupting compound; hEDI, human estimated daily intake; MG, mammary gland; OCP, organochlorine pesticide; PCB, polychlorinated biphenyl; PFAS, perfluoroalkylated substance; POP, persistent organic pollutant; TEB, terminal end bud.

1. Introduction

Persistent organic pollutants (POPs) are of significant concern due to their resistance to degradation and potential toxicity (Secretariat of the Stockholm Convention 2019). This has led to the regulation of production, use and release of POPs by the Stockholm Convention (Secretariat of the Stockholm Convention 2010). A variety of POPs exhibit endocrine disrupting properties as they interfere with the synthesis, transport, metabolism or elimination of naturally occurring hormones in the body (Diamanti-Kandarakis *et al.* 2009, Bopp *et al.* 2018). The hormonal system is of vital importance in the fully functioning organism. Thus, there is a growing concern about endocrine disrupting compounds (EDCs) interfering with the reproductive, metabolic, neuroendocrine or cardiovascular systems (Diamanti-Kandarakis *et al.* 2009, Kabir *et al.* 2015). POPs are known to be transferred from mothers to offspring through the placenta and breast milk (Barr *et al.* 2007, Fenton *et al.* 2009, Berg *et al.* 2010, Bonfanti *et al.* 2014, Vizcaino *et al.* 2014, Winkens *et al.* 2017, Johanson *et al.* 2020). As early stages of development are particularly sensitive to disturbances by EDCs, the placental and lactational transfer of POPs may have devastating consequences (Faustman *et al.* 2000, Barr *et al.* 2005, Landrigan and Goldman 2011).

Mammary gland (MG) development is an intricate process finely regulated by hormones, growth factors and stromal factors, all of which could potentially be disturbed by EDCs (Fenton 2006, Rudel *et al.* 2011). One extensively studied example of such a disturbance is the effect of perfluorooctanoic acid (PFOA) on MG development in rodents (White *et al.* 2007, White *et al.* 2009, Yang *et al.* 2009, Macon *et al.* 2011, White *et al.* 2011, Tucker *et al.* 2015). Other studies have also reported effects in the developing female MG or alterations in ovarian follicle maturation by various POPs (Markey *et al.* 2001, Fenton *et al.* 2002, Baldridge *et al.* 2003, Muto *et al.* 2003, Rayner *et al.* 2005, Lilienthal *et al.* 2006, Shirota *et al.* 2006, Enoch *et al.* 2007, Talsness *et al.* 2008, Kodavanti *et al.* 2010, Miret *et al.* 2017, Tucker *et al.* 2018). Changes in ovarian folliculogenesis could lead to premature ovarian insufficiency and infertility (Vabre *et al.* 2017). Furthermore, exposure to EDCs may increase the risk of developing breast, cervical, uterine or ovarian cancer (Rachoń 2015).

The high sensitivity of fetuses and neonates to POP exposure compared to adults can be partly explained by immature metabolic and excretion systems (Coughtrie *et al.* 1988, Milsap and Jusko

1994). The cytochrome P450 (CYP) family of enzymes (included in phase I metabolism) catalyzes the transformation of xenobiotics to more polar derivatives that can be further metabolized or excreted (Guengerich 1991, Nebert and Russell 2002, Maronpot *et al.* 2010). However, transformation by CYP enzymes may also form free radicals (e.g. reactive oxygen species), or activate procarcinogens, causing potentially more deleterious effects than the original compound (Bondy and Naderi 1994, Ioannides and Lewis 2004, Zangar *et al.* 2004). The expression of CYP enzymes is regulated by different nuclear receptors such as the aryl hydrocarbon receptor (AHR), the pregnane X receptor (PXR) and the constitutive androstane/activated receptor (CAR) (Waxman 1999, Tolson and Wang 2010). Various POPs have been shown to stimulate these receptors and consequently increase the enzymatic activities of CYPs (Sanders *et al.* 2005, Pacyniak *et al.* 2007, Szabo *et al.* 2008, van der Ven *et al.* 2008). Furthermore, upregulation of hepatic CYP transcripts at early life stages has been stated as early indicators of liver toxicity and carcinogenicity (Dunnick *et al.* 2018).

Previously, toxicological studies focused on the use of single compounds or technical mixtures of POPs. However, recent attention has shifted to study more realistic exposure scenarios with multiple compounds that may interact and cause effects not anticipated by dose addition (Hernández *et al.* 2013, Bopp *et al.* 2018). For the present study, a complex mixture of POPs was designed based on information from Scandinavian food basket surveys (Berntsen *et al.* 2017). The mixture included polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs) and perfluoroalkylated substances (PFASs), many of which have previously been studied as single compounds or technical mixtures. The present study aimed to investigate how *in utero* and lactational exposure to the mixture of POPs affected the developing female reproductive organs (the MG and ovary) and liver in CD-1 mice.

2. Materials and Methods

2.1. Ethical considerations

The study was approved by the Institutional Animal Care and Use Committee at the Norwegian University of Life Sciences (NMBU) and the Norwegian Food Safety Authority (application ID: FOTS 7722). It was conducted in accordance with The Norwegian Regulation on Animal Experimentation at the Section for Experimental Biomedicine, NMBU-Faculty of Veterinary Medicine, in Oslo, Norway. The animals were health-monitored according to recommendations

by the Federation of European Laboratory Animal Science Association (FELASA; http://www.felasa.eu/) and kept under Specific Pathogen Free (SPF) conditions.

2.2. Feed design

The POP mixture was designed by Berntsen *et al.* (2017). In brief, the composition of PCBs, OCPs, BFRs and PFASs was chosen based on concentrations in Scandinavian food products stated in publications prior to 2012. Human estimated daily intake (hEDI) levels were estimated and adapted to a 25 g mouse consuming 3 g feed/day. The concentrations were upward adjusted to 5 000x (Low dose) and 100 000x (High dose) hEDI. All polybrominated diphenyl ethers (PBDEs), PCBs and OCPs were purchased from Chiron AS (Trondheim, Norway). Hexabromocyclododecane (HBCD) and all PFASs, except for perfluorohexane sulfonic acid (PFHxS) purchased from Santa Cruz Biotechnology Inc. (Dallas, USA), were obtained from Sigma-Aldrich (St. Louis, USA). Compounds were dissolved in acetone, cyclohexane or chloroform and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey). The solvent was evaporated under N_2-flow and the mixture was incorporated into AIN-93G mouse feed (TestDiets, St.Louis, USA). The control diet contained corn oil from which the solvent had been evaporated. An additional AIN-93G reference diet was also made using corn oil instead of soybean oil. Mixture composition and concentrations of individual compounds (nominal and measured) are presented in Table 1.

134 Table 1. Nominal and measured concentrations (ng/g feed) of the PCBs, OCPs, BFRs and PFASs in AIN-
135 93G mouse feed at the Low and High doses (5 000x and 100 000x human estimated daily intake,
136 respectively). Adapted from Table 1 in Berntsen *et al.* (2017).

Compounds	Nominal concentrations Low	Measured concentrations Low	Nominal concentrations High	Measured concentrations High
Polychlorinated biphenyls (PCBs)				
PCB-28	5.8	3.1	117	46
PCB-52	13.8	15.0	275	182
PCB-101	23.3	25.4	467	377
PCB-118	40.4	37.2	808	612
PCB-138	57.5	53.8	1150	957
PCB-153	57.5	61.4	1150	981
PCB-180	15.4	17.4	308	263
Σ7PCBs	213.7	213.3	4275	3418
Organochlorine pesticides (OCPs)				
HCB	50.0	37.4	1000	588
α-chlordane	37.5	45.0	750	723
Oxychlordane	12.5	9.8	250	297
Trans-nonachlor	12.5	14.9	250	264
α-HCH	21.7	21.2	433	421
β-HCH	17.5	22.3	350	398
γ-HCH (Lindane)	23.8	31.4	475	435
Σ7OCPs	175.5	182.0	3508	3126
p,p'-DDE	119.6	136.0	2392	2390
Dieldrin	75.0	70.4	1500	1470
Brominated flame retardants (BFRs)				
BDE-47	40.4	39.7	808	642
BDE-99	7.9	8.6	158	126
BDE-100	6.3	5.6	125	91
BDE-153	1.3	1.5	25	22
BDE-154	2.5	2.8	50	38
ΣBDE-47-154	58.4	58.2	1166	919
BDE-209	62.5	64.8	1250	1141
HBCD	12.5	9.9	250	203
Perfluoroalkylated substances (PFASs)				
PFHxS	4.9	1.7	98	42
PFOS	10.8	3.2	217	74
PFOA	18.3	6.0	367	121
PFNA	5.8	2.1	117	42
PFDA	7.9	3.1	158	57
PFUnDA	4.0	1.6	80	28
ΣPFASs	51.7	17.7	1037	364

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139 *2.3. Animals, experimental design and sample collection*

140 Timed-pregnant CD-1 mice (F0) were obtained from Charles River Laboratories (Wilmington,
141 USA) and gave birth 4-5 days after arrival to the F1 generation. At 3 weeks of age, F1 females
142 were randomly assigned to a dose: Control (n = 28), Low (n = 27), or High (n = 20). Exposure
143 continued throughout mating (at 10 weeks of age with CD-1 males bred in-house), gestation and
144 lactation. Offspring (F2) produced by the F1 dams were given the AIN-93G reference diet from
145 3 weeks of age and only exposed to the mixture of POPs *in utero*, through lactation and by
146 nibbling on their mothers feed prior to weaning. The study design in illustrated in Supplementary
147 Figure S1.

148 All mice were housed in closed type III individually ventilated cages (IVC) (Allentown Inc,
149 USA) except during mating when animals were moved to open Makrolon Type III cages
150 (Techniplast, Buguggiate, Italy). All cages contained standard aspen bedding, red polycarbonate
151 houses and cellulose nesting material (Scanbur BK, Karlslunde, Denmark). Water and feed were
152 available *ad libitum*. Cages, bedding, nesting material and water bottles were changed weekly.
153 The animal room was on a 12:12 light-dark cycle, with a room temperature of 21 ± 2°C and 45
154 ± 5% relative humidity.

155 Dams (F1) were euthanized at gestation day 17 (pregnant; n = 12, 16 and 8 for the Control, Low
156 and High doses, respectively) or at 21 days post-partum (post-pregnant; n = 14, 10 and 11 for the
157 Control, Low and High doses, respectively) with a total exposure time of 9 or 13 weeks,
158 respectively. Mating was unsuccessful for 3 dams (2 in Control and 1 in High). One dam (exposed
159 to the Low dose) died prior to mating.

160 Female offspring (F2) were sacrificed at 3 (weaning), 6 (pubertal) and 9 (adult) weeks of age (n
161 = 12, 14 and 14 for all doses, respectively). Male offspring (F2) were sacrificed at 9 or 30 weeks
162 of age (n = 15 for all doses) to further investigate the hepatic alternations observed in females.
163 Due to a low number of males produced by dams exposed to the High dose, all males in this dose
164 were sacrificed at 9 weeks of age. Thus, 30-week-old male offspring were only sampled from the
165 Control and Low doses.

166 Mice were sacrificed under anesthesia (isoflurane gas obtained from Baxter, San Juan, Puerto
167 Rico) by cardiac puncture and cervical dislocation. A cross section of the left liver lobe, ovaries,

and the fourth MG on the left side were fixed in 10% neutral buffered formalin for histopathological examinations. The remaining left lobe was frozen on dry ice and stored at -80°C for chemical analysis. The right, caudate and quadrate lobes were also frozen (on dry ice) and stored (-80°C) for cytochrome P450 activity analysis. The fourth MG on the right side was peeled away from the inner skin surface and spread out on a microscope slide for whole mount analysis.

2.4. Chemical analysis

Pieces of the left liver lobe of dams (F1) and female offspring (F2) were pooled according to their respective dose (Control, Low and High) and sampling time (pregnant, post-pregnant, and 3, 6 and 9 weeks of age). Analysis of PCBs, OCPs, BFRs and PFASs was conducted on pooled samples at the Laboratory of Environmental Toxicology, NMBU, Oslo, Norway.

The method for PCB, OCP and BFR analysis is based on Brevik (Brevik 1978) and Polder *et al.* (2014) and accredited by the Norwegian Accreditation for chemical analysis in biota (requirements of the NS-EN ISO/IEC 17025, TEST 137). Briefly, up to 2.5 g liver tissue were homogenized (using a T25 Ika Ultra-Turrax®) and spiked with internal standards (PCB-29, -112 and -207 obtained from Ultra Scientific, North Kingstown, USA, and BDE-77, -119, -181 and $^{13}C_{12}$BDE-209 from Cambridge Isotope Laboratories Inc., Tewksbury, USA). Lipids were extracted twice with cyclohexane and acetone (3:2 ratio, obtained from VWR International S.A.S, Radnor, Pennsylvania) by ultrasonic homogenization, centrifugation and separation. Lipid content was determined gravimetrically in 1 mL aliquots, and lipid removal was performed by using ≥ 97.5% H_2SO_4 (Sigma Aldrich, Missouri, USA). Extracts were concentrated with a factor of approximately 10 by careful evaporation under N_2, before gas chromatography (GC) analyses.

The quantification of PCBs (PCB-52, -101, -118, -138, -153, and -180) and OCPs (*p,p'*-dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB), α-chlordane, oxychlordane and *trans*-nonachlor, and α-, β- and γ-hexachlorocyclohexanes (HCH)) was performed on a high-resolution GC (HRGC) (Hewlett Packard HP 6890 Series, USA) with mass spectrometer (MS) and electron capture (EC) detectors (Agilent Technologies, 5975c inert XL EI/CI MSD triple axis detector, USA). Quantification of BFRs (BDE-28, -47, -99, -100, -153, -154 and -183, and HBCD) was conducted on a HRGC (Agilent 6890 Series GC system, USA) coupled with a low-resolution MS (LRMS) (Agilent 5973 Network Mass Selective Detector,

USA) configured with a split/split-less injector (Agilent Technologies, Santa Clara, USA). For BDE-206, -207, -208 and -209, a HRGC (Agilent 6890 Series GC system, USA) coupled with a LRMS (Agilent 5973 Network Mass Selective Detector, USA) configured with a programmable temperature vaporization (PTV) injector (Agilent Technologies) was used. BDE-28, -183, -206, -207, and -208 were not originally in the POPs mixture, but measured due to possible de-bromination of the original PBDEs. The analysis of BDE-206, -207, and -208 was not accredited.

The analysis of PFASs (PFHxS, perfluorooctane sulfonate (PFOS), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoid acid (PFDA) and perfluoroundecanoic acid (PFUnDA)) is described by Grønnestad *et al.* (2017). This analysis is validated according to the same procedures and quality control measures as for the PCBs, OCPs and BFRs, although not included in the accreditation. Approximately 0.20-0.55 g homogenized liver tissue was spiked with internal standards (a ^{13}C-labeled perfluoroalkyl mix obtained from Wellington Laboratories, Guelph, Canada) and extracted twice with methanol. Lipids were removed using activated carbon (EnviCarb). Quantification was carried out using a high-performance liquid chromatography (HPLC) with a Discovery C18 column, connected to a C18 pre-column (Supelco, Sigma-Aldrich, Oslo, Norway) and a liquid chromatography (LC) tandem MS (MS-MS) (API 3000, LC/MS/MS system).

Method quality control measures were approved as they were within accreditation requirements (see Supplementary section S3.3). Compounds detected at concentrations outside the accepted range of recovery (70-130%) were corrected for recovery (herein *p,p'*-DDE, BDE-206 and HBCD). Concentrations below limit of detection (LOD) were replaced with the LOD to be included in the ΣPCBs, ΣOCPs, ΣPBDEs and ΣPFASs. Additional method information is presented in Supplementary section S3.

2.5. Histological examination and image analysis of mammary glands

2.5.1. Whole mount preparation, development scoring and Sholl analysis

Whole mounts were used to assess alterations in MG development of female offspring (F2). The fourth MG from the right side was placed with the skin side down on a charged microscope slide and fixed in Carnoy's solution (6:3:1 of ethanol:chloroform:acetic acid) at 4°C overnight. Whole mounts were rinsed in 70% ethanol, gradually transitioned to water and stained overnight in

carmine alum stain (2 g/L carmine and 5 g/L aluminum potassium sulfate). After staining, the whole mounts were dehydrated gradually to 100% ethanol and de-fatted in xylene overnight until visibly clear. Multiple pictures were taken of each whole mount at 10x magnification using a Zeiss Axio Imager light microscope and camera (M2, Oberkochen, Germany), and a mosaic (Preibisch *et al.* 2009) was created using ImageJ (Schindelin *et al.* 2012).

Qualitative development scoring was conducted on a scale from 1 to 4 (1 = poor development, 4 = best development). The following criteria were evaluated: lateral and longitudinal epithelial growth into the surrounding fat pad; branching degree; alveolar budding; lobule formation; and the presence or absence of terminal end buds (TEBs). Scores were assigned by two individuals and repeated 3 times for each whole mount to give a median score from each assessor. The final score was obtained by averaging the two median scores. The assessors were initially not blinded to Control as to establish a baseline of MG development for this strain of mice. After this, the glands were evaluated within each age class blinded to dose. TEBs were defined as ends with a diameter of $\geq$ 100 μm and counted using ImageJ.

MG branching density was quantitatively assessed using ImageJ and the modified Sholl analysis method (Stanko *et al.* 2015). In brief, the color channels were separated, and noise were removed from the images using various methods supplied by ImageJ. The images were then skeletonized, made binary and dilated one time to fill in gaps created by skeletonization. Longitudinal distance of the gland (termed mammary epithelial length) was measured between the base of the epithelial tree and the tip of the most distal branch using the skeletonized images. The mammary epithelial area (MEA) and the lymph node area (LNA) were defined by the periphery of the total skeletonized gland and the area covered by the lymph node, respectively. The total number of intersections (N) within the MEA was determined by Sholl analysis. Branching density was calculated using the formula N/(MEA-LNA). A detailed description of the method is given by Stanko and Fenton (2017).

Two whole mount images (both from 6 weeks Control) were ruined during processing. In addition, three glands were partly torn during whole mount preparation (one from 3 weeks High, one from 9 weeks High and one from 9 weeks Control). Thus, a total of five whole mounts were not included in the analysis.

2.5.2. Image analysis of glandular tissue

The fourth MG from the left side of female offspring (F2) were fixated in formalin for at least 24 hours. Glands were transferred to 70% ethanol prior to paraffin embedding and sectioned horizontally using a microtome (5 µm thick). Gland orientation for sectioning was ensured identical to whole mount orientation. Sections were attached onto glass slides and stained with HE. Sections were examined blindly by a board-certified pathologist.

Digital photomicrographs of the glands were taken at 100x magnification using a Zeiss Axio Imager light microscope, Axiocam 506 color camera and Zen microscope software (Carl Zeiss Microscopy GmbH, Germany). Photomicrographs were stitched and reconstructed digital images of the whole glands were created. Image-Pro Plus (Media Cybernetics Inc., MD, USA) was used to measure area fractions occupied by glandular tissue.

2.6. Histological examination of ovaries

Both ovaries from female offspring (F2) were fixated for 24 hours in 10% neutral buffered formalin. After fixation, samples were transferred to 70% ethanol and embedded in paraffin. Sections (8 µm thick) were made using a microtome, every tenth section was mounted onto glass slides and stained with HE. Histological evaluation was conducted using a light microscope on every second of the glass slides throughout one randomly chosen ovary. Between 9 and 12 females were randomly selected for evaluation from each dose and sampling time. One observer, blinded by treatment, counted the numbers of healthy follicles, including primordial follicles, primary follicles, preantral follicles and antral follicles. Categorization was conducted as previously described by Flaws *et al.* (2001). Primordial follicles were defined as having a single layer of squamous granulosa cells surrounding an oocyte, while primary follicles consisted of an oocyte surrounded by one layer of cuboidal granulosa cells. Preantral follicles were defined as being surrounded by at least two layers of cuboidal granulosa cells and theca cells, and antral follicles consisted of a fluid-filled antral space adjacent to the oocyte and surrounded by many layers of theca cells and cuboidal granulosa cells. The preantral and antral follicles were only registered if the oocyte showed nuclear material. Primordial and primary follicles were counted despite showing a nucleus.

2.7. Histopathological examination of liver

Cross sections of the left lobe from dams (F1), and female and male offspring (F2) were embedded in paraffin after fixation. Sections were made (3 μm thick) using a microtome, transferred to microscope glass slides and stained with haematoxylin and eosin (HE). Examinations were conducted by a board-certified pathologist under light microscope blinded by dose. Classification was based on the liver nomenclature guidelines recommended by the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) (Thoolen *et al.* 2010). Pre-neoplastic and neoplastic lesions were noted as present (1) or absent (0). Non-neoplastic lesions were graded on a severity scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change) and included bile duct hyperplasia, fatty change (diffuse), extramedullary hematopoiesis, inflammation (chronic active), hepatocyte fatty change (diffuse), hepatocyte centrilobular hypertrophy, Ito cell hypertrophy, and oval cell hyperplasia.

2.8. Liver microsomal preparations and cytochrome P450 activity

Liver samples from female offspring (F2) were shipped (on dry ice) to the Department of Molecular Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden, for cytochrome P450 (CYP) activity analysis. Approximately 1 g of liver were used to prepare hepatic microsomes by a calcium aggregation method described by Rasmussen *et al.* (2011). Protein concentrations in the microsomes were measured with a commercially available kit (Bio-Rad Laboratories Inc., Hercules, USA) according to the manufacturer's instructions and using bovine serum albumin as standard. Microsomes were diluted to a protein concentration of 4 mg/mL and stored at -80°C until use.

The activities of CYP1A and CYP2B10 were determined as a rate of 7-ethoxyresorufin (EROD, CYP1A1), 7-methoxyresorufin (MROD, CYP1A) and 7-pentoxyresorufin (PROD, CYP2B10) O-dealkylation as described by Zamaratskaia *et al.* (2010). CYP3A11 activity were established as a rate of and benzyloxyresorufin (BROD) O-dealkylation as in Pilipenko *et al.* (2017). CYP2E1 and CYP2A5 activities were determined as a rate of p-nitrophenol (PNPH) and coumarin-7- hydroxylation (CoH), respectively (Zamaratskaia *et al.* 2009). These isoforms were selected because of their well-known role in xenobiotic metabolism. Experimental conditions for activity assays are reported in Supplementary section S4 and Table S2. Briefly, incubation mixtures were comprised of microsomal protein (0.2 mg for all enzymes except for PNPH with 0.5 mg), phosphate buffer (50 mM, pH 7.4) and the appropriate substrate (1 μM for EROD, 2

μM for MROD and BROD, 10 μM for PROD, and 200 μM for PNPH and CoH). Reactions were initiated by addition of 0.5 mM NADPH. The solutions were incubated in a water bath at 37°C for 5 (EROD), 7 (MROD and BROD), 15 (CoH), 20 (PROD), or 30 (PNPH) min. Reactions were terminated by adding 0.5 mL ice-cold methanol (40% TCA for PNPH) and the mixtures were centrifuged (7500 g at 4°C for 5 min). The amount of formed resorufin in the supernatants (for CYP1A, CYP2B10 and CYP3A11) was measured using HPLC with fluorescence detector (560 and 586 nm for excitation and emission wavelengths, respectively). The amount of formed p-nitrochatecol (for CYP2E1) was measured using HPLC with UV detector (345 nm). The amount of formed hydroxycoumarin (for CYP2A5) was measured using HPLC with fluorescence detector (338 and 458 nm for excitation and emission wavelengths, respectively). Formation of all metabolites were linear with microsomal protein concentrations and incubation times. All enzymatic activities were expressed as pmol of reaction product per mg protein and min.

2.10. Statistical analysis

Box and barplots were created using R Studio version 3.6.1 (R Development Core Team 2008) and the packages 'ggplot2' (Wickham 2016) and 'ggpubr' (Kassambara 2020). Statistical analyses were conducted in JMP Pro 13® (SAS, Cary, USA) and a p-value ≤ 0.05 was considered statistically significant.

The measurements of weights, the number of TEBs (at 3 and 6 weeks only) and the branching density were normally distributed and with satisfactory homogeneity in variance. Thus, these variables were analyzed using multivariate linear regression by standard least square personality, which generated least square mean values that were further analyzed using ANOVA. Body, liver and ovaries + uterus weights were analyzed within each sampling time and with dose and weight at weaning (for dam body weight), number of offspring per litter (for offspring body weight) or body weight (for liver and ovaries + uterus weight) as explanatory variables. The number of TEBs (at 3 and 6 weeks only) and branching density were analyzed within each sampling time with dose and body weight as explanatory variables. P-values were generated by performing Dunnett's test on least square mean values.

Contingency table analysis using Chi-square likelihood ratio was conducted on the ratio between males and females, and the severity scores of hepatic histopathology within each sampling time and using the Steel-Dwass method to compare doses. The number of live fetuses and offspring

and the number of ovarian follicles at different stages of development were analyzed for differences between doses within each sampling time by ANOVA followed by Dunnett's or Tukey HSD test, respectively. The MG development scoring, the number of TEBs (at 9 weeks), N, MEA, mammary epithelial length and glandular area, as well as the hepatic enzyme activities, were not normally distributed and analyzed for differences between doses within each sampling time by non-parametric comparison using the Steel or Steel-Dwass methods.

3. Results

3.1. Hepatic internal dosimetry

The concentrations of PCBs, OCPs, BFRs and PFASs were measured in one pooled sample of liver tissue from each dose in dams (F1) and female offspring (F2). Results are presented in Table 2 as ∑6PCBs, *p,p'*-DDE, ∑7OCPs, ∑BDE-28-183, ∑BDE-206-209, HBCD and ∑PFASs. Individual concentrations are presented in Supplementary Tables S3 (ng/g lipid) and S4 (ng/g wet weight). LOD, recovery (%) and fat (%) are presented in Supplementary Tables S1 and S4.

Table 2. Concentrations of ∑6PCBs, *p,p′*-DDE, ∑7OCPs, PBDEs, HBCD and ∑PFASs in pooled liver samples from pregnant (gestation day 17) and post-pregnant (21 days post-partum) dams and female offspring (3, 6 and 9 weeks of age). Dams were dietary exposed to the mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Female offspring were exposed *in utero* and through lactation (ending at 3 weeks). Values are presented as ng/g lipid weight for PCBs, *p,p'*-DDE, OCPs, PBDEs and HBCD, and ng/g wet weight for PFASs.

	Σ6PCBs	Σ7OCPs	*p,p′*-DDE	ΣBDE-28-183	ΣBDE-206-209	HBCD	ΣPFASs
Pregnant dams							
Control	87.1	9.4	<LOD	0.7	0.4	<LOD	2.8
Low	45691.6	2185.9	907.0	373.2	2260.7	<LOD	645.3
High	87012.6	32585.7	13883.9	6923.4	35303.5	<LOD	16183.3
Post-pregnant dams							
Control	21.4	4.2	<LOD	0.7	7.9	<LOD	6.5
Low	7787.9	802.6	922.2	345.5	4534.1	<LOD	1248.5
High	52743.1	15343.4	14189.0	8197.3	35411.8	40.2	22744.6
3 weeks offspring							
Control	48.8	12.3	<LOD	0.7	10.2	<LOD	32.2
Low	19629.9	1779.2	1200.9	515.5	883.1	<LOD	403.7
High	89968.8	38043.8	24412.2	11397.7	16274.1	<LOD	8535.0
6 weeks offspring							
Control	65.3	35.0	<LOD	0.7	0.4	<LOD	5.5
Low	10144.7	502.5	153.6	79.2	30.6	<LOD	170.9
High	50364.8	11392.5	1287.5	1102.7	293.9	<LOD	3490.1
9 weeks offspring							
Control	81.0	38.1	<LOD	0.7	4.1	<LOD	6.3
Low	5318.4	357.0	10.2	38.5	22.7	27.6	142.3
High	30278.5	5068.8	283.7	397.5	165.6	<LOD	2521.8

Limit of detection (LOD), concentrations of individual chemicals and lipid (%) are presented in Supplementary Tables S1, S3 and S4. Σ6PCBs: PCB-52, PCB-101, PCB118, PCB-138, PCB-153 and PCB-180. Σ7OCPs: HCB, α-chlordane, oxychlordane, *trans*-nonachlor, α-HCH, β-HCH and γ-HCH. ΣBDE-28-183: BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-183. ΣPFASs: PFHxS, PFOS, PFOA, PFNA, PFDA and PFUnDA. Abbreviations: PCB (polychlorinated biphenyl); DDE (dichlorodiphenyldichloroethylene); OCP (organochlorine pesticide); PBDE (polybrominated diphenyl ether); HBCD (hexabromocyclododecane); PFAS (perfluoroalkylated substance).

Multiple POPs were detected in dams and offspring exposed to the Control dose. However, all compounds had lower concentrations compared to Low (6-5652x). Furthermore, both dams and offspring had higher concentrations of all compounds in High compared Low (2-25x). PCB-52 was not detected in any samples. BDE-183 and HBCD were only detected in one (post-pregnant

dam, High dose) and two (post-pregnant dam, High dose, and 9 weeks offspring, Low dose) samples, respectively. Debromination of BDE-209 was seen in both dams and offspring.

Σ6PCBs and Σ7OCPs were slightly higher (1.6-6x) in pregnant dams compared to post-pregnant dams. On the other hand, ΣBDE-28-183, ΣBDE-206-209 and ΣPFASs were similar or 2x lower in pregnant dams compared to post-pregnant dams. All compounds (except PCB-52, BDE-183 and HBCD) were detected in 3-week-old offspring from the Low and High doses. Σ6PCBs, Σ7OCPs and ΣBDE-28-183 were 1.3-2.5x higher, while ΣBDE-206-209 and ΣPFASs were 2.2-5.1x lower, in 3-week-old offspring compared to post-pregnant dams. After end of perinatal exposure, all compounds (except BDE-153 and HBCD) had decreasing concentrations with increasing age of offspring.

3.2. Biometrical measurements

The body and liver weights of dams (F1) and offspring (F2) are presented in Table 3. The POPs did not affect the body weight of dams (dietary exposed) or female offspring (perinatally exposed). On the other hand, male offspring perinatally exposed to the Low dose had significantly higher body weights compared to Control at 9 weeks ($p = 0.02$).

Table 2. Body and liver weight of pregnant and post-pregnant dams, and female (3, 6 and 9 weeks of age) and male (9 and 30 weeks of age) offspring. Dams were dietary exposed to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Offspring were exposed *in utero* and through lactation (ending at 3 weeks). Pregnant dams were euthanized on gestation day 17 (n = 12, 16 and 8 for Control, Low and High, respectively). Post-pregnant dams were euthanized 21 days post-partum (n = 14, 10 and n = 11 for Control, Low and High, respectively). Females 3 weeks: n = 12 (all groups). Females 6 and 9 weeks: n = 14 (all groups, except for ovary + uterus weight from 6 weeks Control where n = 13). Males 9 and 30 weeks: n = 15 (all groups, except for liver weight from 9 weeks Low where n = 14). Results are presented as least square mean ± standard error. Bold marks significant differences ($p \leq 0.05$) from Control.

	Body weight (g)	Liver weight (g)
Pregnant dams		
Control	47.40 ± 1.41	2.27 ± 0.06
Low	50.14 ± 1.22	2.36 ± 0.06
High	46.87 ± 1.82	**2.98 ± 0.08**
Post-pregnant dams		
Control	38.43 ± 0.98	2.61 ± 0.07
Low	38.73 ± 1.13	2.75 ± 0.09
High	36.89 ± 0.97	**3.34 ± 0.08**
3 weeks offspring females		
Control	11.82 ± 0.27	0.78 ± 0.04
Low	11.87 ± 0.28	0.83 ± 0.04
High	11.43 ± 0.27	0.87 ± 0.04
6 weeks offspring females		
Control	24.78 ± 0.34	1.26 ± 0.02
Low	24.34 ± 0.34	1.25 ± 0.02
High	24.17 ± 0.35	**1.35 ± 0.02**
9 weeks offspring females		
Control	27.86 ± 0.80	1.34 ± 0.03
Low	27.70 ± 0.88	1.37 ± 0.03
High	27.39 ± 0.82	1.43 ± 0.03
9 weeks offspring males		
Control	37.30 ± 1.06	1.98 ± 0.04
Low	**41.17 ± 1.00**	1.97 ± 0.04
High	40.58 ± 1.02	2.03 ± 0.04
30 weeks offspring males		
Control	48.29 ± 1.29	2.14 ± 0.05
Low	48.67 ± 1.69	2.24 ± 0.05
High	–	–

The High dose caused a significant increase in liver weights of pregnant and post-pregnant dams ($p < 0.01$ and $p = 0.05$, respectively), and in 6-week-old female offspring ($p = 0.02$), compared to Control. No changes in liver weights were seen in 3, 9 and 30-week old offspring.

The number of live offspring and the ratio between male and female offspring were not significantly different from Control (Supplementary Table S5). However, the Low dose led to the production of more males while the High dose produced more females ($p = 0.02$). The collective uterus and ovary weights from 6 and 9-week-old female offspring were not significantly different from Control (Supplementary Table S6).

3.3. Mammary gland histology

The effect of perinatal exposure to the mixture of POPs on MG development was investigated in female offspring (F2). Complete results are presented in Supplementary Table S7.

The development score (of whole mounts) was not affected by exposure. However, a trend towards less developed mammary glands was evident in the High dose at 3 weeks ($p = 0.08$). This trend was supported by a significantly decreased number of TEBs (High dose), compared to Control, at 3 weeks ($p = 0.04$). In addition, less TEBs were seen at 9 weeks in the Low dose, but this decrease was not significant from Control ($p = 0.07$). The number of TEBs was not affected by exposure at 6 weeks.

A visual difference in MG morphology between exposed and control mice was apparent, especially for the High dose. At 6 weeks, the High dose had a significantly higher branching density compared to Control. An increased branching density was also apparent at 9 weeks, but not significantly different from Control ($p = 0.1$). The total number of epithelial intersections was not affected by perinatal POP exposure. On the other hand, the mammary epithelial area was reduced by the Low dose, compared to Control, at 6 and 9 weeks ($p = 0.05$ and $p = 0.01$, respectively). The epithelial length was also reduced by the Low dose at 9 weeks ($p = 0.02$). MG whole mounts representative of each dose and age are shown in Figure 1.

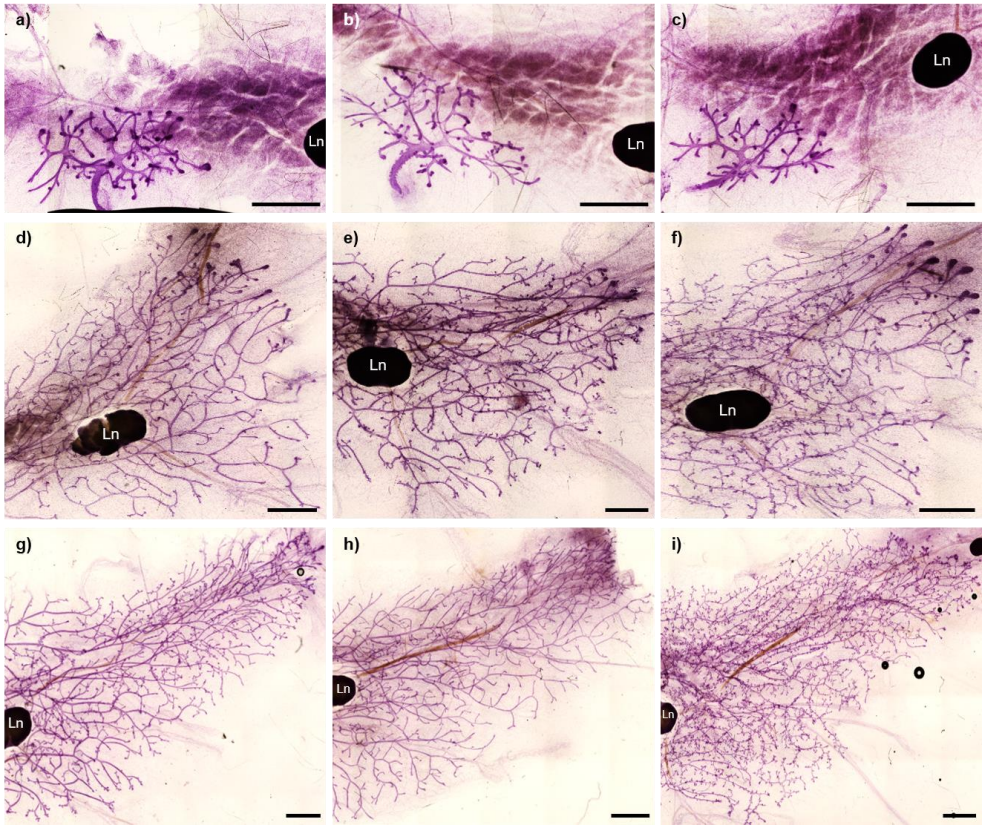

Figure 1. Whole mounts of mammary glands from 3 (a, b and c), 6 (d, e and f) and 9-week-old (g, h and i) female offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Pictures illustrate the morphology representative of each dose; Control (a, d and g), Low (b, e and h) or High (c, f and i). Each whole mount is composed of multiple images taken at 10-fold magnification and put together as a mosaic using ImageJ software. Scale bar = 2000 µm. Ln = Lymph node.

Histological examination of MGs did not reveal any significant effects of perinatal POP exposure. However, a trend towards more glandular tissue occupying the fat pad was seen by the Low dose at 6 weeks ($p = 0.08$).

3.4. Ovarian histology

Alterations in ovarian follicles at different stages in folliculogenesis were investigated in female offspring (F2) after perinatal exposure to the mixture of POPs. Results are presented in Figure 2.

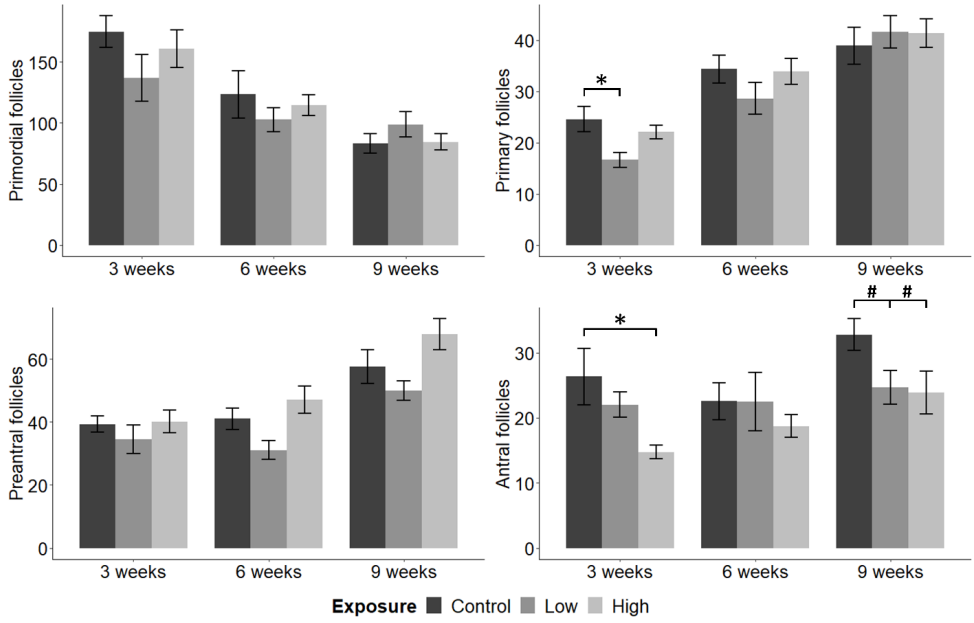

Figure 2. The number of primordial, primary, preantral and antral follicles in ovaries of 3, 6 and 9-week-old female offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Histopathological examination was conducted on every 10th ovarian section. Results are presented as mean ± standard error. 3 weeks: n = 9, 11 and 12 for Control, Low and High, respectively. 6 weeks: n = 11, 10, and 11 for Control, Low and High, respectively. 9 weeks: n = 10, 11 and 9 for Control, Low and High, respectively. *p-values < 0.05. $^{\#}$p-values < 0.1.

The number of primordial follicles was not affected by exposure. The Low dose significantly reduced the number of primary follicles at 3 weeks, compared to Control ($p < 0.01$), but not at 6 and 9 weeks. The number of preantral follicles did not differ in exposed mice compared to controls. However, the Low dose caused the formation of significantly less preantral follicles than the High dose at 6 ($p = 0.01$) and 9 weeks ($p < 0.01$), with the Control showing an

458 intermediate follicle number. The High dose reduced the number of antral follicles at 3 weeks ($p < 0.01$), but not at 6 weeks, compared to Control. At 9 weeks, the number of antral follicles were lower than but not significantly different from Control (with $p = 0.06$ and $p = 0.07$ for High and Low, respectively).

462 *3.5. Hepatic histology*

463 Histopathological examination was conducted on livers from dams (F1) and offspring (F2) dietary or perinatally exposed to the mixture of POPs. No pre-neoplastic or neoplastic lesions were noted. Focal fatty lesions were seen in 5 9-week-old female offspring perinatally exposed to the High dose but did not differ significantly from Control.

467 Dietary exposure to the Low and High doses increased the severity of hepatocellular hypertrophy (of the centrilobular region) in pregnant and post-pregnant dams compared to Control ($p < 0.01$). Perinatal exposure to the High dose caused hypertrophy (with increased severity) in female offspring at 3 weeks ($p = 0.02$). At 6 and 9 weeks, both the Low and High doses caused hepatocellular hypertrophy with increased severity from Control ($p < 0.01$). Hepatocellular hypertrophy was also seen in 9-week-old male offspring with a higher severity than Control in both the Low and High doses ($p < 0.01$). This hypertrophy persisted until 30 weeks in male offspring, which had a significantly higher severity compared to Control (Low dose only, $p < 0.01$). Figure 3 illustrates the hepatocellular hypertrophy representative of each dose in female offspring at 9 weeks.

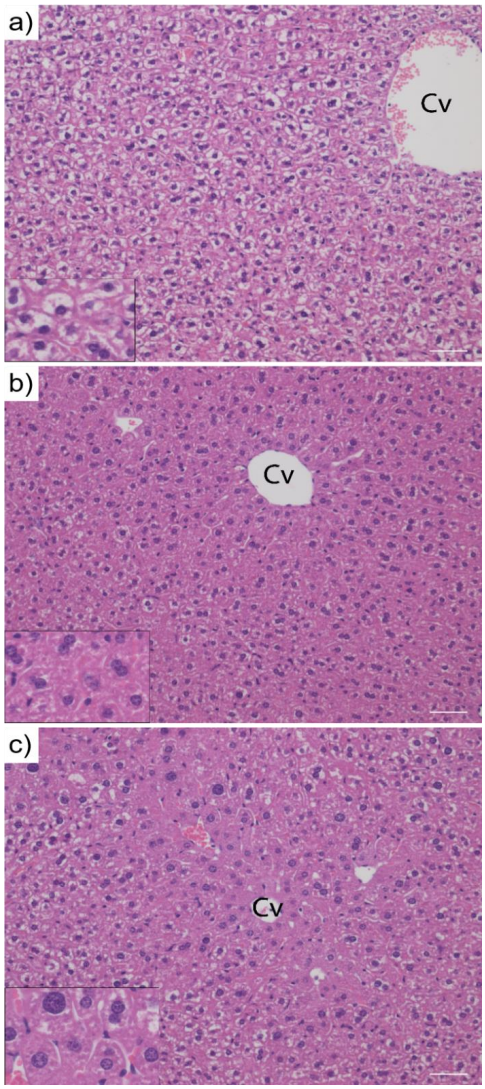

Figure 3. Representative hepatic morphology of 9-week-old female offspring maternally exposed to a mixture of POPs at Control (a), Low (b) or High (c) doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Hepatocytes have increased size and frequently enlarged hyperchromatic nuclei, which were graded on a scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change). The Low (b) and High (c) doses exhibited mild and severe hypertrophy, respectively, compared to the normal morphology of the Control (a). Magnification at 200-fold (inset at 400-fold). Scale bar = 50 µm. Cv = Central vein.

Extramedullary hematopoiesis was seen in female offspring at 3, 6 and 9 weeks with decreasing incidence and severity with increasing age and was not affected by perinatal exposure. Mean severity scores of the hepatocellular hypertrophy and hematopoiesis (± standard error) in dams and offspring are presented in Supplementary Table S8. No other non-neoplastic lesions were seen.

3.6. Hepatic cytochrome P450 activity

The activity of hepatic CYP enzymes were measured in female offspring (F2) perinatally exposed to the mixture of POPs (Figure 4).

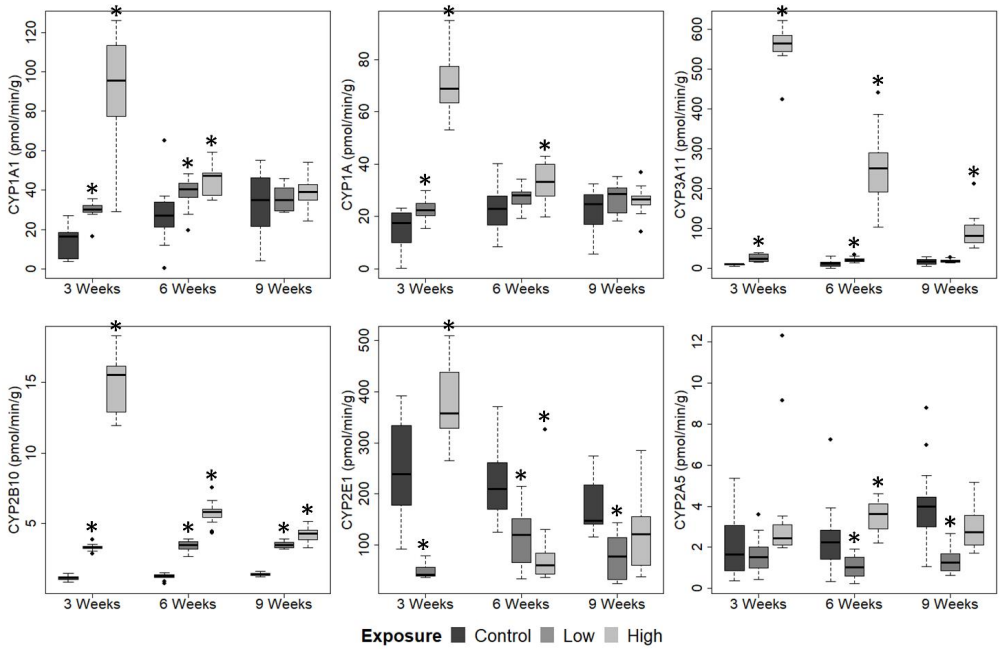

Figure 4. Hepatic enzyme activity of cytochrome P450 (CYP) 1A1, 1A, 3A11, 2B10, 2E1 and 2A5 in female offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Samples were taken at 3 (n = 12), 6 (n = 14) and 9 (n = 14) weeks of age. Activities were measured as a rate of 7-ethoxyresorufin (CYP1A1), 7-methoxyresorufin (CYP1A), benzyloxyresorufin (CYP3A11), 7-pentoxyresorufin (CYP2B10) dealkylation, and p-nitrophenol (CYP2E1) and coumarin-7 (CYP2A5) hydroxylation. Boxplots consist of 25th, 50th (median) and 75th percentiles, whiskers (extending to 1.5 interquartile range) and outliers (•). Significant difference from Control ($p \leq 0.05$) is marked by *.

Perinatal exposure significantly induced the activity of all CYPs (except CYP2A5) at 3 weeks ($p < 0.01$ for all, except for CYP1A for the Low dose with $p = 0.05$), with the High dose inducing the largest change from Control for CYP1A1, CYP1A, CYP3A11 and CYP2B10. For CYP2E1, the activity was decreased by the Low dose, and increased by the High dose, compared to Control ($p < 0.01$).

At 6 weeks, the activities of all CYPs were affected by the High dose ($p \leq 0.01$, except for CYP2A5 with $p = 0.02$). Furthermore, the Low dose caused higher activities of CYP1A1 ($p = 0.02$), CYP3A11 ($p < 0.01$) and CYP2B10 ($p < 0.01$) compared to Control. The activity of CYP2E1 was reduced by both the Low and High doses ($p < 0.01$). The Low dose also reduced the activity of CYP2A5 ($p < 0.01$), while the High dose increased the CYP2A5 activity compared to Control ($p = 0.02$).

At 9 weeks, the CYP1A1 and CYP1A activities were no longer affected by perinatal exposure. However, the High dose still increased the activity of CYP3A11 and CYP2B10 compared to Control ($p < 0.01$). In addition, the Low dose elevated the activity of CYP2B10 ($p < 0.01$). On the other hand, the Low dose reduced the activities of CYP2E1 and CYP2A5 compared to Control ($p < 0.01$).

4. Discussion

The present study demonstrated that *in utero* and lactational exposure to the mixture of POPs altered MG development, ovarian follicle maturation and caused persistent hepatocellular hypertrophy and enzyme induction in CD-1 mice. In dams, the POPs were readily taken up from the diet and distributed to the hepatic tissue. PCBs and OCPs seemed to be more efficiently transferred to offspring during late gestation and lactation compared to BFRs and PFASs. However, all compounds (except PCB-52, BDE-183 and HBCD) were detected in weaning offspring, thus, confirming maternal transfer of the mixture. After the end of lactational exposure, the POPs decreased in concentration over time suggesting dilution due to weight gain, metabolization and excretion.

Previously, Johanson *et al.* (2020) showed that perinatal exposure to the Low dose resulted in 2-35x higher POP concentrations in A/J Min/+ mice (liver and adipose tissue) than the average blood levels reported in the Scandinavian population (Berntsen *et al.* 2017). In the present study,

Low dose female offspring at 9 weeks had comparable (from 2x lower to 5x higher) lipid-adjusted concentrations to those found by Johanson *et al.* (2020) at 20 weeks. This may suggest a slightly lower rate of metabolism and excretion in A/J Min/+ compared to CD-1 mice. However, it demonstrates that the Low dose could be considered relevant to humans.

Results from the traditional qualitative scoring of MG development in female offspring showed a trend towards reduced development in 3-weeks-old mice perinatally exposed to the High dose. In addition, the number of TEBs were significantly lower in these mice. As previously described by Rudel *et al.* (2011), MG development can be regarded as accelerated if the number of TEBs in the treated group is higher than that of the control at 3 weeks of age. Exposure to HCB has previously been shown to increase the number of TEBs in C57BL/6 mice (Miret *et al.* 2017), which could be a possible contributor to increasing the risk of breast cancer as TEBs are highly proliferative structures (Macon and Fenton 2013, Osborne *et al.* 2015, Miret *et al.* 2019). However, the opposite was observed in the present study and, thus, perinatal exposure to the High dose of POPs seemed to restrict MG development in 3-week-old female mice.

In addition, Miret *et al.* (2017) observed enhanced MG branching density after intraperitoneal injection of 3 mg/kg HCB in C57BL/6 mice. This is in accordance with the increased branching density (by the High dose) observed in the present study. As the number of epithelial ducts is coincident with the number of TEBs in a MG gland, and a restricted development leads to fewer TEBs in weaning mice, but a higher number of TEBs in pubertal mice (Rudel *et al.* 2011), the increased branching density indicated that perinatal exposure to the mixture of POPs (High dose) continued to restrict MG development also at 6 and 9 weeks of age. Furthermore, the perinatal exposure to the Low dose reduced (non-significantly) the amount of area occupied by gland epithelium in both 6 and 9-week-old mice, resulting in a higher density of glandular tissue. This could suggest that the enhanced branching observed at 6 weeks resulted in a premature arrest of gland growth and caused the fully developed MGs to cover less area than the unexposed glands.

Restricted MG development has previously been shown following *in utero* exposure to PFOA at concentrations as low as 0.01 mg/kg in CD-1 and C57BL/6 mice (White *et al.* 2009, Macon *et al.* 2011, White *et al.* 2011, Tucker *et al.* 2015). Interestingly, the delayed gland development found by Macon *et al.* (2011) was evident at lower doses of PFOA than those required to increase the liver weight and persisted for a relatively long time after end of exposure. *In utero* and

lactational exposure to DE-71 (a mixture of PBDEs) also delayed MG development in 3-week-old female Long-Evans rats (Kodavanti *et al.* 2010). Furthermore, *in utero* exposures to other EDCs, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, bisphenol A and atrazine metabolites, have been shown to restrict gland development in rats and mice (Markey *et al.* 2001, Fenton *et al.* 2002, Enoch *et al.* 2007). However, as emphasized by Yang *et al.* (2009), caution should be taken when drawing conclusions from studies using only a single mouse strain as differences have been observed in the sensitivity of strains to PFOA-induced alterations in MG development (Yang *et al.* 2009, Tucker *et al.* 2015).

Overall, perinatal exposure to the mixture of POPs affected ovarian folliculogenesis in CD-1 mice. The High dose reduced the number of antral follicles significantly in weaning and non-significantly in adult offspring. This reduction suggests a delayed rate of follicle maturation or an increased rate of follicle atresia. Baldridge *et al.* (2003) showed that *in utero* exposure to Aroclor 1016 (a mixture of low-chlorinated PCBs) decreased the number of preantral and antral follicles in weaning Long-Evan rats. The authors argued that the modulation of follicle maturation was caused by PCB-induced alterations in circulating thyroid hormone levels, as the effect could be (partly) avoided by supplementation of levothyroxine sodium (Baldridge *et al.* 2003). Maternal exposure to PCB-126 has also been shown to reduce the number of antral follicles in Sprague-Dawley rats (Muto *et al.* 2003, Shirota *et al.* 2006). Furthermore, Talsness *et al.* (2008) showed reduced follicle maturation as a result of disruption along the hypothalamic-pituitary-ovarian axis caused by *in utero* exposure to environmentally relevant doses of BDE-47 in Wistar rats. The later stages of follicle maturation are completely dependent on gonadotropins (e.g. luteinizing hormone and follicle-stimulating hormone) secreted by the anterior pituitary in response to stimulation by gonadotropin releasing hormone from the hypothalamus (McGee and Hsueh 2000). As many POPs have endocrine disruptive properties, it is likely that developmental exposure may disturb the finely regulated fluctuations in gonadotropins (and other hormones) and, thus, modulate follicle maturation (Uzumcu and Zachow 2007). However, alterations in hormone levels were not investigated in the present study.

Moreover, the number of primary follicles was reduced by the Low dose in weaning offspring. Previously, Lilienthal *et al.* (2006) and Ema *et al.* (2008) showed reduced numbers of primordial and primary follicles following maternal exposure to BDE-99 and HBCD in rats. The number of

primordial follicles (the follicle pool) is fixed at the time of birth and a reduction in the follicle pool may cause premature ovarian insufficiency and premature menopause (Vabre *et al.* 2017). Various studies have associated exposure to OCPs, PCBs and PFASs with earlier onset of menopause in humans (Cooper *et al.* 2002, Akkina *et al.* 2004, Knox *et al.* 2011, Taylor *et al.* 2014, Grindler *et al.* 2015). Thus, exposure to POPs during early development may alter ovarian folliculogenesis and could possibly lead to reduced female fertility. Unfortunately, no measures to promote estrous cycle homogeneity in offspring were taken. Consequently, the pubertal and adult offspring might have been terminated at different stages of estrus cycle, which could have influenced the results on ovarian follicle numbers.

Dietary exposure to the High dose increased the relative liver weight in dams. However, no effect was seen on the body weight. The enhanced liver weight could have been caused by the hepatocellular hypertrophy observed in the same dams. In addition, hypertrophy of liver cells was seen in dams given the Low dose implying that hypertrophy is a relatively sensitive endpoint to POP exposure. Hepatocellular hypertrophy caused by dietary or oral exposure to various POPs has been shown in multiple other studies (Wade *et al.* 2002, Seacat *et al.* 2003, National Toxicology Programme 2006, van der Ven *et al.* 2008, Wolf *et al.* 2008, Yang *et al.* 2009, Bondy *et al.* 2011, Roos *et al.* 2011, Butenhoff *et al.* 2012). Many of these and other (Zhou *et al.* 2002, Thibodeaux *et al.* 2003, Lau *et al.* 2006, Hansen *et al.* 2019) studies have also reported increased liver weights.

Hepatocellular hypertrophy has been evaluated as a predictor of liver cancer in mice (Allen *et al.* 2004, Hall *et al.* 2012). However, this histopathological feature should not be interpreted as the only precursor of cancer and often occurs together with hepatocellular hyperplasia and increased enzymatic activity (Allen *et al.* 2004, Maronpot *et al.* 2010). Hyperplasia was not detected in the present study. However, dose-dependent increases in hepatic CYP activities and hypertrophy were detected in female offspring following perinatal exposure to the mixture of POPs.

Increased activities of CYPs may lead to the formation of reactive oxygen species and cause hepatotoxicity (Bondy and Naderi 1994, Ioannides and Lewis 2004, Zangar *et al.* 2004). In addition, the enhanced activities of CYP1A, CYP3A and CYP2B10 are commonly used as biomarkers for AHR, PXR and CAR activation, respectively (Whitlock 1990, Waxman 1999, Xie *et al.* 2000, Maglich *et al.* 2002, Yamada *et al.* 2006). In the present study, CYP1A1 and

CYP1A, CYP3A11 and CYP2B10 were dose-dependently increased in weaning offspring, indirectly indicating AHR, PXR and CAR activation. Furthermore, the heightened activities were less prominent with time, which may have been caused by the temporal decline in POP concentrations.

Interestingly, the activities of CYP2E1 and CYP2A5 were (at some points) lower in perinatally exposed mice compared to controls, which might have been due to a reduced capacity for xenobiotic metabolism after POP exposure. Physiological consequences of this should further be studied, as low activity of these enzymes might predispose animals to harmful health effects under conditions where CYP2E1 and CYP2A5 are required for detoxification. CYP2E1 is responsible for the metabolism of xenobiotics of low molecular weight and is known to generate toxic products (Ioannides and Lewis 2004, Bieche *et al.* 2007, Porubsky *et al.* 2008). Thus, alterations in CYP2E1 activity may have contributed to the persistent hepatocellular hypertrophy observed and could lead to hepatotoxicity.

Alterations in hepatic CYP activities following maternal exposure to POPs, such as PBDEs (Zhou *et al.* 2002, Szabo *et al.* 2008, Tseng *et al.* 2008, Dunnick *et al.* 2018), γ-HCH (Johri *et al.* 2008) and compound mixtures (Chu *et al.* 2005, Chu *et al.* 2008, Bonfanti *et al.* 2014), have previously been shown in rodents. As reviewed by Maronpot *et al.* (2010), hepatic enzyme induction generally occurs together with hepatocellular hypertrophy (and hyperplasia), which further leads to liver enlargement as a physiological adaptation to the increased demand of metabolism and detoxification. Hypertrophy of the liver cells was detected, and dose-dependently increased in severity, in perinatally exposed female and male offspring. Furthermore, liver enlargement was seen in 6-week-old females. Other studies have also observed hepatocellular hypertrophy following *in utero* and lactational exposure to mixtures of POPs (Chu *et al.* 2005, Chu *et al.* 2008, Bondy *et al.* 2011, Dunnick *et al.* 2018) or single pollutants (Filgo *et al.* 2015, Quist *et al.* 2015, Dunnick *et al.* 2018).

Normally, resolution of hepatocellular effects occurs when the xenobiotics have been metabolized and excreted from the system (Maronpot *et al.* 2010). This is dependent on the ability of the compound to resist degradation and, consequently, more persistent compounds may cause hepatic effects long after end of exposure. The persistent nature of the POP mixture has recently been demonstrated by Johanson *et al.* (2020), which showed significantly higher (up to 136x)

levels of 20 of the 29 POPs in liver and adipose tissues in A/J Min/+ mice 17 weeks after end of perinatal exposure to the Low dose. Thus, the increased severity of hepatocellular hypertrophy observed in 30-week-old male offspring perinatally exposed to the Low dose of POPs in the present study may have been caused by POPs still present in the hepatic tissue.

Another interesting finding of the present study was that perinatal exposure to the Low dose increased the body weight of male but not female offspring at 9 weeks. Previously, maternal exposure to BDE-47 elevated the body weight of male but not female Sprague-Dawley rats (Gao *et al.* 2019) and stimulated the development of obesity in male ICR mice (Wang *et al.* 2018). Various POPs have also been associated with increased weight gain and obesity in humans (Karmaus *et al.* 2009, Lee *et al.* 2012, Valvi *et al.* 2012, Warner *et al.* 2018). Thus, the increased body weight of male mice observed in the present study could suggest an obesogenic effect of POPs when exposed to relatively low doses during early development.

Notably, extramedullary hematopoiesis was detected in all but one liver of 3-week-old female offspring. This is most probably a residue from the liver serving as the main hematopoietic organ during fetal development (Tavassoli 1991, Sonoda and Sasaki 2012), which was further supported by the decreasing severity scores with increasing age and the lack of significant effect by perinatal POP exposure.

In summary, the present study illustrated that perinatal exposure a human relevant mixture of POPs modulated female mammary gland development and ovarian folliculogenesis in CD-1 mice. Furthermore, the mixture caused hepatocellular hypertrophy, enzymatic induction and slight liver enlargement indicating modest hepatotoxic effects. Further studies are needed to investigate if the present mixture, or other human relevant mixtures of POPs, can affect female reproductive ability or cause mammary or hepatic carcinogenesis.

Acknowledgements

The present study was funded by the Norwegian Research Council (Funding number: NFR 21307/H10 and 204361/H10). CYP analysis was partly supported by the Swedish University of Agricultural Sciences, NJ Faculty. The authors would like to thank Kristine Eraker Aasland Hansen, Christer Wiggen Johnsen and Harry Hjelmseth for excellent technical assistance during the experiment.

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Supplementary material.

Perinatal exposure to a human relevant mixture of persistent organic pollutants: Effects on mammary gland development, ovarian folliculogenesis and liver in CD-1 mice.

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Table of Contents

Materials and Methods

S1. Feed design

Only HCB was detected in the control and 'reference' feed at concentrations of 2.24 and 8.04 ng/g, respectively. A deviation was detected between the nominal (estimated) and measured POP concentration in feed of ± 30% for most of the compounds. However, PCB-28 and the PFASs deviated with approximately 60-70% from nominal concentrations (see more information in Berntsen *et al.* (2017)). The High dose aimed to be 20 times higher than the Low dose, however, the ratio between the High and Low varied from 12 (for PCB-52) to 30 (for oxy-chlordane) times.

The Stockholm Convention on Persistent Organic Pollutants includes all PCBs, OCPs and BFRs in the mixture, in addition to PFOS and PFOA (Secretariat of the Stockholm Convention 2019). PFHxS is currently under consideration for inclusion, but the other PFASs (PFNA, PFDA and PFUnDA) have not yet been incorporated into the treaty, Nevertheless, all PFASs are known to be persistent in the environment and have the potential to bioaccumulate in animals and humans (Houde *et al.* 2011, Olsen 2015).

S2. Animals, experimental design and sample collection

The study timeline is presented in Figure S1. Dams (F1) were exposed to the mixture of POPs at Control, Low or High doses from 3 to 12 or 16 weeks of age (sampled during pregnancy or at weaning of offspring, respectively). Offspring (F2) were only exposed to the mixture *in utero*, during lactation or by nibbling on their mother's feed prior to weaning.

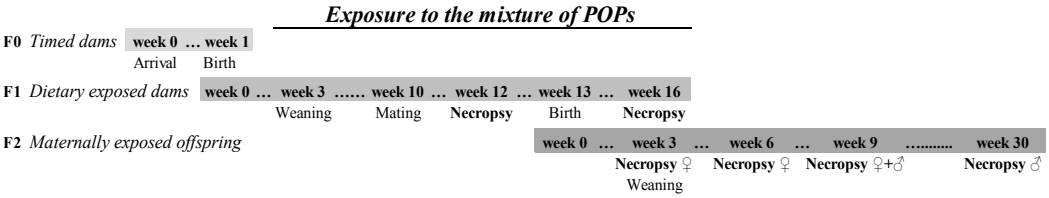

Figure S1. Study design of the experiment. Timed-pregnant CD-1 dams (F0) gave birth to F1 dams at approximately 1 week after arrival. F1 dams were exposed to the mixture of POPs through the diet at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively) from 3 weeks of age until termination during pregnancy (at gestation day 17) or post-pregnancy (at day 21 post-partum). F2 offspring were only exposed to the mixture *in utero*, through lactation and by nibbling on their mother's food prior to weaning, and were terminated at 3 (females), 6 (females), 9 (females and males) or 30 (males) weeks of age.

S3. Chemical analysis

S3.1. Chemicals

Additional chemicals used for the chemical analysis included methanol, cyclohexane and acetone of HPLC quality (VWR International S.A.S, Radnor, Pennsylvania) and purified water was obtained from a Milli-Q Gradient A10 water system (Millipore, Bedford, MA, USA). Certified Reference Materials (CRM 2525, 350) were supplied by Cerilliant Corporation, Round Rock, TX, USA. Interlaboratory tests (human serum) were provided by Arctic Monitoring and Assessment Program (AMAP), Québec, Canada. All chemicals are routinely quality tested according to the accreditation requirements.

S3.2. Analysis of PCBs, OCPs, BFRs and PFASs

The detection of PCBs and OCPs were performed on a DB-5 MS column (60 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific). The temperature program was: 90°C (2 min hold); 25°C/min increase to 180°C (2 min hold); 1.5°C/min increase to 220°C (2 min hold); and 3°C/min increase to 275°C (12 min hold) and 25°C/min increase to 300°C (4 min hold). The total run time was 71.6 min. Detection of BDE-28, -47, -99, -100, -153, -154 and -183, and HBCD were conducted on a DB-5 MS column (30 m, 0.25 mm i.d., 0.25 µm film thickness; J&W

Scientific). The temperature program was: 90°C (1 min hold); 25°C/min increase to 180°C; 2.5°C/min increase to 220°C (1 min hold); and 20°C/min increase to 320°C (10 min hold). The total run time was 36.60 min. For BDE-206, -207, -208 and -209, separation and detection were performed on a DB-5-MS column (10 m, 0.25 mm i.d., 0.10 μm film thickness; J&W Scientific, Agilent Technologies). The temperature program was: 80°C (2 min hold); 30°C/min increase to 315°C (6 min hold). The total run time was 15.83 min. The target ions for all analyzed PCBs, OCPs, BFRs and PFASs are given in Table S1.

Table S1. Overview of target ions (m/z, for PCBs and OCPs), precursor and product ions (m/z, for BFRs and PFASs), limit of detection (LOD; ng/g wet weight) and relative recovery (%) of PCBs, OCPs, BFRs and PFASs measured in pooled liver tissue from CD-1 mice. PCBs, OCPs and BFRs were analyzed by electron capture negative ionization (ECNI) in selected ion monitoring (SIM) mode. PFASs were analyzed in electrospray ionization (ESI) and negative ionization mode.

Compounds	Target ion	LOD	Recovery
Polychlorinated biphenyls (PCBs)			
PCB-52	291.9	0.973	87
PCB-101	325.9	0.088	99
PCB-118	325.9	0.011	96
PCB-138	359.9	0.021	97
PCB-153	359.9	0.007	90
PCB-180	395.8	0.008	102
Organochlorine pesticides (OCPs)			
p,p'-DDE	317.9	0.164	133
HCB	283.8	0.007	98
α-Chlordane	409.8	0.008	99
Oxychlordane	351.8	0.059	123
Trans-nonachlor	443.8	0.007	121
α-HCH	71	0.025	108
β-HCH	71	0.052	121
γ-HCH	71	0.020	116
Brominated flame retardants (BFRs)			
BDE-28	79/81	0.033	86
BDE-47	79/81	0.098	86
BDE-99	79/81	0.069	117
BDE-100	79/81	0.051	106
BDE-153	79/81	0.131	108
BDE-154	79/81	0.132	92
BDE-183	79/81	0.232	94
BDE-206	484/486	0.093	156
BDE-207	484/486	0.069	121
BDE-208	484/486	0.051	129
BDE-209	495/497	0.224	120
HBCD	79/81	1.182	155
Perfluoroalkylated substances (PFASs)			
PFHxS	398.9/80	0.220	97
PFOS	498.9/99	0.130	93
PFOA	413/369	0.240	105
PFNA	463/419	0.180	99
PFDA	513/469	0.150	100
PFUnDA	563/519	0.090	97

S3.3. QA/QC OCPs, PCBs, BFRs and PFASs

One non-spiked and two spiked samples of commercial cod oil, three blanks (solvents) and one harp seal blubber (*Pagophilus groendlandicus*) were included as internal reference material (IRM) for the analysis of OCPs, PCBs and BFRs. For analyses of PFASs, the analytical series included one non-spiked (blind) and two spiked salmon liver samples and three blanks (solvents).

The LOD was defined as three times the noise level for each compound. LOD and relative recovery are presented in Table S1. *p,p'*-DDE, BDE-206 and HBCD were corrected for high relative recovery (> 130%). Percentage fat used for adjusting the concentrations of the lipid-soluble PCBs, OCPs and BFRs are shown in Table S4. Positive procedural blanks were found for some compounds. Results were corrected for blanks, if the blank concentrations were consistent for all solvent samples. The analytical quality was approved by satisfactory quality control measures, and results were within the accepted ranges for the analyzed CRM (2525: Fish containing PCBs, OCPs, BFRs (Cerilliant Corporation, Round Rock, USA) and 350: Fish oil containing PCBs (Commission of the European Communities, Belgium)) and inter-laboratory tests (AMAP: human blood containing PCBs, OCPs, BFRs, PFASs (Québec, Canada)).

S4. Liver microsomal preparations and cytochrome P450 activity

Hepatic microsomes were prepared as described in Rasmussen *et al.* (2011). In brief, liver was homogenized in Tris-sucrose buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4) at a 1:2 ratio (weight of sample:volume of buffer) and centrifuged for 10 min (at 10 000 g and 4°C using an Eppendorf Centrifuge 5417R). The supernatant was extracted and diluted with TRIS-sucrose buffer, containing 8 mM $CaCl_2$, to a final volume of 25 mL. Samples were centrifuged (30 min at 25 000 g and 4°C, Beckman Coulter OptimaTML-80 XP Ultracentrifuge) after 4 min incubation on ice.

Experimental conditions for the activity assay are reported in Table S2.

Table S2. Experimental conditions for cytochrome P450 (CYP) enzyme activity assays.

CYP isoform	Reaction	Substrate	Substrate final concentration (μM)	Microsomal protein (mg)	NADPH (mM)	Incubation time (min)	Terminating reagent
CYP1A	EROD	7-ethoxyresorufin	1	0.2	0.5	5	100 % Methanol
CYP1A	MROD	7-methoxyresorufin	2	0.2	0.5	7	100 % Methanol
CYP3A11	BROD	7-benzyloxyresorufin	2	0.2	0.5	7	100 % Methanol
CYP2B10	PROD	7-pentoxyresorufin	10	0.2	0.5	20	100 % Methanol
CYP2A5	CoH	coumarin	200	0.2	0.5	15	100% Methanol
CYP2E1	PNPH	p-nitrophenol	200	0.5	0.5	30	40% TCA

Abbreviations: EROD = 7-ethoxyresorufin O-deethylase; MROD = 7-methoxyresorufin O-demethylase; BROD = 7-benzyloxyresorufin O-dealkylation; PROD = 7-pentoxyresorufin O-depenthylase; CoH = coumarin hydroxylase; PNPH = p-nitrophenol hydroxylase.

HPLC analyses of CYP enzyme activities were performed on a system comprising a pump (L-7100), autosampler (L-7200), fluorescence (L-7485) or UV-Vis (L-4250) detector, and D-7000 HPLC Manager software (Merck-Hitachi, Tokyo, Japan). A Hypersil ODS (3 μm, 60 x 4.6 mm), with a guard column, was used for quantification of resorufin and coumarin. For p-nitrocatechol, a Lichrosphere RP-18 column (5 μm, 250 x 4 mm) with a guard column, was used.

Results

S5. Hepatic internal dosimetry

The concentrations of individual PCBs, OCPs, BFRs and PFASs in pooled liver samples from dams (F1) and female offspring (F2) are presented in Tables S3 and S4 (lipid adjusted and wet weight, respectively). PCB-28 and dieldrin, however present in the POP mixture, were not measured. Thus, Σ7PCBs is noted as Σ6PCBs in Table 2 and Supplementary Tables S3 and S4.

Similar to the measured POP concentrations in feed, variation in the ratio between High and Low doses were also seen in pooled liver samples from dietary exposed dams (with 1-14x and 4-23x for PCBs, 5-19x and 4-26x for OCPs, 15-26x and 8-24x for BFRs, and 22-28x and 17-21x for PFASs in the pregnant and post-pregnant dams, respectively). In maternally exposed offspring at 3, 6 and 9 weeks, all compounds (except γ-HCH in High, and PCB-52, BDE-183 and HBCD in

both doses) were found at higher concentrations in High compared to Low (with 3-25x, 6-30x, 12-52x and 18-23x higher for PCBs, OCPs, BFRs and PFASs at 3 weeks, respectively). Furthermore, the ratio of PFAS concentrations between High and Low were closer to 20x than for the other POPs (with 19-23x and 17-19x at 6 and 9 weeks, respectively).

Table S3. Concentrations of individual PCBs, OCPs, BFRs and PFASs in pooled liver samples of dietary exposed pregnant (gestation day 17) and post-pregnant (21 days post-partum) dams and maternally exposed female offspring (3, 6 and 9 weeks of age). Mice were exposed to the mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Values are presented as ng/g lipid weight for PCBs, OCPs and BFRs, and ng/g wet weight for PFASs.

	Pregnant dams			*Post-pregnant dams*			*3 weeks offspring*			*6 weeks offspring*			*9 weeks offspring*		
Compounds	Control	Low	High	Control	Low	High	Control	Low	High	Control	Low	High	Control	Low	High
Polychlorinated biphenyls (PCBs)															
PCB-52	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB-101	<LOD	158.2	1725.0	<LOD	124.7	1385.7	<LOD	120.1	1050.5	<LOD	5.7	36.5	<LOD	<LOD	<LOD
PCB-118	1.3	908.9	13030.8	0.9	358.8	7760.9	2.0	663.7	16437.0	1.7	140.2	5450.7	1.8	39.2	2549.8
PCB-138	58.2	35563.2	39569.4	11.6	5104.1	19050.3	31.9	14406.1	36625.9	44.9	7806.7	26077.2	58.6	3912.3	16795.2
PCB-153	8.2	2115.1	22615.9	1.7	682.1	15967.5	5.6	1671.7	27009.3	5.7	656.9	13241.5	6.4	493.4	7559.4
PCB-180	18.3	6945.2	10070.5	6.1	1517.2	8577.7	8.2	2767.3	8845.1	12.0	1534.2	5557.9	13.1	872.4	3373.0
Σ6PCBs	87.1	45691.6	87012.6	21.4	7787.9	52743.1	48.8	19629.9	89968.8	65.3	10144.7	50364.8	81.0	5318.4	30278.5
Organochlorine pesticides (OCPs)															
p,p'-DDE	<LOD	907.0	13883.9	<LOD	922.2	14189.0	<LOD	1200.9	24412.2	<LOD	153.6	1287.5	<LOD	10.2	283.7
HCB	8.5	901.4	14477.2	3.6	281.4	6439.8	8.7	757.5	16181.9	25.4	201.2	4452.5	28.8	132.6	2131.6
α-Chlordane	<LOD	27.7	133.5	<LOD	20.0	71.6	0.2	2.2	14.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Oxychlordane	<LOD	397.7	5894.3	<LOD	96.9	2216.9	<LOD	314.3	7627.2	1.8	108.9	3084.8	2.1	102.7	1563.2
Trans-Nonachlor	0.7	283.6	5414.6	0.4	115.5	3045.6	0.9	238.4	7225.5	0.8	72.4	2185.7	0.7	62.8	924.9
α-HCH	<LOD	76.4	1014.6	<LOD	62.4	873.6	0.9	61.4	854.0	0.8	1.9	11.7	0.8	0.9	3.1
β-HCH	<LOD	491.8	5593.5	<LOD	218.7	2652.1	1.5	404.3	6141.1	6.2	118.1	1657.8	5.7	58.0	446.0
γ-HCH	<LOD	7.3	58.0	<LOD	7.7	43.8	<LOD	1.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Σ7OCPs	9.4	2186.4	32585.7	4.2	802.6	15343.4	12.3	1779.2	38043.8	35.0	502.5	11392.5	38.1	357.0	5068.9
Brominated flame retardants (BFRs)															
BDE-28	<LOD	<LOD	29.6	<LOD	1.1	32.7	<LOD	1.1	54.9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
BDE-47	<LOD	156.7	3199.5	<LOD	189.8	4280.9	<LOD	284.7	7492.3	<LOD	24.5	148.2	<LOD	<LOD	30.1
BDE-99	<LOD	84.5	1465.2	<LOD	83.3	1464.7	<LOD	98.1	1475.2	<LOD	19.6	259.0	<LOD	8.3	78.8
BDE-100	<LOD	79.5	1189.1	<LOD	<LOD	1046.4	<LOD	79.3	1514.4	<LOD	20.0	348.8	<LOD	14.6	122.2
BDE-153	<LOD	23.6	610.9	<LOD	35.3	841.3	<LOD	29.8	585.1	<LOD	10.0	270.6	<LOD	10.8	145.1
BDE-154	<LOD	28.6	428.9	<LOD	35.7	509.4	<LOD	22.3	275.6	<LOD	4.8	75.8	<LOD	4.4	21.0
BDE-183	<LOD	<LOD	<LOD	<LOD	<LOD	21.9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
ΣBDE-28-183	0.7	373.2	6923.4	0.7	345.5	8197.3	0.7	515.5	11397.7	0.7	79.2	1102.7	0.7	38.5	397.5
BDE-206	<LOD	12.5	189.1	<LOD	12.6	86.2	<LOD	5.7	95.6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
BDE-207	<LOD	208.6	2593.0	<LOD	103.4	1379.8	<LOD	99.0	2129.8	<LOD	8.7	131.3	<LOD	5.3	75.6
BDE-208	<LOD	29.1	220.7	<LOD	8.7	86.2	<LOD	5.6	154.8	<LOD	<LOD	6.9	<LOD	<LOD	10.0
BDE-209	<LOD	2010.5	32300.7	7.7	4409.4	33858.7	10.0	772.9	13893.9	<LOD	21.8	155.6	3.9	17.3	79.9
ΣBDE-206-209	0.4	2260.7	35303.5	7.9	4534.1	35411.8	10.2	883.1	16274.1	0.4	30.6	293.9	4.1	22.7	165.6
HBCD	<LOD	<LOD	<LOD	<LOD	<LOD	40.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	27.6	<LOD
Perfluoroalkylated substances (PFASs)															
PFHxS	0.3	13.1	359.7	0.4	22.9	485.3	0.9	7.1	160.6	<LOD	2.9	54.0	<LOD	1.9	35.4
PFOS	0.5	106.9	2968.3	2.3	332.0	6483.3	7.9	63.3	1477.8	1.2	37.7	718.1	1.8	28.9	540.9
PFOA	0.7	151.9	3751.1	1.3	259.6	4400.8	6.0	109.0	2532.0	1.4	30.3	697.8	1.3	22.3	375.5
PFNA	0.4	118.0	3294.1	0.9	198.6	4007.9	5.6	80.1	1738.9	0.9	37.5	760.6	1.1	31.8	614.8
PFDA	0.4	160.1	3570.1	1.0	275.7	4675.8	7.3	100.5	1817.7	1.2	43.2	860.0	1.3	40.9	647.9
PFUnDA	0.5	95.3	2239.8	0.6	159.8	2691.6	4.5	43.6	807.9	0.6	19.3	399.6	0.6	16.6	307.4
ΣPFASs	2.8	645.3	16183.3	6.5	1248.5	22744.6	32.2	403.7	8535.0	5.5	170.9	3490.1	6.3	142.3	2521.8

Σ7OCPs: HCB, α-chlordane, oxychlordane, *trans*-nonachlor, α-HCH, β-HCH and γ-HCH.

Table S4. Concentrations of PCBs, OCPs, BFRs and PFASs (ng/g wet weight) and lipid (%) in pooled liver samples of dietary exposed pregnant (gestation day 17) and post-pregnant (21 days post-partum) dams and maternally exposed female offspring (3, 6 and 9 weeks of age). Mice were exposed to the mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively).

	Pregnant dams			*Post-pregnant dams*			*3 weeks offspring*			*6 weeks offspring*			*9 weeks offspring*		
Compounds	Control	Low	High	Control	Low	High	Control	Low	High	Control	Low	High	Control	Low	High
Lipid %	4.50	3.73	6.13	6.17	6.72	8.93	4.06	4.34	5.54	6.15	5.43	5.52	6.99	7.75	7.01
Polychlorinated biphenyls (PCBs)															
PCB-52	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB-101	<LOD	5.90	105.80	<LOD	8.37	123.72	<LOD	5.21	58.16	<LOD	0.31	2.01	<LOD	<LOD	<LOD
PCB-118	0.06	33.89	799.22	0.06	24.10	692.94	0.08	28.82	910.04	0.10	7.62	301.10	0.13	3.04	178.77
PCB-138	2.62	1326.09	2426.92	0.71	342.85	1700.92	1.30	625.46	2027.80	2.76	424.15	1440.52	4.09	303.05	1177.52
PCB-153	0.37	78.87	1387.11	0.10	45.82	1425.67	0.23	72.58	1495.38	0.35	35.69	731.47	0.45	38.22	529.99
PCB-180	0.83	258.98	617.66	0.37	101.91	765.87	0.33	120.15	489.71	0.74	83.35	307.02	0.92	67.58	236.49
Σ6PCBs	4.94	1704.70	5337.68	2.31	524.02	4710.09	3.00	853.18	4983.12	5.01	512.09	2783.10	6.64	412.95	2123.83
Organochlorine pesticides (OCPs)															
p,p'-DDE	<LOD	33.82	851.55	<LOD	82.39	1684.95	<LOD	69.35	1797.61	<LOD	11.10	71.13	<LOD	0.79	19.86
HCB	0.38	33.61	887.93	0.22	18.90	574.98	0.35	32.89	895.92	1.56	10.93	245.96	2.01	10.27	149.45
α-Chlordane	<LOD	1.03	8.19	<LOD	1.34	6.40	0.01	0.10	0.78	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Oxychlordane	<LOD	14.83	361.52	<LOD	6.51	197.93	<LOD	13.65	422.28	0.11	5.92	170.41	0.15	7.96	109.60
Trans-Nonachlor	0.03	10.58	332.09	0.03	7.76	271.93	0.04	10.35	400.04	0.05	3.93	120.74	0.06	4.87	64.85
α-HCH	<LOD	2.85	62.23	<LOD	4.19	78.00	0.04	2.67	47.12	0.05	0.11	0.64	0.06	0.07	0.21
β-HCH	<LOD	18.34	343.07	<LOD	14.69	236.80	0.06	17.55	340.01	0.38	6.42	91.58	0.40	4.50	31.27
γ-HCH	<LOD	0.27	3.56	<LOD	0.52	3.91	<LOD	0.05	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Σ7OCPs	0.57	81.51	1998.59	0.41	53.91	1369.95	0.58	77.26	2106.17	2.18	27.34	629.36	2.71	27.70	355.41
Brominated flame retardants (BFRs)															
BDE-28	<LOD	<LOD	1.81	<LOD	0.08	2.92	<LOD	0.05	3.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
BDE-47	<LOD	5.84	196.24	<LOD	12.75	382.23	<LOD	12.36	414.81	<LOD	1.33	8.19	<LOD	<LOD	2.11
BDE-99	<LOD	3.15	89.87	<LOD	5.59	130.78	<LOD	4.26	81.67	<LOD	1.06	14.31	<LOD	0.65	5.52
BDE-100	<LOD	2.97	72.93	<LOD	<LOD	93.43	<LOD	3.44	83.85	<LOD	1.09	19.27	<LOD	1.13	8.57
BDE-153	<LOD	0.88	37.47	<LOD	2.37	75.12	<LOD	1.30	32.39	<LOD	0.55	14.95	<LOD	0.84	10.17
BDE-154	<LOD	1.07	26.31	<LOD	2.40	45.48	<LOD	0.97	15.26	<LOD	0.26	4.19	<LOD	0.34	1.47
BDE-183	<LOD	<LOD	<LOD	<LOD	<LOD	1.95	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
ΣBDE-28-183	0.75	14.18	424.85	0.75	23.47	731.90	0.75	22.60	631.26	0.75	4.56	61.17	0.75	3.31	28.11
BDE-206	<LOD	0.47	11.60	<LOD	0.84	7.70	<LOD	0.25	5.30	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
BDE-207	<LOD	7.78	159.04	<LOD	6.95	123.20	<LOD	4.30	117.92	<LOD	0.47	7.25	<LOD	0.41	5.30
BDE-208	<LOD	1.09	13.53	<LOD	0.58	7.77	<LOD	0.24	8.57	<LOD	<LOD	0.38	<LOD	<LOD	0.71
BDE-209	<LOD	74.97	1981.11	0.48	296.19	3023.10	0.41	33.56	769.24	<LOD	1.19	8.60	0.28	1.34	5.60
ΣBDE-206-209	0.44	84.30	2165.28	0.69	304.56	3161.77	0.62	38.34	901.02	0.44	1.80	16.32	0.49	1.89	11.70
HBCD	<LOD	<LOD	<LOD	<LOD	<LOD	3.59	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.14	<LOD
Perfluoroalkylated substances (PFASs)															
PFHxS	0.27	13.09	359.73	0.40	22.94	485.27	0.94	7.15	160.59	<LOD	2.86	53.96	<LOD	1.90	35.41
PFOS	0.54	106.87	2968.33	2.31	331.99	6483.30	7.90	63.35	1477.83	1.24	37.69	718.05	1.77	28.88	540.86
PFOA	0.70	151.93	3751.13	1.27	259.56	4400.77	6.00	109.05	2532.02	1.37	30.30	697.77	1.26	22.29	375.49
PFNA	0.44	118.03	3294.12	0.91	198.59	4007.86	5.55	80.09	1738.92	0.90	37.50	760.65	1.12	31.78	614.79
PFDA	0.38	160.09	3570.14	0.98	275.65	4675.84	7.25	100.45	1817.73	1.16	43.18	860.04	1.26	40.89	647.86
PFUnDA	0.49	95.28	2239.82	0.62	159.76	2691.55	4.54	43.58	807.88	0.58	19.32	399.59	0.61	16.55	307.39
ΣPFASs	2.80	645.28	16183.26	6.48	1248.49	22744.60	32.18	403.67	8534.98	5.45	170.85	3490.06	6.24	142.29	2521.79

Σ7OCPs: HCB, α-chlordane, oxychlordane, *trans*-nonachlor, α-HCH, β-HCH and γ-HCH.

S6. Biometrical measurements

The number of live offspring produced by F1 dams dietary exposed to the mixture of POPs (sampled during pregnancy and post-pregnancy) and the number of male to female offspring (F2) are presented in Table S5.

Table S5. Number of live offspring produced by dams during dietary exposure to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Live offspring of the pregnant dams were counted on gestation day 17. Live offspring of the post-pregnant dams were counted, and gender was determined, at 21 days post-partum. Results are presented as mean ± standard error.

	Live offspring	Males/Females
Pregnant dams		
Control	11.42 ± 0.58 (n = 12)	–
Low	12.81 ± 0.67 (n = 16)	–
High	9.13 ± 1.64 (n = 8)	–
Post-pregnant dams		
Control	12.50 ± 0.65 (n = 14)	83/81
Low	12.10 ± 0.62 (n = 10)	63/47
High	11.09 ± 0.62 (n = 11)	47/65

The combined uterus and ovary weight of 6 and 9-week-old female offspring (F2) maternally exposed to the mixture of POPs is presented in Table S6.

Table S6. Weigh of combined ovaries and uterus (g) in 6 and 9-week-old offspring maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Results are presented as least square mean ± standard error. N = 14, if not otherwise stated.

	Control	Low	High
6 weeks	0.22 ± 0.01 (n = 13)	0.24 ± 0.01	0.20 ± 0.01
9 weeks	0.22 ± 0.02	0.21 ± 0.02	0.21 ± 0.02

S7. Mammary gland histology

Complete results from histological examination of the fourth mammary gland from female offspring (F2) maternally exposed to the mixture of POPs is presented in Table S7.

Table S7. Mammary gland morphology in 3, 6 and 9-week-old female CD-1 mice maternally exposed to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Qualitative development scores were conducted on whole mounts of the 4th mammary gland from the right side using a scale from 1 to 4 (1 = poor development, 4 = best development). Terminal end buds (TEBs) were defined as ends with a diameter of ≥ 100 µm. Branching density, sum of the number of intersections, mammary epithelial area and length were calculated using Sholl analysis in ImageJ software. Glandular area was calculated using ImageJ on one section of fixated 4th mammary gland from the left side. Results are presented as mean ± standard error. At 3 weeks of age, n = 11 for the High group and n = 12 for the Control and Low groups except glandular area were n = 11. At 6 weeks, n = 12 for the Control group and n = 14 for the Low and High groups except glandular area were n = 12. At 9 weeks, n = 13 for the Control and High groups, except Control group glandular area were n = 12. Furthermore, n = 14 for the Low group except glandular area were n =12.Numbers in bold mark significant difference from Control ($p \leq 0.05$). P-values ≤ 0.10 are marked with *.

	Development score	Terminal end buds	Branching density (N/mm^2)	Sum of intersections (N)	Mammary epithelial area (mm^2)	Mammary epithelial length (mm)	Glandular area (%)
3 weeks							
Control	2.63 ± 0.25	10.67 ± 2.40	19.95 ± 0.72	217.92 ± 0.09	11.40 ± 2.19	3.77 ±0.35	0.45 ± 0.19
Low	2.38 ± 0.25	5.08 ± 1.38	19.16 ± 0.67	185.50 ± 18.21	9.91 ± 1.10	3.96 ± 0.25	0.31 ± 0.11
High	1.95 ± 0.23*	**3.17 ± 1.03**	18.64 ± 0.74	146.55 ± 12.46	8.18 ± 0.91	3.45 ± 0.1	0.28 ± 0.11
6 weeks							
Control	2.42 ± 0.16	13.50 ± 1.06	17.63 ± 0.37	3240.92 ± 134.00	188.35 ± 4.83	19.32 ± 0.51	2.03 ± 0.22
Low	2.39 ± 0.25	11.36 ± 1.04	17.70 ± 0.68	2934.29 ± 189.06	**168.79 ± 6.86**	19.90 ± 0.80	2.61 ± 0.31*
High	2.39 ± 0.25	15.86 ± 1.40	**20.68 ± 0.54**	3626.86 ± 198.31	179.73 ± 8.44	20.75 ± 0.48	2.33 ± 0.26
9 weeks							
Control	2.54 ± 0.28	1.62 ± 0.91	23.00 ± 0.67	5757.08 ± 472.73	253.88 ± 17.63	29.36 ± 0.97	2.54 ± 0.45
Low	2.39 ± 0.22	0.00 ± 0.00*	24 58 ± 0.83	4750.14 ± 150.76	**199.20 ± 6.19**	**26.48 ± 0.56**	4.40 ± 0.92
High	2.85 ± 0.21	1.69 ± 0.99	25.26 ± 0.86*	5669.31 ± 301.35	229.81 ± 0.23	27.65 ± 0.51	3.20 ± 0.37

S8. Hepatic histology

Table S8 presents the severity of hepatic extramedullary hematopoiesis and centrilobular hypertrophy in dams (F1) and offspring (F2) dietary or maternally exposed to the mixture of POPs, respectively.

Table S8. Severity of extramedullary hematopoiesis and centrilobular hypertrophy of hepatocytes in livers from dams (pregnant sampled at gestation day 17, and post-pregnant sampled at 21 days post-partum), and female (sampled at 3, 6 and 9 weeks of age) and male (sampled at 9 and 30 weeks of age) offspring. Dams were dietary exposed, and offspring were maternally exposed, to a mixture of POPs at Control, Low or High doses (0x, 5 000x or 100 000x human estimated daily intake, respectively). Results are presented as mean ± standard error. Severity was graded on a scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change). n = 12, 14 and 14 for female offspring in all groups at 3, 6 and 9 weeks, respectively. n = 15 for male offspring in all groups at both sampling times. n = 12, 16 and 8, and n = 14, 10 and 11 for the Control, Low and High groups of pregnant and post-pregnant dams, respectively. Bold indicates significant ($p \leq 0.05$) difference from Control.

	Extramedullary hematopoiesis	Centrilobular hypertrophy
Pregnant dams		
Control	0.00 ± 0.00	0.25 ± 0.13
Low	0.00 ± 0.00	**1.00 ± 0.13**
High	0.13 ± 0.13	**1.75 ± 0.16**
Post-pregnant dams		
Control	0.00 ± 0.00	0.29 ± 0.13
Low	0.00 ± 0.00	**1.30 ± 0.21**
High	0.00 ± 0.00	**3.09 ± 0.21**
3 weeks offspring females		
Control	1.71 ± 0.21	0.00 ± 0.00
Low	1.29 ± 0.23	0.07 ± 0.08
High	1.79 ± 0.23	**1.71 ± 0.21**
6 weeks offspring males		
Control	0.64 ± 0.17	0.00 ± 0.00
Low	0.43 ± 0.17	**2.00 ± 0.21**
High	0.50 ± 0.14	**3.00 ± 0.21**
9 weeks offspring females		
Control	0.00 ± 0.00	0.07 ± 0.07
Low	0.14 ± 0.10	**1.79 ± 0.30**
High	0.00 ± 0.00	**3.79 ± 0.11**
9 weeks offspring males		
Control	0.00 ± 0.00	0.20 ± 0.11
Low	0.00 ± 0.00	**1.67 ± 0.16**
High	0.00 ± 0.00	**2.67 ± 0.16**
30 weeks offspring males		
Control	0.00 ± 0.00	0.20 ± 0.14
Low	0.00 ± 0.00	**0.93 ± 0.21**
High	–	–

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Chemosphere 214 (2019) 534–542

Contents lists available at ScienceDirect

Chemosphere

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

A mixture of Persistent Organic Pollutants (POPs) and Azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model

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HIGHLIGHTS

- Can a mixture of POPs affect intestinal tumorigenesis in the A/J Min/+ mouse?
- Mice were exposed to POPs through the diet and received an injection of Azoxymethane.
- Results show an increased intestinal tumorigenesis in the A/J Min/+ mouse model.

ARTICLE INFO

Article history:
Received 5 July 2018
Received in revised form
19 September 2018
Accepted 20 September 2018
Available online 24 September 2018

Handling Editor: A. Gies

Keywords:
Persistent Organic Pollutants
Colorectal cancer
A/J Min/+ mouse
Azoxymethane
Intestinal tumorigenesis
Synergistic effect

ABSTRACT

A multitude of cancer types, including breast, testicular, liver and colorectal cancer, have associations with exposure to Persistent Organic Pollutants (POPs). The present study aimed to investigate whether a mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse, a model for human colorectal cancer (CRC). Pollutants were selected for their presence in Scandinavian food products and the mixture was designed based on defined human estimated daily intake levels. Mice were exposed through the diet, at control, low and high mixture concentrations, for 10 weeks. In a separate experiment, mice also received one subcutaneous injection of Azoxymethane (AOM) to explore whether this carcinogenic compound influenced the effect of the POPs. Intestinal tumorigenesis was examined by surface microscopy and histopathology. Moderate and dose-dependent increases in tumorigenesis were observed after dietary POP exposure. The AOM treatment alone stimulated the growth of colonic lesions, but did not increase the formation of new lesions. Combined AOM treatment and POP exposure demonstrated a synergistic effect on lesion formation in the colon, and to a lesser extent in the small intestine. This synergy was also evident by an increased number of malignant colonic tumors (carcinomas). In conclusion, the study shows that a mixture of POPs interacted synergistically with a known carcinogen (AOM), causing increased intestinal tumorigenesis in the A/J Min/+ mouse model.

1. Introduction

Persistent Organic Pollutants (POPs) are man-made chemicals that are toxic to humans and wildlife, resistant to degradation and have the potential to bioaccumulate and biomagnify in living organisms (UNEP, 2015). The compounds have adverse health effects and have been associated with an increased risk of breast cancer (Hoyer et al., 2000; Cameron and Foster, 2009), testicular cancer (McGlynn et al., 2008; Giannandrea et al., 2011), liver cancer (Filgo et al., 2015), and colorectal cancer (Howsam et al., 2004; Song et al.,

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https://doi.org/10.1016/j.chemosphere.2018.09.126

2014). The main route of non-occupational exposure to POPs in humans is through ingestion (Darnerud et al., 2006; Vestergren et al., 2012), which makes the GI tract the first organ of exposure. Traditional animal experiments only assess the impact of POPs using single compounds (Sethi et al., 2017) or compounds belonging to the same chemical group (Colter et al., 2018). However, carcinogenesis is a multistep process, so focus on individual compounds may prevent the discovery of potential synergism between multiple chemicals.

Colorectal cancer (CRC) is the third most common cancer in humans worldwide and exposure to carcinogens through the diet is an essential risk factor (IARC, 2016). CRC develops as a result of several genetic and epigenetic changes that cause a transformation of intestinal epithelium from normal tissue, via benign neoplasms, into carcinomas (Kinzler and Vogelstein, 1996; Sancho et al., 2004). Up to 85% of CRC cases are considered sporadic and 1% are attributed to the hereditary CRC syndrome known as familial adenomatous polyposis (FAP) (Burt, 2000). Mutations in the tumor-suppressor gene adenomatous polyposis coli (*APC*) are responsible for FAP, and patients develop a vast number of adenomatous polyps in the intestine, which are likely to progress into malignant tumors (Kinzler and Vogelstein, 1996). In addition, dysfunctional *APC* alleles have been found in the majority of sporadic colorectal lesions (Fodde, 2002). Research on CRC caused by *APC* mutations is therefore highly relevant to human health.

The most widely used animal model for human CRC is the multiple intestinal neoplasia (Min/+) mouse. This mouse has a heterozygous mutation in the *Apc* gene, resulting in a truncated gene product at amino acid 850 (Su et al., 1992). Inactivation of the remaining functional allele in the intestinal epithelium appears to be the rate-limiting step in tumorigenesis (Luongo et al., 1994). Loss of *Apc* inhibits the formation of the β-catenin destruction complex, leading to accumulation of β-catenin in the cytoplasm and subsequent translocation to the nucleus. Here, it interacts with the transcription factor Tcf-4, creating an active complex that transcribes specific target genes (Fodde, 2002; Kretzschmar and Clevers, 2017). The conventional Min/+ mouse model, bred on a C57BL/6 genetic background (Moser et al., 1990), develops lesions primarily in the small intestine (Mollersen et al., 2004). The A/J Min/+ mouse, on the other hand, also develops a large number of lesions in the colon, many of which progress to carcinomas over time (Sødring et al., 2016b). Therefore, the A/J Min/+ mouse model more closely resembles CRC development in humans and was therefore chosen for the present study.

The A/J strain has been shown to be more susceptible to the induction of colorectal cancer by Azoxymethane (AOM) than its C57BL/6 counterpart (Nambiar et al., 2003; Meunier et al., 2011). AOM is a genotoxic chemical used to mimic sporadic CRC and to study the underlying mechanisms of sporadic colorectal carcinogenesis (Venning FA, 2013). Following metabolic activation by cytochrome P450 enzymes (mostly CYP2E1), AOM reacts with DNA and causes adduct formation, leading to DNA mutations initiating colorectal carcinogenesis (Takahashi and Wakabayashi, 2004).

The aim of this study was to investigate whether dietary POP exposure, alone or following AOM treatment, could affect intestinal tumorigenesis in the A/J Min/+ mouse model. The mixture was designed to simulate a real-life exposure scenario relevant to humans (Berntsen et al., 2017).

2. Animals, materials and methods

2.1. Ethics statement

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (https://www.aaalac.org/). The animal experiment was approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and the Food Safety Authority (application ID: FOTS 8127) and executed in compliance with the local and national regulations associated with laboratory animal experiments. The rodent and rabbit section of the facility is a Specific Pathogen Free (SPF) unit and follows a health monitoring program recommended by Federation of European Laboratory Animal Science Associations/FELASA (http://www.felasa.eu/). The care of the animals was carried out by two veterinary nurses with FELASA B certification and the study was performed by a veterinarian with FELASA C certification.

2.2. Chemicals and experimental diet

A thorough description of the design and preparation of the POP mixture can be found in Berntsen et al. (2017). A list of the individual compounds can be found in Table 1. In brief, compounds occurring in Scandinavian food products reported in studies prior to 2012 were selected for the POP mixture. Human estimated daily intake (hEDI) levels were defined and adjusted to a 25 g mouse consuming 3 g feed/day. However, due to the possibility of background exposure and interspecies differences in compound metabolism, concentrations were adjusted up to 5000× (low dose) and 100 000× (high dose) hEDI. All polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and other organochlorines were purchased from Chiron AS (Trondheim, Norway). All perfluorinated compounds (PFCs) and hexabromocyclododecane (HBCD) were obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of perfluorohexane sulfonic acid (PFHxS) potassium salt which was purchased from Santa Cruz (Dallas, US). All chemicals were dissolved in an appropriate solvent and added to corn oil (Jasmin, fully refined, Yonca Gida San A.S., Manisa, Turkey) intended for human consumption. Solvents were thoroughly evaporated under N_2-flow and the remaining oil was incorporated in AIN-93G mouse feed (TestDiets, St.Louis, MO) at the low and high mixture concentrations. The control diet contained only corn oil from which the solvent had been evaporated.

2.3. Study design

In Experiment 1, 66 mice were used and each litter was randomly divided into 3 exposure groups (control, low and high POP diet) at weaning and exposed for 10 weeks (Fig. 1). In Experiment 2, 21 mice were exposed to the mixture of POPs in the same way, but in addition, these mice were also given one subcutaneous injection of 8.5 mg/kg AOM (Sigma-Aldrich, St. Louis, MO, USA) during their second week after birth. After 10 weeks of POP exposure, all mice were sacrificed and sampled. Because of high offspring mortality after the AOM injection, the breeding of mice for Experiment 2 was terminated for animal welfare reasons prior to completion of breeding the individuals for the study. This resulted in a lower number of animals compared to Experiment 1.

2.4. Animal model

The A/J Min/+ mouse model was established by backcrossing the Min/+ trait onto the genetic background of the A/J strain for >12 generations (Sødring et al., 2016b). In the present study, a total of 87 A/J Min/+ mice were used. The animals were bred in-house. Female A/J +/+ mice were mated with male A/J Min/+ mice and their A/J Min/+ offspring were used in the present study. The pups

Table 1
A mixture of persistent organic pollutants (POPs) based on a literature review on estimated daily intake (EDI) values in the Scandinavian population (Berntsen et al., 2017). Average EDI values for a 70 kg human and corresponding values for a 25 g mouse are shown. EDI values for a 25 g mouse consuming 3 g of feed designed to provide daily doses of POPs corresponding to the low (5000× human EDI) and high (100,000× human EDI) doses are shown in grey, and are based on measured feed concentrations. The table is adapted from Berntsen et al. (2017).

Compound	Average EDI [a]	Daily intake human	EDI [b] 25 g	EDI [c] 25 g mouse	EDI [d] 25 g mouse	Feed measured [e]	Feed measured [f]	EDI [g] 25 g mouse	EDI [h] 25 g mouse
	70 kg person ng/day	ng/kg/day	mouse pg/day	5000× ng/day	100,000× ng/day	5000× ng/g feed	100,000× ng/g feed	5000× ng/day	100,000× ng/day
Chlorinated									
PCB 28	10	0.14	3.5	18	350	3.1	46	9	138
PCB 52	23	0.33	8.3	41	825	15.0	182	45	546
PCB 101	39	0.56	14.0	70	1400	25.4	377	76	1131
PCB 118	68	0.97	24.3	121	2425	37.2	612	112	1836
PCB 138	97	1.38	34.5	173	3450	53.8	957	161	2871
PCB 153	97	1.38	34.5	173	3450	61.4	981	184	2943
PCB 180	26	0.37	9.3	46	925	17.4	263	52	789
∑PCBs	360	5.13	128.4	642	12,825	213.3	3418	640	10,254
p,p'-DDE	201	2.87	71.8	359	7175	136.0	2390	408	7170
HCB	84	1.20	30.0	150	3000	37.4	588	112	1764
a-Chlordane	63	0.90	22.5	113	2250	45.0	723	135	2169
Oxychlordane	21	0.30	7.5	38	750	9.8	297	29	891
trans-Nonachlor	21	0.30	7.5	38	750	14.9	264	45	792
a-HCH	36	0.52	13.0	65	1300	21.2	421	64	1263
b-HCH	29	0.42	10.5	53	1050	22.3	398	67	1194
g-HCH (Lindane)	40	0.57	14.3	71	1425	31.4	435	94	1305
Dieldrin	126	1.80	45.0	225	4500	70.4	1470	211	4410
∑OCPs	621	8.88	222.1	1112	22,200	388.4	6986	1165	20,958
∑PCBs + OCPs	981	14.01	350.5	1754	35,025	601.7	10,404	1805	31,212
Brominated									
PBDE 47	68	0.97	24.3	121	2425	39.7	642	119	1926
PBDE 99	13	0.19	4.8	24	475	8.6	126	26	378
PBDE 100	11	0.15	3.8	19	375	5.6	91	17	272
PBDE 153	2	0.03	0.8	4	75	1.5	22	5	67
PBDE 154	4	0.06	1.5	8	150	2.8	38	8	114
PBDE 209	105	1.50	37.5	188	3750	64.8	1141	194	3423
HBCD	21	0.30	7.5	38	750	9.9	203	30	609
∑BFRs	224	3.2	80.2	402	8000	132.9	2263	399	6789
Perfluorinated									
PFHxS	1.2	0.017	0.4	2	43	1.7	42	5	125
PFOS	18	0.26	6.5	33	650	3.2	74	10	222
PFOA	31	0.44	11.0	55	1100	6.0	121	18	363
PFNA	9.5	0.14	3.5	18	350	2.1	42	6	127
PFDA	13	0.19	4.8	24	475	3.1	57	9	172
PFUnDA	6.7	0.096	2.4	12	240	1.6	28	5	84
∑PFAAs	79.4	1.14	28.6	144	2858	17.7	364	53	1094

Abbreviations: PCBs (polychlorinated biphenyls); OCPs (organochlorine pesticides); BFRs (brominated flame retardants); PFAAs (perfluoroalkyl acids).
[a] Average EDI (Estimated daily intake) values of POPs for a 70 kg human e based on a literature review of Scandinavian EDI values (Berntsen et al., 2017).
[b] EDI values for a 25 g mouse corresponding to human EDI values.
[c] EDI values for a 25 g mouse corresponding to human EDI values * 5000
[d] EDI values for a 25 g mouse corresponding to human EDI values * 100,000.
[e] Measured concentrations of the various compounds in the 5000× feed.
[f] Measured concentrations of the various compounds in the 100,000× feed.
[g] EDI values for a 25 g mouse consuming 3 g of the 5000× feed/day e based on concentrations measured in the feed of the current project.
[h] EDI values for a 25 g mouse consuming 3 g of the 100,000× feed/day e based on concentrations measured in the feed of the current project.

were marked with ear punches and genotyped at weaning, as previously described in Sødring et al. (2015).

2.5. Housing and husbandry

During mating, animals were housed in groups in open type III cages (Tecniplast, Buguggiate, Italy). During exposure and AOM injection animals were housed in closed type III IVC-cages (Allentown Inc, USA) for health and safety reasons. All cages contained standard aspen bedding, cellulose nesting material and red polycarbonate houses (Tecniplast, Buguggiate, Italy). The animals were given their assigned feed, and tap water in standard drinking bottles (Tecniplast, Buguggiate, Italy), *ad libitum*. The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 ± 2 °C with 20 air changes per hour and 45 ± 5% relative humidity. The cages, bedding, nesting material and water bottles were changed once a week.

2.6. Sample collection and identification of intestinal lesions

The A/J Min/+ offspring were sacrificed at 13 weeks of age. They were anesthetized with isoflurane gas (Isoflurane Baxter, San Juan, Puerto Rico), bled by cardiac puncture and euthanized by cervical dislocation. The small intestine and colon were collected, fixed and dyed as previously described in Sødring et al. (2016a). Briefly, the intestines were rinsed with PBS, fixed flat, and stored in 10% neutral buffered formalin for at least 24 h, before being stained with 0.2% methylene blue dissolved in formalin. The liver was collected and

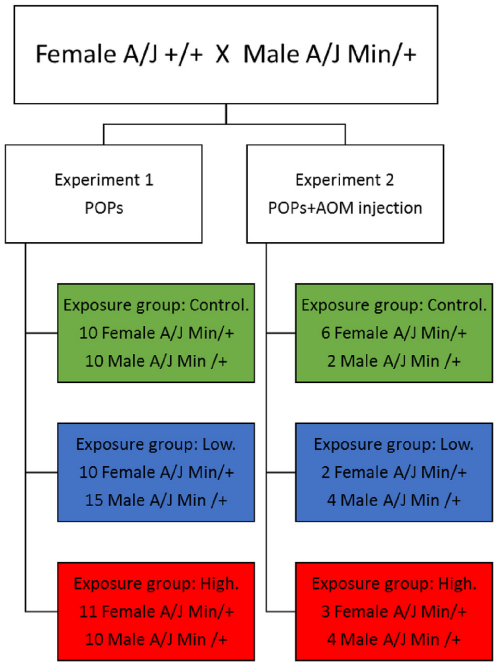

Fig. 1. Study design of the two experiments, including exposure groups (control, low and high), breeding of A/J Min/+ mice and the number of animals (females and males) in each group. In both experiments, A/J Min/+ mice were exposed to a mixture of POPs through feed for 10 weeks. In addition, mice in Experiment 2 received one subcutaneous injection of AOM (8.5 mg/kg) during the second week after birth.

weighed. All tumors that were found (one in the liver, one from the forelimb, one sub-mandibular and one from the abdomen) were also collected and fixed in 10% neutral buffered formalin. The blood, cecum, spleen and retroperitoneal adipose tissue were collected and stored for analysis in another project. For surface microscopy and transillumination of the intestines, an inverted light microscope (CKX41, Olympus Inc., Hamburg, Germany) with a digital color camera (DP25, Olympus) was used. In the colon, lesions were identified as either flat aberrant crypt foci (flat ACF; <30 crypts) or tumors (>30 crypts covering more than approximately 0.4 mm^2) as explained by Sødring et al. (2015).

2.7. Histology

After scoring, the intestines were prepared using the Swiss roll technique as described earlier by Sødring et al. (2016b). The Swiss rolls were embedded in paraffin and 3 μm thick histological sections were cut and stained with haematoxylin eosin (HE) and periodic acid Schiff (PAS). All Swiss rolls were sectioned at three different random levels in the paraffin block. Examination was conducted in a microscope and lesions were identified, counted and classified as preneoplastic lesions (hyperplastic and dysplastic cells), adenomas or carcinomas. Tumors with distinct infiltrative growth through the muscularis mucosa and into the submucosa were classified as carcinomas, whereas tumors confined to the mucosa without infiltrative growth were classified as adenomas. Tumors that were found outside the intestine were also embedded in paraffin, sectioned and stained with HE and PAS, and examined in the microscope.

2.8. Statistical analyses

Statistical analyses were performed using JMP Pro 13® (SAS, Cary, NC, USA). Least squares analyses were used to analyze data on body measures. Experiment 1 and 2 were analyzed separately by the following model:

$$Y_{ijpmn} = \mu + G_i + E_j + e_{ij}$$

where:

Y_{ij} = observation of either body weight, relative liver weight, relative colon length or relative small intestine length.
μ = overall mean of body weight, relative liver weight, relative colon length and relative small intestine length.
G_j = effect of sex, $i = 1$ (Male) or 2 (Female).
E_j = effect of exposure group, $j = 1$ (control), $j = 2$ (low), $j = 3$ (high).
e_{ij} = error term.

Measures of histological changes and visually scored lesions did not meet the assumption of normality. Log transformation provided an improved, but not satisfactory, fit to the normal distribution. Initially least squares analyses were performed on log-transformed data with sex and exposure group as explanatory variables. Some sex differences were noticed, but few interactions were found between the exposure group and the sex of the animal. Thus, exposure effects were not dependent on the sex. In the final analyses, univariate non-parametric tests were used. Differences between exposure groups and the control were assessed using Steel's test, which controls for the overall experiment wise error rate (Type I). Differences between sexes were investigated using the Wilcoxon two-sample test. The level of significance was set to 5%. Size and location distribution figures were produced using Excel 2013®.

3. Results

3.1. Effects on body weight, liver weight and intestinal length

The high mixture concentration of POPs significantly decreased the terminal body weight of both the mice who only were exposed to POPs (Experiment 1) and also the mice that were injected with AOM (Experiment 2), compared to the control group (Table 2). In addition, there was a significant increase in liver weight, relative to body weight, in the high group of both experiments. Colon length, relative to body weight, was not affected by AOM or POPs. However, the length of the small intestine was significantly increased by the high concentration of POPs after the AOM injection (Experiment 2). Notably, AOM alone did not change any of the parameters measured.

3.2. Scoring of intestinal lesions

The effects of dietary exposure to the mixture of POPs on intestinal tumorigenesis was examined in mice by scoring of intestinal lesions (Table 3). High levels of POPs (Experiment 1) significantly increased the number of flat ACF in colon, when compared to the control group. Although not significant, a trend was observed towards increased flat ACF load in the high and the low groups ($p = 0.051$ and $p = 0.058$, respectively). The low mixture concentration increased the number of colonic tumors, compared to the control group. However, this was not evident after exposure to the high mixture concentration ($p = 0.096$). No other parameters

Table 2
Least square mean (±SE) of body weight (BW), relative liver weight (LW), relative colon length and relative small intestine (SI) length at necropsy in Experiment 1 (POP exposure) and Experiment 2 (POP exposure + AOM injection). The table included effects of exposure group control, low and high in both experiments. Bold letters indicate significant difference from the control group (Dunnet's test; $p \leq 0.05$).

Exposure		BW at necropsy (g)	Relative LW	Relative colon length (cm/g)	Relative SI length (cm/g)
Experiment 1	Control	23.54 ± 0.71	$0.05 \pm 0.08 \times 10^{-2}$	0.31 ± 0.01	1.41 ± 0.03
POPs	Low	23.26 ± 0.79	$0.05 \pm 0.07 \times 10^{-2}$	0.31 ± 0.01	1.46 ± 0.03
	High	$\mathbf{21.47 \pm 0.49}$	$\mathbf{0.07 \pm 0.08 \times 10^{-2}}$	0.33 ± 0.01	1.49 ± 0.03
Experiment 2	Control	24.68 ± 1.09	$0.05 \pm 0.12 \times 10^{-2}$	0.31 ± 0.01	1.36 ± 0.05
POPs + AOM	Low	25.43 ± 1.44	$0.05 \pm 0.14 \times 10^{-2}$	0.31 ± 0.03	1.49 ± 0.05
	High	$\mathbf{20.99 \pm 1.47}$	$\mathbf{0.07 \pm 0.13 \times 10^{-2}}$	0.33 ± 0.02	$\mathbf{1.62 \pm 0.05}$

Table 3
Summary of results from scoring of lesions in colon and small intestine (SI) of A/J Min/+ mice from Experiment 1 (POP exposure) and Experiment 2 (POP exposure + AOM injection). Colonic lesions are categorized as either flat ACF (<30 aberrant crypts) or tumors (>30 aberrant crypts). Load equal the total area of intestine covered by lesions. Results are presented as means (±SE). Differences between exposed groups (low and high) and control were assessed with Steel's test and indicated in bold when significant ($p \leq 0.05$). Trends with $p \leq 0.07$ are denoted *.

Exposure		Colon						SI		
		Number of flat ACF	Average size of flat ACF (mm^2)	Load of flat ACF (mm^2)	Number of tumors	Average size of tumors (mm^2)	Tumor load (mm^2)	Number of tumors	Average size of tumors (mm^2)	Tumor load (mm^2)
Experiment	Control	4.75 ± 0.98	$0.01 \pm 0.03 \times 10^{-1}$	0.06 ± 0.02	0.70 ± 0.24	0.75 ± 0.23	1.07 ± 0.37	27.95 ± 5.12	0.62 ± 0.04	20.39 ± 4.77
1	Low	8.60 ± 1.97	$0.02 \pm 0.04 \times 10^{-1}$	$0.18 * \pm 0.04$	$\mathbf{2.04 \pm 0.47}$	0.65 ± 0.15	2.52 ± 0.93	25.56 ± 4.50	0.58 ± 0.04	17.92 ± 4.76
POPs	High	$\mathbf{8.38 \pm 1.42}$	$0.02 \pm 0.02 \times 10^{-1}$	$0.13 * \pm 0.03$	1.24 ± 0.23	0.47 ± 0.11	0.81 ± 0.23	26.57 ± 3.76	0.55 ± 0.03	15.85 ± 3.51
Experiment	Control	2.25 ± 0.84	0.05 ± 0.02	0.15 ± 0.06	3.63 ± 0.78	0.77 ± 0.20	3.47 ± 1.37	18.75 ± 5.44	0.70 ± 0.03	13.70 ± 4.39
2	Low	6.17 ± 1.30	0.06 ± 0.01	0.35 ± 0.09	12.33 ± 3.33	1.72 ± 0.50	25.80 ± 9.67	18.33 ± 2.33	0.63 ± 0.05	11.52 ± 1.57
POPs + AOM	High	$\mathbf{11.57 \pm 2.03}$	0.06 ± 0.01	$\mathbf{0.69 \pm 0.15}$	$\mathbf{29.86 \pm 4.83}$	$\mathbf{1.99 \pm 0.17}$	$\mathbf{56.62 \pm 6.70}$	$\mathbf{48.00 \pm 9.85}$	0.74 ± 0.10	40.85 ± 13.44

measured in the small intestine and colon were affected by dietary POPs alone.

In combination with the AOM injection (Experiment 2), POPs significantly increased the number of flat ACF, flat ACF load, number of tumors, average tumor size and tumor load in the colon of mice in the high group (Table 3). In addition, the number of tumors in the small intestine was significantly higher in the high group compared to the controls. No significant changes were observed after exposure to the low mixture concentration of POPs in Experiment 2. However, there were clear trends towards increases in several parameters, including a 7-fold increase in the colonic tumor load.

3.3. Size distribution and location of intestinal lesions

To demonstrate the distribution of size, lesions were divided into five different size categories. Fig. 2a presents the number of lesions per size category for each exposure group in both experiments and clearly illustrates the shift towards larger lesions observed in AOM treated animals. The AOM treatment alone did not appear to give any new lesions, but instead stimulated the growth of the colonic lesions. Notably, the increase in the number of lesions provoked by POPs seemed to be more pronounced in AOM treated animals than in untreated animals, particularly in the colon. This implies a synergistic effect of AOM and POPs. In the

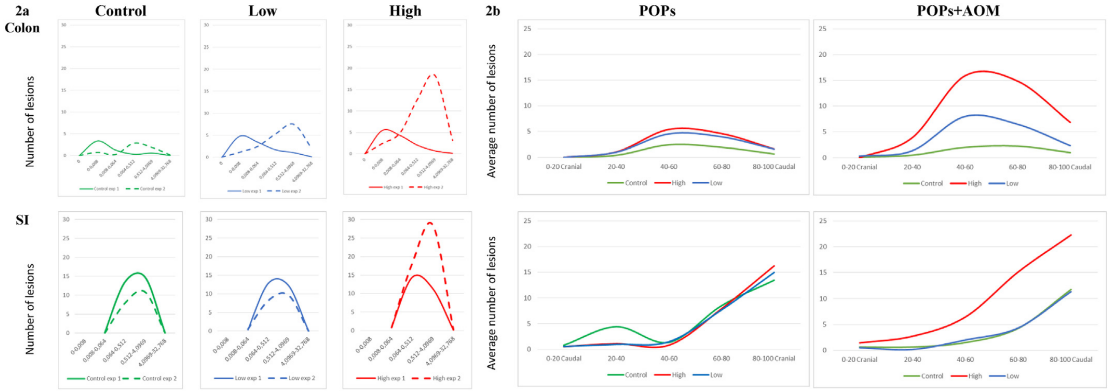

Fig. 2. a). Size distribution of lesions in colon and small intestine (SI) of A/J Min/+ mice exposed to POPs in control, low and high mixture concentrations, without AOM (Exp 1) and with AOM injection (Exp 2). Size categories (mm^2) are described by Sødring et al 2015 and represented on the X axis. The Y axis shows the number of lesions. b). Location of lesions in colon and small intestine (SI) of A/J Min/+ mice exposed to POPs in control, low and high mixture concentrations, without AOM (Exp 1) and with AOM injection (Exp 2). Location categories (20% sections) are represented on the X axis. The Y axis shows the average number of lesions.

small intestine, AOM alone did not induce any apparent changes, but a moderate synergistic effect on tumor formation seemed to occur between AOM and the high level of POPs.

Location of lesions along the intestine (Fig. 2b) shows an increased number of lesions in the middle and caudal parts of the colon and caudally in the small intestine, in both experiments. In addition, the figure illustrates how dietary exposure to POPs enhances the number of lesions in both experiments, represented by more lesions in the high and low groups compared to the control group.

3.4. Histopathology

Histology from tumors collected from non-intestinal tissue showed no metastases originating from the intestinal lesions. Instead, they were either hyperplastic lesions or metastases from the local tissue.

In Experiment 1, lesions were found in the intestines of animals from all exposed groups. The total number of lesions in the small intestine was higher than that of the colon (Table 4). No significant differences were observed between the control group and the exposed groups. Preneoplastic changes and adenomas were the most frequent lesions, and only a few animals had carcinomas. Fig. 3 illustrates the types of lesions in the colon in Experiments 1 and 2.

In Experiment 2, the mice fed the high concentration of POPs had significantly more colonic lesions of all types compared to the control group (Table 4). A trend was also evident towards increases in the number of small intestinal preneoplastic lesions ($p = 0.067$). Interestingly, this increase of lesions appeared to be due to the synergistic effect between AOM and the high level of POPs, as suggested above.

4. Discussion

In the present study, we investigated whether a mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse model. In a separate experiment, we also investigated whether a sub-carcinogenic exposure of AOM could influence the effect of POPs. We found that POPs alone increased the intestinal tumorigenesis moderately and in a dose-dependent manner. Comparing the two experiments, AOM alone did not seem to increase the formation of new lesions, or have a deleterious effect on the mice. However, the growth of colonic lesions was stimulated by AOM treatment. A strong synergistic effect was apparent between POPs and AOM on the formation of colonic lesions, and to a lesser extent on lesions in the small intestine. Interestingly, this synergy was also associated with a significant increase of malignant tumors

Table 4
Histopathological examination of lesions (preneoplastic, adenoma or carcinoma) in colon and small intestine (SI) of A/J Min/+ mice from Experiment 1 (POP exposure) and Experiment 2 (POP exposure + AOM injection). Results are presented as mean (±SE). Differences between exposed groups (low and high) and control were assessed with Steel's test and indicated in bold when significant ($p \leq 0.05$). Trends with $p \leq 0.07$ are denoted *.

Exposure		Colon			SI		
		Preneoplastic	Adenoma	Carcinoma	Preneoplastic	Adenoma	Carcinoma
Experiment 1	Control	0.70 ± 0.25	0.35 ± 0.25	0.05 ± 0.05	9.05 ± 1.32	6.80 ± 1.33	0.90 ± 0.42
POPs	Low	0.84 ± 0.29	0.32 ± 0.19	0.12 ± 0.09	7.00 ± 1.21	3.96 ± 1.04	0.44 ± 0.22
	High	1.05 ± 0.30	0.05 ± 0.05	0.00 ± 0.00	6.86 ± 1.58	3.86 ± 1.09	0.29 ± 0.16
Experiment 2	Control	1.38 ± 0.42	0.75 ± 0.31	0.00 ± 0.00	4.50 ± 1.59	5.13 ± 1.75	0.50 ± 0.27
POPs + AOM	Low	2.00 ± 0.58	2.17 ± 0.70	0.67 ± 0.49	6.00 ± 1.21	2.67 ± 0.67	0.00 ± 0.00
	High	**5.57 ± 1.11**	**6.57 ± 1.51**	**2.57 ± 0.92**	10.29 * ± 2.35	9.43 ± 2.06	0.71 ± 0.36

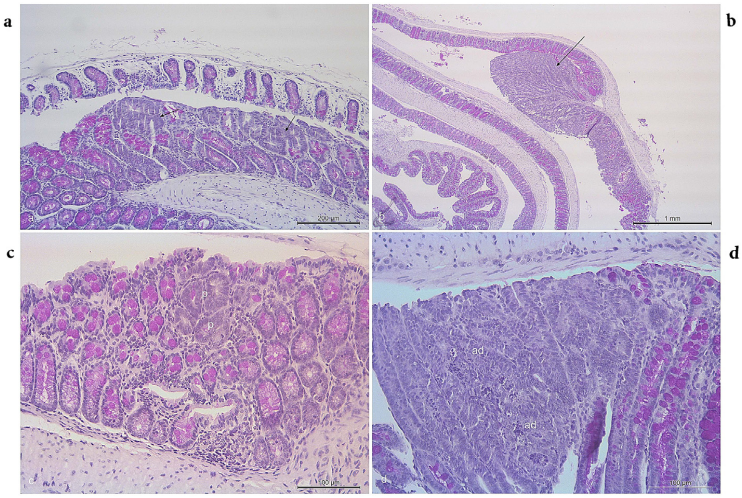

Fig. 3. Histological lesions observed in colon. a. Preneoplastic lesions (dysplasia and hyperplasia) are present in the luminal part of crypts (arrows) of a mouse of the low exposure group of Experiment 1. **b**. A carcinoma (arrow) in the mucosa infiltrates Muscularis Mucosae and Submucosa. Mouse of the low exposure group of Experiment 1. **c**. A small focus with preneoplastic crypt lesions in a mouse of the low exposure group of Experiment 2. **d**. Mucosal adenoma (ad) in a mouse from the high exposure group of Experiment 2.

(carcinomas) in the colon.

4.1. Effects of POPs on body weight and liver weight

The concentration of each compound in the high dose was generally below the No Observed Adverse Effect Level (NOAEL), where such a level was available (Berntsen et al., 2017). Although we did not observe any clinical signs in the animals during the present study, we did observe apparent adverse effects at the end of the study, indicated by reduced body weight and increased relative liver weight in both experiments. These effects were seemingly unrelated to AOM treatment and may have been caused by additive or synergistic effects between individual POPs in the mixture. In another experiment using the same mixture but a different mouse strain (129:C57BL/6F0), there was no significant effect of the high POPs feed on body weight (Hudecova et al., 2018). This suggests there are mouse strain differences in sensitivity to POPs.

Aberrant *Apc* expression as a consequence of the germline mutation in *Apc* has been shown to affect the ability of the liver to metabolize xenobiotics (Benhamouche et al., 2006), and may lead to degrees of pollutant tolerance. In addition, the large number of intestinal lesions in the mice exposed to the high dose of POPs in our experiment may have contributed to a lower absorption rate of nutrients, which could have reduced the body weight of mice in this group.

Our findings of increased relative liver weights is in line with other studies where animals have been exposed to perfluorinated compounds (Seacat et al., 2003; Tan et al., 2013). These chemicals have been thoroughly investigated for hepatotoxicity, because of their high affinity to serum proteins and subsequent accumulation in the liver (Jones et al., 2003).

The highest concentration of POPs in our study is relatively large, but the low mixture concentration could potentially be considered more relevant for humans when taking life-long exposure and slow pollutant metabolism into account (Martignoni et al., 2006; Hudecova et al., 2018).

The occurrence of high mortality in offspring after neonatal AOM treatment (Experiment 2) was surprising, as the dosage used has not previously been associated with increased mortality. It is therefore unclear whether the lethality observed was caused by an abnormally high sensitivity to AOM, either alone or in combination with stress. However, we can conclude that the event was not caused by dietary POPs, since the AOM injection was given prior to weaning.

4.2. Effects of POPs on intestinal lesions

The process of cancer is divided into three phases; initiation, promotion and progression (Farber and Cameron, 1980). Depending on their mode of action, compounds may interfere with the molecular processes within each of these phases, and ultimately affect the carcinogenic process. Initiation is the irreversible heritable change in DNA, while promotion is the non-genotoxic advantages of mutated cell growth (Ludewig and Robertson, 2013). In the present study, the high mixture concentration of POPs initiated the formation of new lesions in the colon of A/J Min/+ mice, which was reflected by a significantly larger intestinal area covered by flat ACF. The low concentration of POPs did not affect the number of newly formed lesions, but promoted intestinal tumorigenesis by resulting in more colonic tumors of a larger diameter (>30 crypts). This initiating and promoting effect was not visible in the small intestine.

Previous studies have reported that some POPs affect both carcinogenic initiation and promotion *in vivo*. Liver tumorigenesis was initiated by a mixture of PCBs (Kanechlor 500) in mice (Ito et al., 1973). The same study also showed a promotional effect of the PCBs when administered together with hexachlorobenzene (HCB, α or β). Developmental exposure (*in utero* and via lactation) to dieldrin initiated the formation of mammary, ovarian and liver tumors in a transgenic mouse model for mammary tumorigenesis (Cameron and Foster, 2009). *In utero* exposure to perfluorooctanoic acid (PFOA) induced hepatocellular adenomas in CD-1 mice (Filgo et al., 2015). However, PFOA and PFOS did not increase the formation of intestinal lesions (Ngo et al., 2014). HCB was shown to promote mammary, liver and lung tumorigenesis in xenograft mouse models, without having initiating effects (Pontillo et al., 2013). The organochlorine metabolite *p,p'*-DDE has been suggested as a promoting agent in mammary tumorigenesis (Johnson et al., 2012). In addition, its parental compound *p,p'*-DDT (*p,p'*-dichlorodiphenyltrichloroethane) has been shown to promote CRC growth in mice injected with a suspension of the human colorectal adenocarcinoma cell line DLD1 (Song et al., 2014). The study also demonstrated that the CRC promotion by *p,p'*-DDT was achieved through the Wnt/β-catenin signaling pathway mediated by oxidative stress. *p,p'*-DDT elevated the production of reactive oxygen species (ROS), inhibited enzymes and reduced antioxidants levels in intestinal cells. Subsequently, there was an accumulation of β-catenin and the consecutive expression of target genes, which induced the proliferation of colorectal cancer cells and thus promoted CRC growth. The study also demonstrated that an increased production of ROS could affect colorectal carcinogenesis by interacting with specific pathways or by damaging DNA.

Furthermore, the metabolic activation of compounds may create products or intermediates that can interfere directly with DNA. PCBs have been shown to form highly reactive products and by-products that have the ability to mutate DNA, as reviewed by Ludewig and Robertson (2013). PBDEs are structurally similar to PCBs and have been shown to induce ROS formation, leading to chromosomal breakage (Ji et al., 2011). POPs may therefore have the ability to affect DNA and to increase tumorigenesis by inducing mutations in oncogenes or tumor suppressor genes such as *KRAS*, *p53* and *APC*. Changes in these genes are necessary for the development of colorectal cancer (Fodde, 2002). It has also been shown that most intestinal lesions in the Min/+ mouse have lost their remaining functioning *Apc* allele (Luongo et al., 1994). In the present study, mutations in *Apc* might have caused the formation of new lesions and enhanced the growth from flat ACF to tumors in the A/J Min/+ mice. However, this remains to be investigated.

4.3. Effects of AOM and POPs on intestinal lesions

AOM is converted to methylazoxymethanol (MAM) by cytochrome P450 enzymes (CYP450) located in both the liver and the intestines (Sohn et al., 2001). This highly reactive metabolite causes DNA mutations that are thought to initiate colorectal carcinogenesis (Takahashi and Wakabayashi, 2004). Different strains of mice vary in their susceptibility to AOM-induced CRC, and the A/J strain is known to be highly sensitive (Rosenberg et al., 2009). In addition, Min/+ mice exposed to AOM during their first two weeks of life have been shown to be particularly susceptible to induced and spontaneous intestinal carcinogenesis (Paulsen et al., 2003).

In the present study, neonatal mice in Experiment 2 were given one injection of AOM. This treatment did not seem to initiate the formation of new colonic lesions. Instead, it promoted the growth of already existing lesions, as evident from the increased number of tumors and colonic lesions of the larger size classes in mice from the control group. Combined exposure to AOM and POPs both initiated and promoted colorectal carcinogenesis and resulted in a severe lesion burden, especially in mice exposed to the high mixture concentration of POPs. This large effect on tumorigenesis,

compared to the relatively moderate initiation and promotion by POPs alone, indicates a synergistic effect between AOM and POPs. The high group exhibited the most extreme outcomes, which could be explained by the relatively high concentration of pollutants. However, the numerical differences from the control group demonstrate that the low mixture concentration also displayed initiating and promoting effects in the colon, as shown by a 7-fold increase in colonic tumor load. As with AOM, POPs are metabolized by CYP450 (Docea et al., 2017) and CYP450 has been shown to be a strong biomarker for the presence of POPs in animal tissue (Bachman et al., 2015). This similarity could be the origin of the synergistic effect observed between AOM and POPs, but this remains to be investigated. Previous studies in mice (Swiss and B6129SF2/J strains) have shown that PCBs promote carcinogenesis in lung and liver tissues when the tumors were initiated by N-nitrosodimethylamine (Anderson et al., 1994; Strathmann et al., 2006). The same promotional effect of PCBs was seen in A/J mice when given together with 1-Nitropropane to induce lung tumorigenesis (Nakanishi et al., 2001). The synergistic effect seen in the present study emphasizes the importance of anticipating synergistic effects between compounds which individually have the ability to initiate or promote cancer development. In addition, due concern should be given to chemical mixtures that not individually cause cancer, but which are disruptive in a manner that collectively provokes carcinogenesis (Goodson et al., 2015).

4.4. Histopathology

In the present study, the histopathological characterization of intestinal lesions differentiated between preneoplastic lesions, adenomas and carcinomas. The preneoplastic lesions included both hyperplastic and dysplastic cells. Dysplasia is a known hallmark of malignant potential and is closely related to *APC* mutations (Jen et al., 1994). Moderate to severe dysplasia has previously been shown in flat ACF from both traditional (C57BL/6) and A/J Min/+ mice (Paulsen and Alexander, 2001; Paulsen et al., 2006; Sødring et al., 2016a). In addition, flat ACF have been shown to be reliable surface biomarkers of *Apc*-driven colorectal carcinogenesis (Sødring et al., 2016a).

The initiating and promoting effect observed by intestinal scoring was not evident from the histopathological examination of mice only exposed to POPs (Experiment 1). However, carcinomas were observed in both the colon and small intestine, which could be an indication of the promotional effect explained above. In mice from Experiment 2, AOM and the high mixture concentration increased all types of lesion, which further confirms the synergistic effect observed between AOM and POPs. Interestingly, one injection of AOM alone did not cause the formation of colonic carcinomas. Instead, it induced the formation of preneoplastic lesions and adenomas, suggesting that AOM did not act as a tumor promotor in the A/J Min/+ mice after only one injection.

Because of the difference in method of analysis between intestinal scoring and histopathology, identical results could not be expected. However, similar trends were observed and both methods showed the same synergistic effect between AOM and POPs. It is important to emphasize that the histopathological examination was conducted on only three slides from each intestine. Thus, this method only investigates a small part of the area in question, compared to the scoring of the whole surface of the intestine, which could explain the discrepancy in findings between the two different methods.

5. Conclusion

The present study indicates that a mixture of POPs designed on the basis of human exposure, together with an injection of AOM, increased intestinal tumorigenesis in A/J Min/+ mice. Furthermore, a synergistic effect was observed between POP exposure and one injection of AOM. The results emphasize the importance of anticipating synergies when assessing the carcinogenic potential of compound mixtures.

Acknowledgements

The authors would like to thank the staff at Section for Experimental Biomedicine at The Norwegian University of Life Sciences for excellent care for the animals and support for the researchers. The study was funded by The Research Council of Norway (project name MultiPOP, project number 204361/H10).

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Chemosphere 252 (2020) 126484

Contents lists available at ScienceDirect

Chemosphere

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

Maternal exposure to a human relevant mixture of persistent organic pollutants reduces colorectal carcinogenesis in A/J Min/+ mice

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HIGHLIGHTS

- A/J Min/+ mice were maternally exposed to a mixture of PCBs, OCPs, BFRs and PFASs.
- Exposure through gestation and lactation reduced colorectal carcinogenesis.
- Exposure affected amino acid, lipid, glycerophospholipid and energy metabolism.
- Alterations were observed in intestinal microbiota composition.

ARTICLE INFO

Article history:
Received 24 January 2020
Received in revised form
10 March 2020
Accepted 12 March 2020
Available online 13 March 2020

Handling Editor: A. Gies

Keywords:
Persistent organic pollutants
Colorectal cancer
Metabolomics
Gut microbiota

ABSTRACT

An increased risk of developing colorectal cancer has been associated with exposure to persistent organic pollutants (POPs) and alteration in the gut bacterial community. However, there is limited understanding about the impact of maternal exposure to POPs on colorectal cancer and gut microbiota. This study characterized the influence of exposure to a human relevant mixture of POPs during gestation and lactation on colorectal cancer, intestinal metabolite composition and microbiota in the A/J Min/+ mouse model. Surprisingly, the maternal POP exposure decreased colonic tumor burden, as shown by light microscopy and histopathological evaluation, indicating a restriction of colorectal carcinogenesis. ^{1}H nuclear magnetic resonance spectroscopy-based metabolomic analysis identified alterations in the metabolism of amino acids, lipids, glycerophospholipids and energy in intestinal tissue. In addition, 16S rRNA sequencing of gut microbiota indicated that maternal exposure modified fecal bacterial composition. In conclusion, the results showed that early-life exposure to a mixture of POPs reduced colorectal cancer initiation and promotion, possibly through modulation of the microbial and biochemical environment. Further studies should focus on the development of colorectal cancer after combined maternal and dietary exposures to environmentally relevant low-dose POP mixtures.

Abbreviations: ACF, aberrant crypt foci; *APC*, *adenomatous polyposis coli*; ASV, amplicon sequence variant; CRC, colorectal cancer; FAP, familial adenomatous polyposis; hEDI, human estimated daily intake; Min, multiple intestinal neoplasia; NMR, nuclear magnetic resonance; POP, persistent organic pollutant.

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https://doi.org/10.1016/j.chemosphere.2020.126484

1. Introduction

Persistent organic pollutants (POPs) are recognized as a global threat to human health and ecosystems due to their persistence in the environment, accumulation, long-range transport and deleterious effects (Secretariat of the Stockholm Convention, 2008; United Nations Environment Programme, 2019). Many POPs have been shown to cross the placental barrier (Barr et al., 2007; Vizcaino et al., 2014; Winkens et al., 2017). Thus, exposures to these compounds are initiated during the early prenatal period. After birth, exposure continues through breast-feeding (Fenton et al., 2009; Polder et al., 2009; Thomsen et al., 2010; Nyberg et al., 2018; Lenters et al., 2019), and POP exposure during gestation and lactation has been shown to negatively impact development of the nervous, immune and endocrine systems (Lai et al., 2001; Ribas-Fitó et al., 2001; Wade et al., 2002; Bowers et al., 2004; Winans et al., 2011; Lenters et al., 2019). Furthermore, human exposure to POPs has been associated with an increased risk of developing breast (Høyer et al., 2000; Arrebola et al., 2015; Morgan et al., 2016), testicular (McGlynn et al., 2008; Giannandrea et al., 2011) and colorectal cancer (CRC) (Howsam et al., 2004; Lee et al., 2018; Abolhassani et al., 2019). In rodents, maternal POP exposure has been shown to increase mammary, ovarian and hepatic tumorigenesis (Cameron and Foster, 2009; Filgo et al., 2015), and direct exposure increased the growth of colorectal tumors (Song et al., 2014; Hansen et al., 2018).

In 2018, more than 1.8 million people were diagnosed with CRC, and Norwegian women had the highest incidence rate in the world (39.3 age-standardized rate per 100 000) (World Cancer Research Fund, 2019). The multiple intestinal neoplasia (Min) mouse, originally with the C57BL/6J strain background (Moser et al., 1990), is much used as a model for human CRC development. This mouse has a truncation of the *adenomatous polyposis coli* (*apc*) gene product caused by a heterozygous mutation at amino acid 850 (Su et al., 1992). The *APC* gene is categorized as a tumor-suppressor gene and considered to have a gate-keeping role in CRC formation and progression (Powell et al., 1992). Furthermore, germline mutations in human *APC* are responsible for the dominantly inherited autosomal condition known as familial adenomatous polyposis (FAP) (Nishisho et al., 1991). Patients with FAP develop numerous adenomatous intestinal polyps, some of which continue the progression to malignancy (Kinzler and Vogelstein, 1996). Mutation in the *APC* gene is also apparent in a high proportion of sporadic CRC cases (Fearnhead et al., 2001).

The gut microbiome has numerous metabolic, protective and structural functions in the intestinal epithelium (Gagnière et al., 2016; Rooks and Garrett, 2016). In addition, the gut microbiota is involved in the development of CRC through interaction with the immune system, induction of oxidative stress, or production and release of metabolites associated with cancer (Louis et al., 2014; Gagnière et al., 2016; Dahmus et al., 2018). Indeed, microbiota has been shown to play a vital role in the formation of CRC in Min/+ mice (Li et al., 2012). Furthermore, intestinal microbes are sensitive to pollutants and exposure has been shown to alter microbial diversity and community structures (Choi et al., 2013; Liu et al., 2017; Xu et al., 2017; Chi et al., 2018a; Li et al., 2018; Petriello et al., 2018). However, little is known about the long-term changes in gut microbiota following maternal POP exposure.

Metabolomics provides a snapshot of the metabolic state of the organism at the time of sampling and holistically measures metabolites involved in a broad range of metabolic processes (Bouhifd et al., 2013; Patti et al., 2013). Using this approach, the multifaceted biochemical response to a stimulus and their implications for the overall health of the organism can be studied. The value of metabolomic analysis in characterizing possible toxic effects of POPs has been shown by multiple studies (Zhang et al., 2012; Jones et al., 2013; O'Kane et al., 2013; Wang et al., 2016; Carrizo et al., 2017; Pikkarainen et al., 2019).

Humans are exposed to complex mixtures of pollutants and assessing the effects of low and environmentally relevant doses is of great importance (Kortenkamp, 2014; Bopp et al., 2018; Kortenkamp and Faust, 2018). Previously, a complex mixture of polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs) and perfluoroalkylated substances (PFASs) was constructed to simulate a realistic human exposure scenario (Berntsen et al., 2017). Indeed, maternal exposure to the mixture previously resulted in plasma concentrations in mice similar to those reported in the general Scandinavian population (Hudecova et al., 2018, Berntsen et al. In prep). Furthermore, dietary exposure to the mixture was shown to promote CRC in A/J Min/+ mice (Hansen et al., 2018). In the present study, an identical animal model and POPs mixture were used to characterize the effects of maternal exposure on CRC. In addition, long-term alterations in gut metabolite composition and microbiota were investigated.

2. Animals, materials and methods

2.1. Ethical considerations

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Norwegian University of Life Sciences (NMBU) and the Norwegian Food Safety Authority (application ID: FOTS 11549). It was conducted in strict accordance with the local and national regulations for laboratory animal experiments at the Section for Experimental Biomedicine, Faculty of Veterinary Medicine, NMBU, Oslo, Norway. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/). The rodents were kept under strict Specific Pathogen Free (SPF) regulations and followed a health-monitoring program recommended by the Federation of European Laboratory Animal Science Association (FELASA; http://www.felasa.eu/).

2.2. Feed design and chemicals

The design of the POP mixture is described in Berntsen et al. (2017). In brief, the chosen composition of PCBs, OCPs, BFRs and PFASs was designed to represent concentrations in Scandinavian food products reported in studies between 2004 and 2012. Human estimated daily intake (hEDI) levels were identified for the compounds, adjusted to the mouse (weighing 25 g and consuming 3 g feed/d), and increased approximately 5000 times in concentration to account for possible background exposure and interspecies differences in xenobiotic metabolism (Walton et al., 2001). The POPs were dissolved in appropriate solvent (acetone, cyclohexane or chloroform) and added to corn oil (Jasmin fully refined, Yonca Gida San A.S., Manisa, Turkey). The solvent was evaporated under N_2-flow and oil containing the POPs was incorporated into AIN-93G mouse feed. The control diet contained corn oil from which the solvents had been evaporated. Corn oil was also used in the standard AIN-93G diet (referred to as the reference feed in Berntsen et al. (2017)). All polybrominated diphenyl ethers (PBDEs) and chlorinated compounds (including PCBs and other OCPs) were purchased from Chiron AS, Trondheim, Norway. Hexabromocyclododecane (HBCD) and all PFASs, except for perfluorohexane sulfonic acid (PFHxS, which was purchased from Santa Cruz Biotechnology Inc., Dallas, USA), were obtained from Sigma-Aldrich, St. Louis, USA. Nominal and measured concentrations in AIN-93G feed are presented in Supplementary Table A1.

2.3. Animals and husbandry

An inbred colony of A/J mice heterozygous for the Min trait (Min/+) were used for the study (Sødring et al., 2016a), as embryos with mutations in both *Apc* alleles are not viable (Moser et al., 1995). Female A/J +/+ mice were randomly assigned to an exposed (n = 26) or control (n = 28) diet at 3 weeks of age and housed pairwise in closed Type III IVC cages (Allentown Inc., Allentown, USA) awaiting breeding. Water and feed were available *ad libitum*. A/J Min/+ males were introduced at 9 weeks of age and removed after three days. Due to a low number of offspring obtained from the first round of mating, males were reintroduced at 15 and 21 weeks of age to allow for two additional periods of mating. These timepoints were chosen as the total gestation and lactation time for mice is 6 weeks. Female A/J +/+ mice were exposed to the diet containing the mixture of POPs during the entirety of the three consecutive periods of mating, gestation and lactation, and all A/J Min/+ offspring were included in the experimental groups (n = 14 and 28 for the Control and Exposed groups, respectively). An illustration of the study design is presented in Supplementary Fig. A1, and the number of offspring produced by each mating are shown in Supplementary Table A2. The genotype of the resulting offspring was determined by allele-specific polymerase chain reaction (PCR) on DNA extracted from ear punch samples (method described in Supplementary section S2). Min/+ mice showed a PCR product at 327 base-pair (bp), in addition to the +/+ allele consisting of 618 bp (Dietrich et al., 1993), visualized by gel electrophoresis.

A/J Min/+ offspring (n = 38 in total, 25 females and 13 males) were weaned at 3 weeks of age, housed in groups (3–6 mice per cage) in open Makrolon Type III cages (Techniplast, Buguggiate, Italy) and given AIN-93G feed (reference diet) and tap water *ad libitum*. All closed and open cages contained standard aspen bedding, red plastic houses and cellulose nesting material (Scanbur A/S, Karlslunde, Denmark). Open and IVC cages were changed every week or fortnight, respectively. The animal room had a 12:12 light-dark cycle, room temperature of 20 ± 2 °C, and 45 ± 5% relative humidity.

Three A/J +/+ mothers (2 from Control and 1 from the Exposed groups) were euthanized prior to completion because of wounds gained due to excessive grooming. Of the A/J Min/+ offspring, 2 females had to be sacrificed at 16 weeks of age due to large tumors in the skin of the pelvic area (both from the Exposed group). An additional 3 offspring (all females from the Control group) showed clinical signs of disease at 20 weeks of age, where one of these had a large tumor in the cecocolic junction.

2.4. Sample collection

A/J +/+ mothers were sacrificed at 27 weeks of age. Weights were recorded and ceca were collected, frozen on dry ice and stored at −80 °C for metabolomic analysis. Maternally exposed A/J Min/+ mice were sacrificed at 20 weeks of age. Approximately two pellets of feces were sampled prior to euthanasia, frozen on dry ice and stored at −80 °C until microbiota analysis. The intestines were excised and rinsed with ice-cold phosphate buffered saline solution (PBS) before being slit open longitudinally. Ceca were removed, weighed, frozen on dry ice and stored at −80 °C awaiting metabolomic analysis. The small intestine was divided into three segments of approximately equal length (proximal, middle and distal), while the colon was left intact. Fixation was done by placing the intestinal segments flat between two filter papers soaked in PBS and submerging in 10% neutral buffered formalin for no more than 24 h. Once fixed, the intestine was stained with 0.1% methylene blue (MB; M9140, Sigma-Aldrich, St. Louis, USA) dissolved in formalin for approximately 20 s and stored in 70% ethanol at 4 °C. Some of the intestines were re-stained for an additional 5–10 s due to heavy leakage of MB during storage. Liver and abdominal fat tissue were collected, frozen on dry ice and stored at −80 °C for chemical analysis. One section of the liver (left lobe) was stored in formalin for histopathological examination. Tumors from the pelvic area of two mice (Exposed group) were excised and fixed in formalin prior to histopathological evaluation.

2.5. Chemical analysis

2.5.1. Analysis of PCBs, OCPs, BFRs and PFASs

The chemical analysis of pollutants was performed at the Laboratory of Environmental Toxicology, NMBU, Oslo, Norway. The laboratory is accredited by the Norwegian Accreditation for chemical analysis of PCBs, OCPs and BFRs in biota according to the requirements of the *NS*-EN ISO/IEC 17025 (TEST 137). PFAS analysis is not included in the accreditation but is validated according to the same procedures and quality control measures. PCBs, OCPs and BFRs, or PFASs were analyzed in individual abdominal fat or liver tissue, respectively, from A/J Min/+ mice (n = 14 and n = 24 for the Control and Exposed groups, respectively).

The analytical method for PCBs, OCPs and BFRs is based on Brevik (1978) and Polder et al. (2014), while the method for PFASs is described by Grønnestad et al. (2017). Details regarding method modification for the present study is described in Supplementary section S3. The analytical quality control measures were within the accreditation requirements and, thus, approved (see details in Supplementary section S3.4.).

2.5.2. Data treatment

For compounds with detected levels in more than 60% of the samples, levels below limit of detection (LOD) were replaced with the LOD for the respective compound and further included in statistical evaluation. Compounds detected in less than 60% of the samples are only given with range in Supplementary Table S5 (PCB-52, α-chlordane and HBCD).

2.6. Identification of intestinal lesions

An inverted light microscope (CKX41, Olympus Inc., Hamburg, Germany) with a digital color camera (DP25, Olympus Inc., Hamburg, Germany) was used to examine the fixed and stained intestinal segments for lesions. Lesion size (mm^2) was calculated by measuring the diameter of the lesion using an eyepiece graticule. Tumor incidence was calculated by dividing the number of mice with tumors by the total number of mice in the group. Tumor load (mm^2) was defined as the total area of the intestine covered by lesions. The lesion scoring method has previously been described elsewhere (Sødring et al., 2016b). Colonic lesions were characterized as either flat aberrant crypt foci (flat ACF) or tumors as defined by Sødring et al. (2016a) and Sødring et al. (2016b), and further described in Supplementary section S4. Flat ACF have been shown as reliable surface biomarkers of *apc*-driven carcinogenesis in the colon (Sødring et al., 2016b). As the small intestine does not contain flat ACF, all tumors were classified as lesions. The identification of lesions was done blindly by one observer.

2.7. Histopathology

Swiss rolls were made of the intestinal segments after surface examination, as originally described by Moolenbeek and Ruitenberg (1981) and modified by Sødring et al. (2016a). Briefly, each segment was rolled lengthwise from proximal to distal, with the mucosa facing inwards, and embedded in paraffin. Colon and

small intestine were embedded in separate paraffin blocks, and sections (3 μm thick) were made at three different depths (top, middle, bottom). The sections were stained with haematoxylin and eosin (HE) and examined blindly by a certified pathologist using a light microscope. Lesions were classified as hyperplasia/dysplasia, adenomas (tumors restricted to the mucosa) or carcinomas (tumors with distinct infiltrative growth through the mucosa into the submucosa).

The liver sections were embedded in paraffin, sectioned (3 μm thick) and stained with HE. Examination was conducted blinded to exposure group using a light microscope. Pre-neoplastic and neoplastic lesions were noted as present (1) or absent (0). Non-neoplastic lesions included bile duct hyperplasia, diffuse fatty change, extramedullary hematopoiesis, chronic active inflammation, diffuse hepatocyte fatty change, hepatocyte centrilobular hypertrophy, ito cell hypertrophy and oval cell hyperplasia. Severity was scored on a scale from 0 to 4 (0 = no, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe change).

The fixed tumors from the pelvic area were embedded in paraffin, sectioned (3 μm thick), stained with HE and examined by light microscopy.

2.8. Precursor animal experiment

Ceca were excised from A/J Min/+ mice in a precursor experiment by Hansen et al. (2018) and frozen at −80 °C until metabolomic analysis. Here, mice were exposed to the mixture of POPs (identical to the one described in section 2.2) directly through feed for 10 weeks from 3 weeks of age. For more detailed information see Hansen et al. (2018).

2.9. Metabolomic analysis

2.9.1. Sample preparation

Ceca from the present study and the precursor animal experiment were stored at −80 °C and shipped on dry ice to Imperial College, London (UK) for ^{1}H nuclear magnetic resonance (NMR) spectroscopy. On the day of tissue extraction, ceca were thawed on ice. The content was transferred to a new Eppendorf tube and stored at −20 °C until sample preparation. Cecal tissue was rinsed in distilled water and 75–80 mg tissue was finely homogenized (6500 rpm 2 × 45 s) in 300 μL chloroform:methanol (2:1) using a Precellys 24 lysis machine (Bertin technologies, Montigny-le-Bretonneux, France). The homogenate was mixed with 300 μL water and spun at 13 000 rpm for 10 min at 4 °C (Micro Star 17R, VWR International, Radnor, USA). Following centrifugation, the aqueous (upper) and organic (lower) phases were transferred to individual Eppendorf tubes. The separation step was repeated by adding 300 μL of water and 300 μL chloroform:methanol to the remaining pellet. Samples were mixed and centrifuged before separating the two phases a second time. A SpeedVac (Concentrator plus, Eppendorf, Hamburg, Germany) was used to evaporate water and methanol from the aqueous phase and pelleted metabolites were kept at −20 °C until NMR spectroscopy. On the day of analysis, the aqueous phase was reconstructed in 700 μL phosphate buffer (pH 7.4) with 9:1 D_2O:H_2O containing 1 mM of the internal standard 3-(trimethylsilyl)-[2,2,3,3,-2H4]-propionic acid (TSP). Samples were spun for 10 min at 13 000 rpm (4 °C), to pellet out any debris, before 600 μL of the supernatant was transferred to a 5 mm (outer diameter) NMR tube (Bruker, Billerica, USA).

Cecal content (70–75 mg) was homogenized in 700 μL phosphate buffer (pH 7.4) with 9:1 D_2O:H_2O containing 1 mM TSP and centrifuged at 13 000 rpm for 15 min at 4 °C. A volume of 550 μL supernatant was transferred to a 5 mm NMR tube.

2.9.2. ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy on cecal content and tissue extracts was performed using a 600 MHz Bruker NMR spectrometer (600 UltraShield™) operating at 300 K. A 1D nuclear overhauser enhancement spectroscopy (1D NOESY) experimental setup was used for the tissue extracts, while a CPMG-presat was used for the content samples. All experiments were performed using 32 scans and 8 dummy scans. For a more detailed description of the procedure see the protocol by Beckonert et al. (2007).

2.9.3. Data treatment

^{1}H NMR spectra were digitized into consecutive integrated spectral regions of equal width (0.00055 ppm). Spectral regions corresponding to TSP (δ −0.2-0.2) and water (δ 4.7–4.9) were removed from all spectra (both cecal tissue and content). The region δ 3.345–3.375 was also removed from tissue spectra as it caused strong outliers in the principal component analysis (PCA). Manual alignment of the NMR spectra was performed using the recursive segment-wise peak alignment (RSPA) algorithm to reduce peak position variation between individual spectra (Veselkov et al., 2009) and spectra were normalized using a probabilistic quotient-based approach. Groupings and outliers were visualized by PCA.

2.10. Microbiota analysis

2.10.1. DNA extraction and bacterial SSU rRNA gene amplification

The analysis of microbiota was conducted at the Department of Food Safety and Infection Biology, NMBU Oslo, Norway. Genomic DNA extraction from fecal samples was performed using QIAamp PowerFecal DNA Kit (Qiagen, GmbH, Hilden, Germany), which includes a beat beating step for mechanical disruption of cells in addition to chemical lysis. The extracted DNA was measured by Qubit® 3.0 fluorometer using dsDNA Broad Range Assay Kit (Invitrogen, Eugene, USA) and sent to Eurofins Genomics (Ebersberg, Germany) for the library preparation and sequencing. The V3–V4 region of bacterial SSU rRNA gene was amplified using the primers 347F (5′-TACGGGAGGCAGCAG-3′) and 800R (5′-CCAGGGTATCTAATCC-3′). The 2x 300 bp paired-end sequencing was performed on Illumina MiSeq instrument (Illumina, San Diego, USA). The fastq files have been deposited in the NCBI Sequence Read Archive (SRA) database (SRA Accession: PRJNA565126).

2.10.2. Data treatment

The paired-end reads from sequencing were processed and analyzed using Quantitative Insights Into Microbial Ecology 2 (Qiime2) version 2018.8 (Bolyen et al., 2019). The demultiplexed paired-reads (with primers trimmed) were joined using VSEARCH (Rognes et al., 2016) Qiime2 plug-in (q2-vsearch) and quality filtering was applied on joined sequences using the default parameters. The remaining sequences were denoised using Deblur (Amir et al., 2017) at a trim length of 401 bp, which was decided based on the quality scores plots obtained in the previous steps. Overall 398 amplicon sequence variants (ASV) were obtained. The ASV method was chosen as a replacement for the operational taxonomic units (OTUs) method as it can distinguish sequences that vary with only one bp, compared to the 3% radius of variability in OTUs (Callahan et al., 2017; Caruso et al., 2019). The ASV table was filtered to include the ASVs that are present in at least 3 of the samples to remove very rare, possibly noisy sequences. From this, a total of 367 ASVs remained. Multiple sequence alignment of the representative sequences was performed using MAFFT program (Katoh et al., 2002) and the alignment was masked to remove highly variable regions and positions that were all gaps. FastTree (Price et al., 2010) was applied to the masked alignment to generate

a phylogenic tree. The tree was used to generate an unweighted UniFrac distance metric (Lozupone and Knight, 2005), which included the calculated distances between samples based on their ASV composition, and the metric was visualized by principle coordinate analysis (PCoA). Alpha diversity indices: Shannon index, Faith-phylogenetic diversity index and evenness were calculated. For alpha and beta diversity computing, a sampling depth of 37 838 was used (see the rarefaction curve in Supplementary Fig. A2). Taxonomic classification was made using q2-feature-classifier plug-in (Bokulich et al., 2018) and Greengenes 16S rRNA gene database (13_8 release) (McDonald et al., 2012). A core microbiome analysis was run with the most stringent definition that required the presence of an ASV in all the samples affiliated to one group.

2.11. Statistical analysis

All univariate statistical analyses, in addition to the creation of boxplots and stacked barplot, were conducted in R studio version 3.5.1 and 3.5.2 (R Development Core Team, 2008) using the packages 'lawstat' (Hui et al., 2008), 'reshape 2' (Wickham, 2007) and 'ggplot2' (Wickham, 2016). Because of the low number of males in the Control group (only 3 replicates), the decision was made to pool both genders for further analysis. Previous studies using the A/J Min/+ mouse model have showed no differences between genders in the formation or progression of intestinal cancer (Sødring et al., 2016a; Steppeler et al., 2017; Hansen et al., 2018).

Variable distribution and variance homogeneity were tested using Shapiro-Wilk normality test and Levene's test, respectively. For variables showing a satisfactory fit to the normal distribution (before or after transformation), a Welch two-sample *t*-test was used to test for differences between treatments. For the non-normally distributed variables (including the ordinal histopathological data), a non-parametric Mann-Whitney *U* test was conducted on untransformed data.

Liver and cecal weight, and intestinal length (small intestine and colon) were analyzed with regards to the body weight. One measurement of relative cecal weight (female, Control group) was excluded from the analysis due to the presence of a large tumor in the cecum. Body weight gain was calculated from weaning (3 weeks) until adulthood (11 weeks).

Multivariate analysis of ^{1}H NMR spectra was carried out in MATLAB version 2017a and 2018a (MathWorks, Natick, USA) using scripts developed at Imperial College (London, UK). Orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed on mean centered data (both cecal tissue and content, independently) with individual ^{1}H NMR spectra as the feature matrix and group classification (e.g. Control versus Exposed) as the predictor matrix. OPLS regression models were constructed using the ^{1}H NMR spectral data as the descriptor matrix and the scores for intestinal lesions or the relative abundance of microbial ASVs for each individual animal as the response vector. The regression models were created for each group separately. Model predictive performance (Q^2Y) was determined by 7-fold internal cross-validation and the validity and robustness of this was assessed through permutation testing (1000 permutations) (Westerhuis et al., 2008). OPLS-DA coefficients plots were used to identify discriminatory metabolic features between the groups. Significant differences between groups were found by extracting the maximum correlation coefficients (R) of identified metabolites from the valid model.

Permutational multivariate analysis of variance (PERMANOVA) was applied on the unweighted UniFrac distance metric to compare the composition of microbiota between groups. Alpha group significance method in Qiime2 was used to compare the alpha diversity indices between groups. Differential abundance testing was performed using ANCOM (Mandal et al., 2015) at different taxonomic levels.

All statistical tests had a threshold for significance at 0.05.

3. Results

3.1. Chemical concentrations

The concentrations of PCBs, OCPs, BFRs and PFASs in 20-week-old A/J Min/+ mice are presented in Table 1 and in Supplementary Table A5. LOD, recovery (%) and fat (%) are presented in Supplementary Tables A3, A4 and A5.

Overall, 20 of the 29 compounds had significantly higher concentrations in the maternally exposed mice, compared to controls. Of the PCBs, PCB-118, -138, −153 and −180 were detected in significantly higher (25–88 times) concentrations in the Exposed group, compared to Control. PCB-28, -52 and −101 did not differ in concentration between groups, and PCB-52 was not detected in any of the mice.

HCB, oxy-chlordane, *trans*-nonachlor, β-HCH and dieldrin had significantly higher (2–28 times) concentrations in the Exposed group, compared to Control. The concentrations of *p,p*'-DDE, α-

Table 1
Chemical concentrations. Concentrations of PCBs, OCPs and BFRs in abdominal fat and PFASs in liver samples from control and maternally exposed A/J Min/+ mice (sampled at 20 weeks of age). Mice were exposed during gestation and lactation to a mixture of POPs at concentrations simulating 5000 times human estimated daily intake levels. Values are presented as mean ± standard error of ng/g lipid for PCBs, OCPs and BFRs, and ng/g wet weight for PFASs. n = 13 (n = 14 for PFASs) and n = 24 for the Control and Exposed groups, respectively. Bold indicate a significant difference ($p \leq 0.05$) between groups.

Compound	Control	Exposed
Polychlorinated biphenyls (PCBs)		
PCB-28	0.448 ± 0.073	0.431 ± 0.090
PCB-101	0.459 ± 0.066	0.506 ± 0.0646
PCB-118	**3.234 ± 1.199**	**44.631 ± 12.023**
PCB-138	**18.840 ± 10.417**	**769.930 ± 101.660**
PCB-153	**20.572 ± 11.210**	**784.766 ± 99.772**
PCB-180	**6.252 ± 3.384**	**261.949 ± 31.754**
Σ 7PCBs	**49.806 ± 26.284**	**1862.214 ± 242.270**
Organochlorine pesticides (OCPs)		
p,p'-DDE	3.697 ± 1.078	7.183 ± 1.918
HCB	**78.894 ± 18.569**	**127.957 ± 10.977**
Oxy-chlordane	**8.767 ± 4.627**	**107.116 ± 16.670**
Trans-nonachlor	**3.980 ± 1.405**	**90.630 ± 11.318**
α-HCH	1.171 ± 0.057	1.298 ± 0.074
β-HCH	**3.342 ± 0.785**	**14.869 ± 1.570**
γ-HCH (Lindane)	0.209 ± 0.062	0.207 ± 0.020
Σ HCHs	**4.722 ± 0.878**	**16.374 ± 1.623**
Dieldrin	**8.259 ± 1.679**	**42.525 ± 6.419**
Σ OCPs	**108.320 ± 27.292**	**391.786 ± 43.753**
Σ OCPs + 7PCBs	**158.127 ± 53.473**	**2254.000 ± 283.595**
Brominated flame retardants (BFRs)		
BDE-47	0.474 ± 0.097	0.682 ± 0.201
BDE-99	**0.480 ± 0.139**	**7.965 ± 1.634**
BDE-100	**0.460 ± 0.195**	**12.625 ± 3.160**
BDE-153	**0.227 ± 0.138**	**18.919 ± 2.104**
BDE-154	**0.048 ± 0.008**	**5.372 ± 0.794**
BDE-209	**2.077 ± 1.444**	**3.909 ± 0.393**
Σ BFRs	**3.816 ± 1.978**	**49.473 ± 7.569**
Perfluoroalkylated substances (PFASs)		
PFHxS	**0.979 ± 0.074**	**1.718 ± 0.111**
PFOS	**5.359 ± 0.661**	**58.993 ± 2.687**
PFOA	**0.956 ± 0.221**	**8.485 ± 1.093**
PFNA	**1.713 ± 0.314**	**55.795 ± 4.448**
PFDA	**1.761 ± 0.303**	**87.294 ± 6.095**
PFUnDA	**0.659 ± 0.093**	**25.844 ± 2.143**
Σ PFASs	**11.426 ± 1.427**	**238.130 ± 13.482**

PCB-52, α-Chlordane and HBCD were detected in less than 60% of the samples and not subjected to statistical testing.

chlordane, α- and γ-HCH did not differ significantly between groups, and α-chlordane was only detected in 6 mice (all from the Control group) in concentrations below 3xLOD.

All BFRs, except BDE-47 and HBCD, had significantly higher (6–136 times) concentrations in the Exposed group, compared to Control. HBCD was only detected in 19% of the samples (2 and 5 mice from the Control and Exposed groups, respectively).

The PFASs were detected in 100% of the mice and had significantly higher (2–72 times) concentrations in the Exposed group, compared to Control.

3.2. Biometric measurements

Dietary exposure to the mixture of POPs did not change body, liver or cecal weight of A/J +/+ mothers (data not shown). Maternal exposure did not significantly affect the body weight, body weight gain, liver weight, cecal weight, or the length of the small intestine and colon in A/J Min/+ offspring. Results are presented in Supplementary Fig. A3.

3.3. Intestinal scoring

Results from scoring of colonic lesions in A/J Min/+ mice after maternal exposure to the mixture of POPs are shown in Fig. 1. The exposure significantly increased the average size of flat ACF in the colon ($p = 0.03$). However, it did not change flat ACF load or number. In contrast, the number, load and average size of colonic tumors were significantly decreased by maternal exposure ($p = 0.04$, $p < 0.01$ and $p = 0.01$, respectively). In addition, the incidence rate of colonic tumors was higher in control mice, with 78.6% and 48.8% in the Control and Exposed groups, respectively. The same trend of more lesions in the Control group was evident from the size and location distribution graphs shown in Supplementary Fig. A4. No significant differences were detected between the Control and Exposed groups for small intestinal lesion number, load or size (data not shown).

3.4. Histopathology

Swiss rolls were made from the intestines of A/J Min/+ mice and examined for hyperplastic/dysplastic lesions, adenomas and carcinomas. Results are presented in Table 2. The maternal POP exposure decreased the number of carcinomas in the small intestine ($p < 0.01$) and adenomas in the colon ($p = 0.04$). No colonic carcinomas were detected, and the maternal exposure did not change the number of intestinal hyperplasia/dysplasia.

In the liver, no pre-neoplastic or neoplastic lesions were identified in any of the mice. Furthermore, non-neoplastic lesions

Table 2
Histopathological examination of intestinal lesions. Lesions in the small intestine and colon from 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. Lesions were classified as hyperplasia/dysplasia, adenomas or carcinomas. Results are shown as mean ± standard error of lesion numbers. n = 14 and n = 24 in the Control and Exposed groups, respectively. Bold marks significant differences ($p \leq 0.05$) between groups.

	Small intestine		Colon	
	Control	Exposed	Control	Exposed
Hyperplasia/Dysplasia	10.21 ± 2.53	5.52 ± 0.94	2.43 ± 1.34	1.16 ± 0.62
Adenoma	12.43 ± 4.88	3.79 ± 1.07	**0.71 ± 0.38**	0.08 ± 0.08
Carcinoma	**1.79 ± 0.94**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

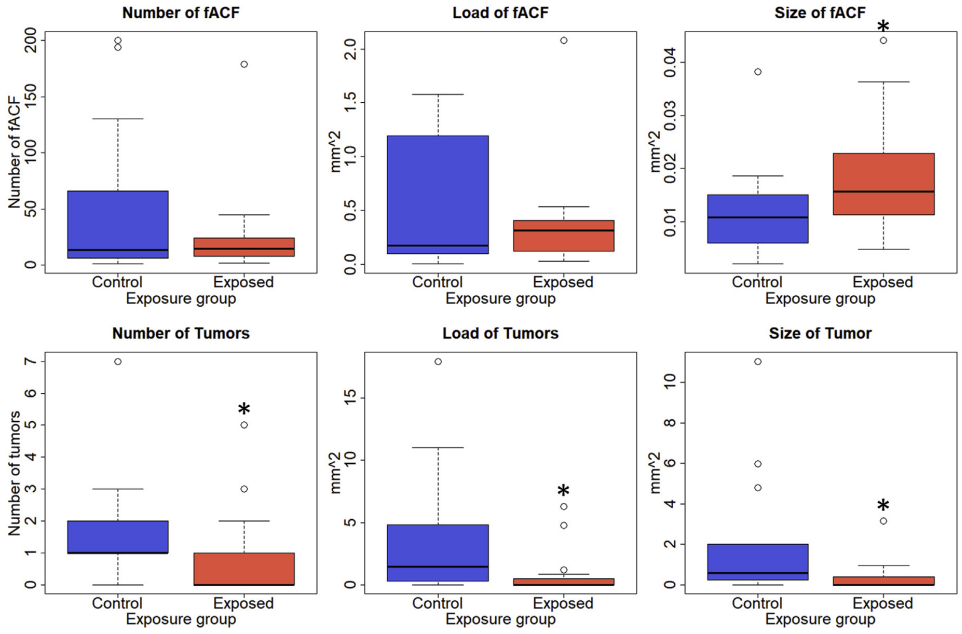

Fig. 1. Scoring of colonic lesions. Number, load (total area covered by lesions) and average size of colonic lesions in 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. Lesions were categorized as either flat aberrant crypt foci (fACF; < 30 abnormal crypts) or tumors (>30 abnormal crypts). 25th, 50th (median) and 75th percentiles constitute the boxes. Whiskers extend to 1.5 interquartile range and outliers are displayed as open circles. n = 14 and n = 24 in the Control and Exposed groups, respectively. Significance ($p \leq 0.05$) from Control is marked by *.

(extramedullary hematopoiesis and chronic active inflammation) were only found in a few individuals and were not significantly affected by maternal exposure.

Two mice from the Exposed group had tumors in the skin of the pelvic area. Histological examination identified these lesions as squamous cell carcinomas that showed infiltrative growth into the skeletal muscle. No metastases originating from the intestinal lesions were found.

3.5. Metabolic profiles of cecal tissue and content

Dietary exposure to the mixture of POPs for 10 weeks (samples from Hansen et al. (2018)) did not significantly change the metabolic profiles of either cecal tissue or content in A/J Min/+ mice. Furthermore, dietary exposure did not change the profiles of cecal metabolites (tissue and content) in A/J +/+ mothers (exposed for 24 weeks). PCA scores plots of metabolic profiles in dietary exposed A/J Min/+ and A/J +/+ mice are presented in Supplementary Fig. A5 and Fig. A6.

Interestingly, maternal exposure to the mixture of POPs changed the composition of metabolites associated with the cecal tissue in A/J Min/+ mice ($Q^2Y = 0.1127$, $p = 0.028$). The OPLS-DA coefficients plot with metabolic features for the significant model is showed in Fig. 2. Furthermore, PCA and OPLS-DA scores plots are shown in Supplementary Fig. A7.

Maternal POP exposure elevated the abundance of isovalerate ($p < 0.01$), 3-hydroxyisobutyrate ($p < 0.01$), propylene glycol ($p = 0.02$) and phosphorylcholine ($p = 0.03$), and reduced the abundance of lactate ($p = 0.02$), ethanolamine ($p < 0.01$), glycerol ($p < 0.01$) and S-adenosyl homocysteine (SAH, $p < 0.01$) in cecal tissue. Three unknown metabolites (singlets at δ 3.46 and δ 3.48 and a doublet at δ 7.68) were also significantly reduced in the cecal tissue following maternal exposure ($p < 0.01$). A heatmap of the metabolites that were significantly increased or decreased by maternal exposure is presented in Supplementary Fig. A8. In addition, multiplicities and chemical shifts of the altered metabolites are presented in Supplementary Table A6.

Maternal exposure did not affect the metabolite composition in cecal content (PCA plot shown in Supplementary Fig. A9).

3.6. Microbial diversity and composition

Maternal exposure to the mixture of POPs did not change the microbial diversity (i.e. alpha diversity) in fecal samples from A/J Min/+ mice. However, there was a significant difference in fecal bacterial composition (i.e. beta diversity) between the Control and Exposed groups ($p < 0.01$), which is displayed by a slight categorization in the PCoA scores plot (Fig. 3a).

As shown in Fig. 3b, *Bacteroidetes* and *Firmicutes* were the two main microbial phyla in the maternally exposed mice. Together these phyla accounted for 94.8% ± 1.3% and 96.4% ± 0.4% (mean ± standard error) of the total fecal microbiome in the Control and Exposed groups, respectively. The third most represented phylum was the *Proteobacteria*, with 3.1% ± 0.7% and 2.4% ± 0.3% of the relative microbial abundance, respectively. Furthermore, close to all the remaining microbial species could be classified into the phyla *Actinobacteria* and *Deferribacteres*. No significant differences in relative abundance where detected between the groups at phylum level.

Three ASVs were registered as core microbiome members in feces from the Exposed group, however, not as core members in the Control group. These included one ASV affiliated to the *Bilophila* genus, one ASV affiliated to the *Peptococcaceae* family, and one ASV affiliated to the *Mogibacteriaceae* family. In addition, two ASVs affiliated to the *Adlercreutzia* genus were identified as core members in the Exposed group, compared to only one ASV of the same genus in the Control group. All core ASVs are presented in Supplementary Table A7. When analyzing the differences in relative bacterial abundances, only one bacterial species (*Unclassified Sutterella*) was found to be significantly less abundant in the Exposed group compared to Control.

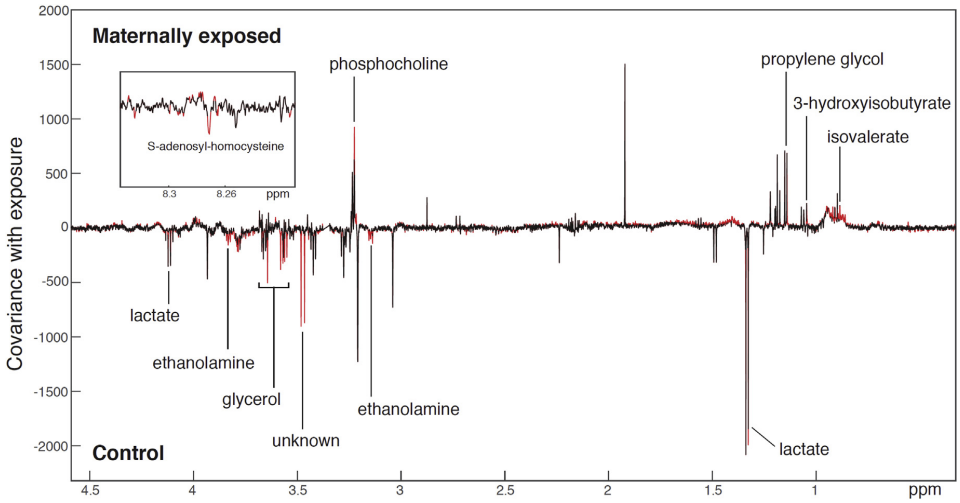

Fig. 2. OPLS-DA coefficients plot of cecal tissue extracts. Metabolomic analysis (by ^{1}H nuclear magnetic resonance spectroscopy) on cecal tissue extracts from 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. The coefficients plot is extracted from the significant model, showing a difference in metabolic profiles between the control (n = 14) and maternally exposed (n = 24) mice, and plotted back scaled onto the spectral data. Metabolites that were significantly more abundant in either groups are marked (features below or above 0 covariance with exposure for the control and maternally exposed mice, respectively). Unknown features at 3.46 and 3.48 ppm (singlets).

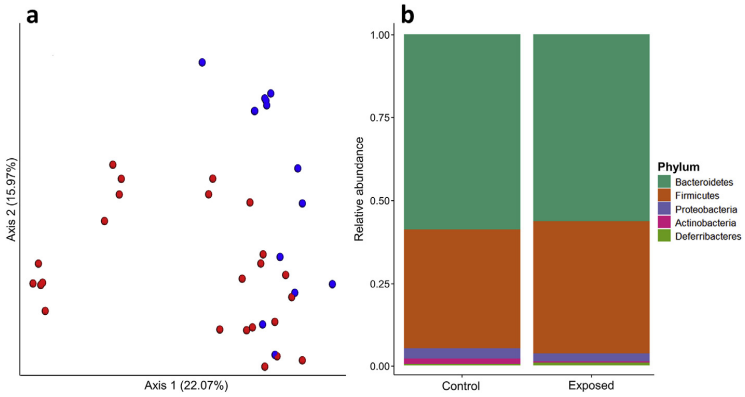

Fig. 3. Microbiota composition and relative abundance. Principal coordinates analysis (PCoA) scores plot of microbial composition (a) and relative abundance of the top 5 bacterial taxa at phylum level (b) in feces from control (blue, n = 14) or exposed (red, n = 24) 20-week-old A/J Min/+ mice. The mice were maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. The PCoA visualize the unweighted UniFrac distance metric, which includes the difference in amplicon sequence variant composition between samples.

3.7. Correlation between metabolic profiles and intestinal lesions or microbiota

A significant regression model was found between the ^{1}H NMR spectral data of cecal content and the number of colonic tumors in maternally exposed A/J Min/+ mice ($Q^2Y = 0.3728$, $p = 0.009$). When investigating the metabolic features, ethanol (quartet at δ 3.65) showed a significant positive correlation with tumor number ($p < 0.01$).

For the microbiota, a significant model was acquired in the maternally exposed mice between the ^{1}H NMR spectral data of cecal content and one of the identified ASV affiliated to the *Adlercreutzia* family ($Q^2Y = 0.1494$, $p = 0.041$). After identifying the metabolic features, butyrate (triplet at δ 0.88, $p < 0.01$), acetate (singlet at δ 1.91, $p = 0.05$) and trimethylamine (TMA, singlet at δ 2.87, $p = 0.02$) were positively correlated, while ethanol was negatively correlated ($p = 0.05$), with the abundance of the ASV affiliated to the *Adlercreutzia* family.

No other significant models were obtained between the metabolic profiles of cecal tissue or content and the scores for intestinal lesions or microbiota.

4. Discussion

The present study investigated if maternal exposure to a human relevant mixture of POPs could influence the development of colorectal cancer, intestinal metabolite composition and microbiota in A/J Min/+ mice. Maternal exposure did not cause the development of more intestinal lesions, but rather decreased the initiation and promotion of colonic tumors. Alterations were identified in metabolites associated with the amino acid, lipid, glycerophospholipid and energy metabolisms in intestinal tissue. Furthermore, maternal exposure modified fecal bacterial composition.

4.1. Chemical concentrations

POPs are known to be readily transferred from mothers to offspring (Barr et al., 2007; Fenton et al., 2009; Vizcaino et al., 2014; Winkens et al., 2017). In the present study, PCBs, OCPs and BFRs, and PFASs were detected in fat and liver tissue, respectively, from the 20-week-old A/J Min/+ mice. Twenty of the compounds had significantly higher concentrations, with levels up to 136 times higher, in the Exposed group compared to Control. This confirms that the compounds were transferred from mothers to offspring, and that the majority of the POPs accumulated and persisted in offspring until 17 weeks after end of exposure.

It is important to note that the inclusion of more than one round of mating, gestation and lactation on the dietary exposed A/J +/+ mothers might have resulted in relatively higher maternal exposure concentrations for compounds with longer half-lives in the offspring from the later pregnancies, compared to the offspring born from the first mating. However, due to unknown factors, the first round of mating produced very few A/J Min/+ offspring (1 and 4 for the Control and Exposed groups, respectively) and more replicates were need. Thus, the decision was made to include additional rounds of mating, pregnancy and lactation on the dietary exposed mothers.

As previously mentioned, the concentrations incorporated into mouse feed were approximately 5000 times hEDI levels. Recently, maternal exposure to the same mixture and dose resulted in 1–58x higher plasma concentrations of PCBs, OCPs and PFASs in 129:C57BL/6F1 mice (at 9–10 weeks of age) compared to human blood levels reported in the Scandinavian population (Hudecova et al., 2018, Berntsen et al. In prep). In the present study, lipid-adjusted values of PCBs, OCPs and BFRs were 2–35x higher than the average human blood levels (ng/g lipid) reported by Berntsen et al. (2017). Furthermore, the levels of PFOS and PFOA were only 2-fold higher in livers of maternally exposed mice than in human blood, and PFHxS had higher levels in humans than in the present study (Berntsen et al., 2017). PFOS and PFOA have also been reported as 2–7x higher in breast milk from Norwegian mothers sampled prior to 2 months post-partum than in the present study (Iszatt et al., 2019; Lenters et al., 2019). Although a comparison between PFAS concentrations in human blood and breast milk, and murine liver should be interpreted with caution, the above-mentioned studies indicate that the exposure scenario in the present study should be regarded as human relevant.

4.2. Biometric measurements

Exposure to the mixture of POPs did not affect the body or liver

weights in mice (both in dietary exposed A/J +/+ mothers and maternally exposed A/J Min/+ offspring). This result is in accordance with the results presented by Hansen et al. (2018) after 10 weeks of dietary exposure to the same mixture and dose (Low dose in Hansen et al. (2018)). However, an approximately 20 times higher dose of the same mixture decreased body weights and increased liver weights in A/J Min/+ mice (Hansen et al., 2018). Other studies have shown growth suppression in Sprague-Dawley rats caused by maternal exposure to environmentally relevant mixtures of POPs (Chu et al. 2005, 2008). However, in the present study, the dose of POPs used seemed to be too low to cause growth suppression or increased liver weights in the A/J Min/+ mice.

4.3. Intestinal cancer

Although maternal POP exposure increased the size of the smaller colonic lesions (flat ACFs), mice in the Control group had more and larger tumors in the colon. Histopathological evaluations also showed more carcinomas and adenomas in the small intestine and colon, respectively, of control mice. Thus, maternal exposure to the mixture of POPs reduced the formation and growth of colonic tumors. To the authors knowledge, this is the first study to show reduced colonic carcinogenesis due to maternal exposure to a mixture of POPs (including multiple compound classes).

Previously, PFOS has been shown to reduce intestinal tumor burden after exposure through the drinking water (8–9 weeks) and the authors speculated that PFOS both reduced tumor initiation and promotion in C57BL/6J Min/+ mice (Wimsatt et al., 2016). In addition, a mouse xenograft model showed reduced tumor growth after treatment with PFOS (Wimsatt et al., 2018), and a strong negative association was found between the likelihood of CRC diagnosis and PFOS serum concentrations in humans inhabiting the Appalachia region (Innes et al., 2014). Furthermore, Ngo et al. (2014) showed no increase in intestinal lesion formation after maternal exposure to PFOS and PFOA (in C57BL/6J Min/+ mice). Thus, the indication is that some perfluoroalkylated substances may have a protective effect and possibly reduce colorectal lesion formation and growth.

Dietary exposure (for 10 weeks) to the same mixture of POPs previously increased the formation of lesions in the colon (both flat ACFs and tumors) of A/J Min/+ mice (Hansen et al., 2018). Furthermore, Hansen et al. (2018) found a synergistic increase in CRC when combining POPs with the carcinogenic compound azoxymethane (AOM). The difference between the present study and the study by Hansen et al. (2018) is mainly the route of exposure. Exposure to pollutants during early development might alter the risk of developing cancer later in life, as this period is considered particularly sensitive to disturbances (Heindel, 2007, Heindel and vom Saal, 2009). In the present study, CRC initiation and promotion were reduced due to maternal POP exposure. However, continuous dietary exposure throughout life might increase the risk of developing CRC (as shown by Hansen et al. (2018)). Thus, the total carcinogenic potential of the POP mixture is still of concern.

There is limited knowledge about the relationship between maternal POP exposure and CRC. Apart from the studies mentioned above, only a few other studies have assessed how exposure to POPs affect CRC development in rodents. Song et al. (2014) showed that relatively low concentrations of *p,p'*-DDT promoted CRC growth through Wnt/β-catenin signaling and oxidative stress in a mouse xenograft model. Furthermore, the protective effect of fish oil on the formation of colonic ACFs in Sprague-Dawley rats was inhibited when combined with a mixture of POPs (including PCBs and OCPs) at environmentally relevant concentrations (Hong et al., 2017). Epidemiological studies have showed conflicting results between the concentrations of various POPs and the risk of developing CRC. Positive correlations between CRC risk and the levels of OCPs (such as *p,p'*-DDE, HCHs, oxy-chlordane) and PCBs (including PCB-28, -118, −138 and −180) have been found by Howsam et al. (2004), Lee et al. (2018) and Abolhassani et al. (2019). On the other hand, no associations between PCBs, OCPs or PBDEs and CRC were found in Egyptian patients (Abdallah et al., 2017), and the weight-of-evidence review by Alexander et al. (2012) concluded that there was no causal relationship between pesticide exposure and CRC.

There seem to be a complex relationship between exposure to environmentally relevant combinations of POPs and the development of CRC. Additive, antagonistic or synergistic interactions between the compounds should be expected (Bopp et al., 2018). Moreover, the relationship becomes even more complex when investigating different routes of exposure, as shown in the present study compared to the study by Hansen et al. (2018). Further studies should be conducted to explore how environmentally relevant mixtures and concentrations of POPs contribute to intestinal cancer after maternal and successive dietary exposure.

4.4. Histopathology of the liver

Maternal exposure to the mixture of POPs did not affect hepatic morphology in the A/J Min/+ mice. However, other studies have observed histopathological changes caused by mixed POP exposures. A low dose mixture of PCBs and OCPs have been shown to promote hepatic steatosis in Ob/Ob mice (Mulligan et al., 2017). In addition, hepatic hypertrophy, inflammation and vacuolation have been observed in Sprague-Dawley rats after maternal exposure to environmentally relevant mixtures of POPs (Chu et al. 2005, 2008). Although we did not see an effect in the present study, differences in sensitivity between strains and species are plausible. Thus, hepatic morphological changes should not be excluded as possible effects of maternal exposure to environmentally relevant POP mixtures.

4.5. Metabolic features of cecal tissue

Alterations in the metabolic features of cecal tissue from A/J Min/+ mice were detected after maternal exposure to the mixture of POPs. Maternal exposure increased the cecal abundance of isovalerate, 3-hydroxyisobutyrate and phosphorylcholine, which are involved in the metabolism of amino acids and phospholipids. Furthermore, the levels of propylene glycol were higher in the maternally exposed mice. Propylene glycol is commonly used as a food additive, readily absorbed over the intestines and metabolized by alcohol dehydrogenase in the liver. In addition, approximately half of the absorbed propylene glycol is excreted by the kidneys (Agency for Toxic Substances and Disease Registry, 2013; McGowan et al., 2017). Because propylene glycol is considered safe to use, it is likely that this compound occurred in the reference feed. Hence, the significantly higher concentrations in the Exposed group might indicate perturbation of metabolism (reduced function of hepatic alcohol dehydrogenase) or excretion (possible renal dysfunction) caused by early-life exposure to POPs.

The maternally exposed mice were characterized by having lower abundances of ethanolamine, glycerol, lactate and S-adenosyl homocysteine (SAH). These compounds are components in the metabolism of glycerophospholipids, lipids, energy and amino acids. Thus, early-life exposure to the mixture of POPs caused long-term perturbation in the metabolism of amino acids, lipids and energy. Furthermore, the results indicate a disruption of essential cell membrane components and possible effects on the liver and kidneys in the A/J Min/+ mice.

Recently, Pikkarainen et al. (2019) showed long-lasting and

dose-dependent alterations in metabolic pathways associated with glycerophospholipids, amino acids and carnitines in serum of adult Sprague-Dawley rats maternally exposed to PCB-180, which indicated effects on liver, neurodevelopment and behavior that were likely of developmental origin (Pikkarainen et al., 2019). Early-life exposure (postnatal days 1–5) to BDE-209 altered metabolites associated with amino acid, carbohydrate and lipid metabolism in ICR mice (Eguchi et al., 2016). Furthermore, dietary BDE-209 exposure affected the amino acid, carbohydrate and energy metabolism pathways and was associated with neurodevelopmental toxicity in adult Sprague-Dawley rats (Yang et al., 2014; Jung et al., 2016). Early-life exposure to HBCD (postnatal day 10) altered metabolites involved in aerobic energy, lipid and amino acid metabolism, and neurodevelopment in C57BL/6 mice (Szabo et al., 2017). Similar alterations were also found in adult mice after oral HBCD exposure (Wang et al., 2016). In addition, dietary exposure to PCBs (Aroclor 1254) in rats has been shown to affect cell membranes (O'Kane et al., 2013), alter fatty acid metabolism and cause mitochondrial dysfunction (Lu et al., 2010). Furthermore, combined Aroclor 1254 and phthalate (DEHP) exposure disturbed the metabolism of lipids, tryptophan and phenylalanine in serum of Kunming mice (Zhang et al., 2012). Thus, exposure to POPs has been shown to affect cell membranes and impact multiple metabolic pathways including lipid, amino acid and energy.

Maternal exposure to the mixture of POPs did not alter the abundance of metabolites in cecal content. This could be explained by the identical diet given to the Control and Exposed groups after weaning, as the content metabolites would strongly reflect components in the feed. Furthermore, no differences were detected in cecal metabolic profiles (both tissue and content) following dietary exposure to the same mixture and dose in either A/J Min/+ mice (samples from Hansen et al. (2018)) or A/J +/+ mothers. Thus, the intestinal tissue seemed to be sensitive to POPs when exposed during early developmental periods.

In summary, maternal POP exposure affected amino acid, lipid, glycerophospholipid and energy metabolism in the intestinal tissue of A/J Min/+ mice. Furthermore, the results indicate a modulation of hepatic metabolism and renal extraction of foreign compounds. Alterations in pathways of energy, lipids, and amino acids have previously been seen in CRC patients (Zhang et al., 2017). In addition, exposures to various POPs have showed effects on cell membranes in humans and rodents (O'Kane et al., 2013; Carrizo et al., 2017; Pikkarainen et al., 2019). Thus, the results presented herein underline that exposure to human relevant concentrations of POPs during early development can cause perturbations of multiple metabolic pathways. Furthermore, the affected pathways might indicate possible mechanistic connections between POP exposure and CRC development.

It is relevant to mention that the predictive performance of the model showing a significant difference in the metabolic features of cecal tissue between control and maternally exposed mice should be considered relatively low ($Q^2Y = 0.1127$). However, a significant p-value ($p = 0.028$) was obtained from permutation testing and, thus, the model was accepted as valid. The relatively low predictive performance can be explained by only small differences in metabolic features between the two groups, a plausible explanation seeing that the present study investigated dissimilarities in cecal metabolites in samples collected 17 weeks after end of exposure.

4.6. Microbial diversity and composition

Fecal microbial diversity was not affected by maternal exposure to the mixture of POPs. On the other hand, maternal exposure changed the bacterial composition in A/J Min/+ mice.

Maternally exposed mice had a lower relative abundance of one *Unclassified Sutterella* species (phylum *Proteobacteria*), compared to controls. Previously, *Proteobacteria* has been shown to be over-represented in CRC rats (Zhu et al., 2014) and human adenoma samples (Shen et al., 2010). Thus, as the control mice had more and larger colonic tumors than the maternally exposed mice, the increased abundance of this bacteria might have been caused by the CRC and not the exposure.

The relative abundance of bacteria at phylum level or the ratio between *Bacteroidetes* and *Firmicutes* were not changed by maternal POP exposure. *Bacteroidetes* and *Firmicutes* are the predominant phyla in the gut of both mice (Ley et al., 2005) and humans (Gagnière et al., 2016). Previously, dietary POP exposure has been shown to alter the ratio between *Bacteroidetes* and *Firmicutes* (Liu et al., 2017; Chi et al. 2018a, 2018b; Petriello et al., 2018), and it is well known that microbial unbalance is involved in the development of CRC (Louis et al., 2014; Gagnière et al., 2016; Dahmus et al., 2018).

Interestingly, maternal exposure to the mixture of POPs changed the core microbiome in A/J Min/+ mice. One ASV affiliated to the *Bilophila* genus (phylum *Proteobacteria*) was represented as a core member in maternally exposed mice, but not in controls. This genus includes the sulfidogenic bacteria *Bilophila wadsworthia*, and hydrogen sulfide has been suggested as a contributing factor to the development of CRC, especially when combined with an already existing DNA mutation (Attene-Ramos et al., 2006; Dahmus et al., 2018). It is a possibility that *B. wadsworthia* might represent the ASV affiliated to the *Bilophila* genus, however, identification at lower classification level was not possible due to insufficient resolution of the 16S rRNA sequencing method.

Furthermore, mice from the Exposed group had an additional ASV affiliated to the *Adlercreutzia* genus (of the subclass *Coriobacteridae*), compared to the Control group. One study has identified *Coriobacteridae* as increased in human CRC tissue, although bacteria in this subclass generally live in symbiosis with the host (Marchesi et al., 2011). The maternally exposed mice also had core members from the *Peptococcaceae* and *Mogibacteriaceae* families (of the order *Clostridiales*). Previously, a negative correlation has been shown between *Clostridiales* and intestinal tumor burden (Baxter et al., 2014), possibly due to the production of the anti-tumorigenic compound butyrate. Thus, the differences in core microbiota caused by maternal exposure to the mixture of POPs might have contributed to the lower colonic tumor burden seen in the Exposed group.

To the authors knowledge, no studies have yet investigated the long-term effects of maternal exposure to large mixtures of POPs on microbiota. However, a few studies have investigated the effects of maternal exposure on gut microbial diversity and composition. Rude et al. (2019) showed that maternal exposure to a mixture of 12 PCBs increased the relative abundances of the *Proteobacteria* phylum, *Bacteroidales S7-24* genus and *Alistipes* genus, in 4-week-old Sv129:C57BL/6 mice. Furthermore, Iszatt et al. (2019) showed that PCB-167, BDE-28, PFOS and PFOA altered gut bacterial composition and function in one-month old infants.

More studies have assessed alterations in microbial diversity and community structures after direct exposures to various POPs. A mixture of PCB-138, -153 and −180 has been shown to decrease the overall abundance of intestinal bacterial species (Choi et al., 2013), and exposure to only PCB-153 altered the microbial composition (Chi et al., 2018b), in C57BL/6 mice. Cheng et al. (2018) showed that oral administration of the Fox River Mixture of PCBs (mimicking contamination in fish from the Fox River) changed the microbial composition in C57BL/6 mice. Furthermore, an imbalance of the intestinal microbiota (dysbiosis) was found after oral exposure to an environmentally relevant concentration of PCB-126 (Chi et al.,

2018a), and PCB-126 has been linked to alterations in gut microbiota similar to those seen under the condition of chronic inflammatory diseases (Petriello et al., 2018). BDE-47 and -99 exposure decreased the alpha diversity and changed the bacterial composition of colonic microbiota in C57BL/6 mice in ways that influenced xenobiotic metabolism, caused intestinal inflammation and disrupted the absorption and metabolism of essential micronutrients (Li et al. 2017, 2018). In addition, Li et al. (2018) showed that BDE-47 and -99 perturbed bile acid metabolism (by increasing secondary bile acids) and argued the possibility of a positive correlation between PBDE exposure and an increased risk of CRC. Perturbation of microbial composition associated with bile acid metabolism has also been seen after dietary exposure to *p,p'*-DDE or β-HCH (Liu et al., 2017). Moreover, PFOS has been shown to cause dose-dependent alterations in the abundance and composition of intestinal bacteria in ERβ knockout and CD-1 mice (Xu et al., 2017; Lai et al., 2018).

Modulations of whole microbiota communities might determine carcinogenesis just as much as the presence or absence of individual bacterial populations (Arthur et al., 2012; Zackular et al., 2013). As POPs have been shown to alter the composition of intestinal microbiota, a connection could be argued between pollutant exposure and CRC through changes in the microbial community. The importance of evaluating microbiota when investigating the deleterious effects of POPs has previously been highlighted by Jin et al. (2017). However, the present study emphasize that the gut microbiota is sensitive to early-life POP exposure and that this exposure modulated the bacterial composition in a way that possibly reduced colorectal carcinogenesis. Further studies should be conducted to eluate how real-life exposure scenarios affect the formation of the microbial community.

4.7. Correlation between metabolic profiles and intestinal lesions or microbiota

A higher abundance of ethanol in cecal content was found to be positively correlated with the number of colonic tumors in A/J Min/+ mice maternally exposed to the mixture of POPs. The consumption of large amounts of ethanol increases the risk of developing several types of cancers, including CRC (World Cancer Research Fund/American Institute for Cancer Research, 2018). Furthermore, ethanol can be produced by microbial organisms and is metabolized by alcohol dehydrogenase in the liver (Holford, 1987; Louis et al., 2014). Thus, the high levels of ethanol, together with the increased amount of propylene glycol (reported in section 3.5), might indicate that early-life POP exposure reduced the function of hepatic alcohol dehydrogenase. Further studies should investigate how POPs might affect hepatic metabolism of deleterious or foreign compounds.

Maternally exposed mice with higher relative abundances of one ASV affiliated to the *Adlercreutzia* family had higher levels of butyrate, acetate and TMA, and lower levels of ethanol in cecal content. An elevation in the abundance of *Adlercreutzia* spp. has previously been positively associated with alcohol intake in mice (Xu et al., 2019). Thus, indicating a preference for ethanol as a source of energy for bacterial species in the *Adlercreutzia* family and explaining the negative correlation observed in the present study.

Butyrate is a short-chain fatty acid (SCFA), a major fermentation product in healthy adults, and heavily influenced by microbiota (Louis et al., 2014). Butyrate is known for inhibiting intestinal tumor formation. However, it might also stimulate cell proliferation due to its role as the preferred energy source for colonocytes (Roediger, 1982; O'Keefe, 2016). Acetate is another SCFA, essential in the tricarboxylic acid (TCA) cycle, and recognized as an anti-tumorigenic agent (Louis et al., 2014). Thus, both butyrate and acetate might have contributed to the lower colonic tumor burden in maternally exposed mice.

The relative abundance of microbiota with the ability to produce TMA, a component in energy metabolism, has recently been linked to increased CRC development (Xu et al., 2015; Thomas et al., 2019). Hence, higher levels of TMA in maternally exposed mice (possibly together with higher levels of butyrate) might have increased CRC development, as shown by the larger size of small colonic lesions (flat ACFs), and further complicated the relationship between microbiota and CRC.

5. Conclusions

The present study showed that maternal exposure to a mixture of POPs, resulting in concentrations comparable to humans, lowered the burden of colonic tumors in A/J Min/+ mice. In addition, maternal exposure caused long-lasting alterations in fecal bacterial composition and affected the intestinal metabolism of amino acids, lipids, glycerophospholipids and energy in ways that may have reduced CRC initiation and promotion. The results emphasize that early developmental periods are sensitive to pollutant exposures, which may alter the risk of developing CRC. Through further studies, the effects on mechanisms of intestinal carcinogenesis should be investigated after combined maternal and dietary exposures to low-dose POP mixtures. In addition, studies should be conducted to examine how early-life exposures to POPs alter hepatic metabolism and renal excretion of foreign compounds.

Declaration of competing interest

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

CRediT authorship contribution statement

Silje M. Johanson: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration. **Jonathan R. Swann:** Formal analysis, Resources, Writing - review & editing, Visualization, Supervision. **Özgün C.O. Umu:** Formal analysis, Resources, Writing - review & editing, Visualization. **Mona Aleksandersen:** Formal analysis, Resources. **Mette H.B. Müller:** Formal analysis, Resources, Writing - review & editing. **Hanne F. Berntsen:** Resources. **Karin E. Zimmer:** Resources. **Gunn C. Østby:** Investigation, Resources. **Jan E. Paulsen:** Methodology, Resources. **Erik Ropstad:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgments

The authors would like to thank the staff at the Section for Experimental Biomedicine, NMBU, for excellent support during the animal experiment, and Kristine Eraker Aasland Hansen for providing samples of cecum from dietary exposed A/J Min/+ mice for metabolomic analysis. The study was funded by NMBU.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126484.

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Appendix A. Supplementary information

Maternal exposure to a human relevant mixture of persistent organic pollutants reduces colorectal carcinogenesis in A/J Min/+ mice.

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Materials and Methods

S1. Feed design and chemicals

The chosen persistent organic pollutants (POPs), with nominal and measured concentrations in AIN-93G mouse feed, are presented in Table A1. The measured concentrations deviated with approximately 30% from nominal levels, except for the perfluoroalkylated substances (PFASs), which showed a variability of 60-70% probably due to the method of feed preparation. All nominal concentrations were within the range of the maximum allowed levels used by feed companies.

Of the compounds in the mixture, all polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and brominated flame retardants (BFRs), as well as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), are included in the Stockholm Convention on Persistent Organic Pollutants (UNEP 2008). At the present time, the remaining PFASs (perfluorohexane sulfonate [PFHxS], perfluorononanoic acid [PFNA], perfluorodecanoid acid [PFDA] and perfluoroundecanoic acid [PFUnDA]) have not yet been incorporated into the treaty. However, these compounds have been reported to bioaccumulate in humans and wildlife (Houde *et al.* 2011, Olsen 2015).

Table A1. Nominal and measured concentrations of the POPs mixture containing 29 different PCBs, OCPs, BFRs and PFASs. Nominal concentrations aimed to represent 5000 times human estimated daily intake levels calculated for a 25 g mouse consuming 3 g feed/d. The measured concentrations were analyzed after the mixture was incorporated into AIN-93G mouse feed and somewhat deviated from nominal concentrations. The table is modified from Table 1 in Berntsen *et al.* (2017).

Compound	Nominal concentrations (ng/g feed)	Measured concentrations (ng/g feed)
Polychlorinated biphenyls (PCBs)		
PCB-28	5.8	3.1
PCB-52	13.8	15.0
PCB-101	23.3	25.4
PCB-118	40.4	37.2
PCB-138	57.5	53.8
PCB-153	57.5	61.4
PCB-180	15.4	17.4
Σ 7PCBs	213.7	213.3
Organochlorine pesticides (OCPs)		
p,p'-DDE	119.6	136.0
HCB	50.0	37.4
α-chlordane	37.5	45.0
Oxychlordane	12.5	9.8
Trans-nonachlor	12.5	14.9
α-HCH	21.7	21.2
β-HCH	17.5	22.3
γ-HCH (Lindane)	23.8	31.4
Σ HCHs	63.0	74.9
Dieldrin	75.0	70.4
Σ OCPs	370.1	388.4
Σ OCPs + PCBs	583.8	601.7
Brominated flame retardants (BFRs)		
BDE-47	40.4	39.7
BDE-99	7.9	8.6
BDE-100	6.3	5.6
BDE-153	1.3	1.5
BDE-154	2.5	2.8
BDE-209	62.5	64.8
HBCD	12.5	9.9
Σ BFRs	133.4	132.9
Perfluoroalkylated substances (PFASs)		
PFHxS	4.9	1.7
PFOS	10.8	3.2
PFOA	18.3	6.0
PFNA	5.8	2.1
PFDA	7.9	3.1
PFUnDA	4.0	1.6
Σ PFASs	51.7	17.7

S2. Animals and husbandry

The study design is illustrated in Fig. A1. Female A/J +/+ mothers were exposed to the mixture of POPs through the diet from 3 weeks of age (n = 28 and 26 for the Control and Exposed groups, respectively). Mating with A/J Min/+ males occurred at 9, 15 and 21 weeks of age, during which the mothers were continuously exposed to the POPs. All maternally exposed A/J Min/+ offspring were included in the experimental groups (n = 14 and 24 for Control and Exposed respectively).

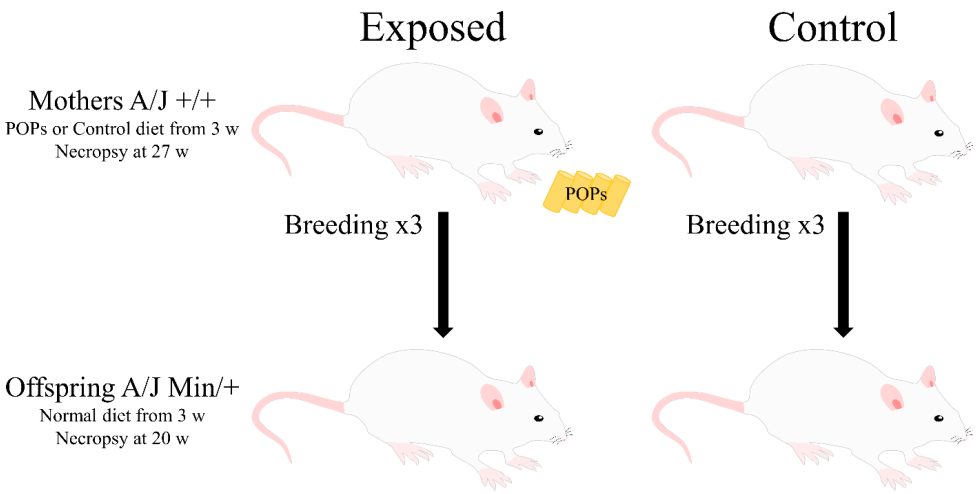

Figure A1. Study design including the two experimental groups (Exposed and Control) and the two generations of mice. A/J +/+ mothers (n = 14 and 24 for Control and Exposed respectively) were exposed to a mixture of POPs (resembling 5000 times human estimated daily intake levels) through the diet from 3 weeks of age. The mothers went through 3 consecutive periods of breeding (mating, pregnancy and lactation) at 9, 15 and 21 weeks of age, and were terminated at 27 weeks of age. The offspring (harboring the Min/+ genotype, n = 14 and 24 in the Control and Exposed groups, respectively) produced were given normal mouse diet (AIN-93G) from 3 weeks of age until necropsy at 20 weeks of age.

Due to random factors, the first mating of A/J +/+ mothers did not result in many surviving offspring. Thus, the decision was made to continue breeding as to increase the number of replicates in the experimental groups. The number of offspring produced by each mating is shown in Table A2.

Table A2. Number of A/J Min/+ offspring produced by each A/J +/+ mother pair after mating with A/J Min/+ males. A/J +/+ mothers were exposed from 3-27 weeks of age to a mixture of POPs through the diet at concentrations approximate to 5000 times human estimated daily intake levels. Mating 1, 2 and 3 occurred at 9, 15 and 21 weeks of age, respectively. By genetic inheritance, there is a 50% chance of the offspring having the Min/+ genotype. Numbers in parenthesis are the total number of offspring.

Mother pair	Exposure group	Mating 1	Mating 2	Mating 3
1	Control	0	0	0
2	Control	0	0	0
3	Control	0	5 (11)	2 (3)
4	Control	0	0	0
5	Control	0	0	0
6	Control	0	1 (5)	0
7	Control	0	0	0
8	Control	1 (4)	5 (7)	0
9	Control	0	0	0
10	Control	0	0	0
11	Control	0	0	0
12	Control	0	0	0
13	Control	0	0	0
14	Control	0	0	0
15	Control	0	0	0
1	Exposed	0	0	1 (3)
2	Exposed	0	0	0
3	Exposed	0	0	0
4	Exposed	0	0	3 (7)
5	Exposed	3 (4)	3 (6)	0
6	Exposed	0	0	0
7	Exposed	0	0	0
8	Exposed	0	5 (6)	0
9	Exposed	1 (4)	0	0
10	Exposed	0	4 (6)	2 (3)
11	Exposed	0	0	0
12	Exposed	0	0	0
13	Exposed	0 (3)	3 (5)	0

Determination of mouse genotype was conducted using allele-specific polymerase chain reaction (PCR) on DNA extracts from ear punch samples as follows. The tissue samples were suspended in 60 µL TE-buffer (10 mM Tris pH 7.4 and 0.1 mM EDTA pH 8.0) with 0.05% SDS and incubated at 95 °C for 10 min. Aliquots of Proteinase K (6 µL of 10 mg/mL, PanReac AppliChem

ITW Reagents, Chicago, USA) were added to the samples prior to incubation over night at 56 °C. The next day, samples were again incubated at 95 °C for 10 min to inactivate the enzyme. The PCR was executed using a BIO-RAD T100 Thermal cycler (BIO-RAD, Hercules, USA) with the amplification conditions at 36 cycles of 3 min at 94 °C, 15 s at 94 °C and 15 s at 56.5 °C, followed by 20 s and 7 min at 72 °C. DNA solutions (5 µL of 1:50 diluted isolated DNA) was combined with three primers (0.8 µM MAPC MT [5'-TGAGAAAGACAGAAGTTA -3'], 0.4 µM MAPC 15 [5'-TTCCACTTTGGCATAAGGC-3'] and 0.2 µM MAPC 9 [5'-GCCATCCCTT- CACGTTAG-3']) all purchased from Eurogentec (Seraing, Belguim). In addition, 0.2 mM of dNTP mix (consisting of dCTP, dGTP, dTTP and dATP, purchased from VWR International, Radnor, USA), 2.5 mM $MgCl_2$ (Applied Biosystems, Foster City, USA), PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl, Applied Biosystems, Foster City, USA) and 0.017 units GoTaq DNA polymerase (Promega Corporation, Madison, USA) were added for a final reaction volume of 10 µL. The PCR product was visualized by gel electrophoresis through a 2.2% agarose gel (Lonza FlashGel system, Basel, Switzerland).

S3. Analysis of PCBs, OCPs, BFRs and PFASs

S3.1. Chemicals

Cyclohexane, acetone and methanol of HPLC quality were supplied from VWR Chemicals, VWR International S.A.S, Radnor, USA. Purified water was obtained from a Milli-Q Gradient A10 water system (Millipore, Bedford, USA). Primary standards were supplied from Ultra Scientific, North Kingstown, USA (PCB-29, -112 and -207) and Cambridge Isotope Laboratories Inc., Tewksbury, USA (BDE-77, -119 and -181 and 13C12-BDE-209). H_2SO_4 (≥ 97.5%) was supplied from Sigma-Aldrich, Saint-Louis, USA. Certified Reference Materials (CRM) (CRM 2525, 350) were supplied by Cerilliant Corporation, Round Rock, USA. Interlaboratory tests (human serum) were supplied by Arctic Monitoring and Assessment Program (AMAP), Québec, Canada. According to the accreditation requirements, all chemicals are routinely quality tested.

S3.2. Analysis of PCBs, OCPs and BFRs

The analytical method is based on Brevik (Brevik 1978) and Polder *et al.* (2014), with further modifications described herein. During the analytical procedure the samples were protected from daylight to avoid degradation of the BFRs. One gram of homogenized fat tissue (homogenized

using a T25 Ika Ultra-Turrax®) was weighed in centrifuge tubes. The following internal standards were added: PCB-29, -112 and -207 (Ultra Scientific, North Kingstown, USA); and brominated diphenyl ether (BDE)-77, -119 and -181 and 13C12-BDE-209 (Cambridge Isotope Laboratories Inc., Tewksbury, USA). After adding solvents and distilled water, lipids were extracted twice with cyclohexane and acetone (3:2) using an ultrasonic homogenizer followed by centrifugation and separation. The lipid determination was done gravimetrically using 1 mL aliquot of the lipid extract. The removal of lipids was performed using ≥ 97.5% H_2SO_4 (Sigma Aldrich, Missouri, USA). All extracts were concentrated approximately with a factor 10 by careful evaporation under N_2, before gas chromatography (GC) analyses.

Detection of the OCPs (*p,p'*-dichlorodiphenyldichloroethylene [*p,p'*-DDE], hexachlorobenzene [HCB], α-chlordane, oxy-chlordane and *trans*-nonachlor, α-, β- and γ-hexachlorocyclohexanes [HCHs] and dieldrin) and PCBs (IUPAC nos.: PCB-28, -52, -101, -118, -138, -153, -180,) were performed on a high resolution (HR) GC (Hewlett Packard HP 6890 Series, USA) with MS and ECD detectors (Agilent Technologies, 5975c inert XL EI/CI MSD triple axis detector, USA). The separation and identification of the compounds were performed on a DB-5 MS column (60 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific). The temperature program was: 90 °C (2 min hold); 25 °C/min increase to 180 °C (2 min hold); 1.5 °C/min increase to 220 °C (2 min hold); and 3 °C/min increase to 275 °C (12 min hold) and 25 °C/min increase to 300 °C (4 min hold). The total run time was 71.6 min. The target ions are given in Table A3.

Table A3. Overview of target ions (m/z), lowest level of detection (LOD; ng/g wet weight) and relative recovery (%) of PCBs, OPCs and BFRs in abdominal fat from 20-week-old A/J Min/+ mice. All compounds were analyzed in electron capture negative ionization (ECNI) in selected ion monitoring (SIM) mode.

Compounds	Target ion	LOD	Recovery
Polychlorinated biphenyls (PCBs)			
PCB-28	291.9	0.029	99
PCB-52	291.2	1.47	83
PCB-101	325.9	0.016	110
PCB-118	325.9	0.008	98
PCB-138	359.9	0.008	96
PCB-153	359.9	0.008	98
PCB-180	395.8	0.008	97
Organochlorine pesticides (OCPs)			
p,p'-DDT	71	0.045	114
p,p'-DDE	317.9	0.034	111
p,p'-DDD	248	0.225	109
HCB	283.8	0.008	96
α-Chlordane	409.8	0.008	101
Oxychlordane	351.8	0.016	60
Trans-nonachlor	443.8	0.008	101
α-HCH	71	0.015	113
β-HCH	71	0.026	117
γ-HCH (Lindane)	71	0.019	112
Dieldrin		0.114	142
Brominated flame retardants (BFRs)			
BDE-47	79/81	0.008	90
BDE-99	79/81	0.011	96
BDE-100	79/81	0.012	101
BDE-153	79/81	0.016	98
BDE-154	79/81	0.012	99
BDE-209	495/497	0.045	131
HBCD	79/81	0.114	231

The detection of BFRs (BDEs -47, -99, -100, -153, -154 and hexabromocyclododecane [HBCD]), were performed on a HRGC (Agilent 6890 Series GC system, USA) coupled with a low resolution (LR) mass spectrometer (MS; Agilent 5973 Network Mass Selective Detector, USA) configured with a split/splitless injector (Agilent Technologies, Santa Clara, USA). The separation and identification of the compounds were performed on a DB-5 MS column (30 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific). The temperature program was: 90 °C (1 min hold); 25 °C/min increase to 180 °C; 2.5 °C/min increase to 220 °C (1 min hold); and 20 °C/min increase to 320 °C (10 min hold). The total run time was 36.6 min.

For detection of BDE-209, 10 µL were injected on a HRGC–MS (Agilent 6890 Series/5973Network) configured with a programmable temperature vaporization (PTV) injector (Agilent Technologies). The separation and identification were performed on a DB-5-MS column (10 m, 0.25 mm i.d., 0.10 µm film thickness; J&W Scientific, Agilent Technologies). The temperature program was: 80 °C (2 min hold); 30 °C/min increase to 315 °C (6 min hold). The total run time was 15.83 min. Target ions for all analyzed BFRs are presented in Table A3.

S3.3. Analyses of PFASs

PFASs were analyzed in liver tissue as these compounds have a higher affinity to proteins than lipids (Klevens and Ellenbogen 1954, Qin *et al.* 2010). The analytical procedure is described by Grønnestad *et al.* (2017). In brief, 0.5 g of homogenized liver tissue were weighed in Falcon centrifuge tubes (VWR International, LLC Radnor, USA) and spiked with internal standards containing a ^{13}C-labeled perfluoroalkyl mix (Wellington Laboratories). The samples were extracted twice with methanol (MeOH). For lipid removal, active carbon (EnviCarb) was used. Separation and detection of PFAS (PFHxS, PFOS, PFOA, PFNA, PFDA and PFUnDA) were performed using a high-performance liquid chromatography (HPLC) with a Discovery C18 column, connected to a C18 pre-column (Supelco, Sigma-Aldrich, Oslo, Norway) and a liquid chromatography (LC) tandem mass spectrometry (MS-MS) (API 3000, LC/MS/MS System). The target ions are given in Table A4.

Table A4. Overview of precursor and product ion (m/z), lowest level of detection (LOD; ng/g wet weight) and relative recovery (%) of PFASs in liver tissue from 20-week-old A/J Min/+ mice. All compounds were analyzed in electrospray ionization (ESI) and negative ionization mode.

Compounds	Precursor/Product ion	LOD	Recovery
PFHxS	398.9/80	0.23	103
PFOS	498.9/99	0.15	113
PFOA	413/369	0.19	106
PFNA	463/419	0.15	113
PFDA	513/469	0.097	116
PFUnDA	563/519	0.066	118

187 *S3.4. QA/QC PCBs, OCPs, BFRs and PFASs*

188 For analyses of PCBs, OCPs and BFRs, the analytical batches included one non-spiked and two
189 spiked samples of swine fat, three blanks (solvents), and one harp seal blubber (*Pagophilus*
190 *groendlandicus*) as internal reference material (IRM). For analyses of PFASs, the analytical
191 series included one non-spiked (blind) and two spiked swine liver samples and three blanks
192 (solvents).

193 For PCBs, OCPs, BFRs, PFASs, the lowest level of detection (LOD) for individual compounds
194 was defined as three times the noise level for each compound. The LOD (ng/g wet weight) and
195 relative recoveries are given in Table A3 and Table A4. If the relative recoveries were outside
196 the range of 70-130%, the compounds were corrected for recoveries (herein, this applied to oxy-
197 chlordane, dieldrin, BDE-209 and HBCD). Positive procedural blanks were found for some
198 compounds. If the blank concentrations were consistent for all blank samples, results were
199 corrected for blanks. The analytical quality was approved by satisfactory quality control
200 measures, and results were within the accepted ranges for the analysed Certified Reference
201 Materials (CRM 2525: Fish containing PCBs, OCPs, BFRs (Cerilliant Corporation, Round Rock,
202 USA) and CRM 350: Fish oil containing PCBs (Commission of the European Communities,
203 Belgium)) and interlaboratory tests (AMAP: human blood containing PCBs, OCPs, BFRs,
204 PFASs (Québec, Canada)).

205 The percentage of fat was used for adjusting the concentrations of the lipid-soluble PCBs, OCPs
206 and BFRs (shown in Table A5). One sample from the Control group was lost during preparation
207 and not analysed for PCBs, OCPs and BFRs. Thus, the Control group had 13 replicates for these
208 analytes.

209 *S4. Identification of intestinal lesions*

210 As previously described by Sødring *et al.* (2016a) and Sødring *et al.* (2016b), flat aberrant crypt
211 foci (flat ACF) were recognized by the difference in color when compared to surrounding
212 epithelia. In addition, flat ACFs have compressed luminal openings and enlarged crypts (which
213 gives them a gyrus-like presentation) and they usually lie flat against the surrounding epithelium.
214 The gyrus-like pit patterns are important because of the similarity in coloration of flat ACFs with
215 other structures, such as lymphoid aggregates, after MB staining.

Colonic lesions were characterized as either flat ACF or tumors, with tumors being lesions containing more than 30 abnormal crypts (covering more than approximately 0.4 mm^2), and usually prominent from the intestinal wall. The smallest size lesions had only 1-4 crypts, and one crypt has previously been measured to 0.002 mm^2 (Sødring *et al.* 2016b). Therefore, lesions with sizes ranging from 0.002 to 0.008 were identified as the first size class. Using a logarithmic scale, the remaining lesions were classified into four size classes; 0.009-0.064 mm^2, 0.065-0.512 mm^2, 0.513-4.096 mm^2, and > 4.097 mm^2.

S5. Microbiota analysis

The rarefaction curve from alpha and beta computing of gut microbiota is presented in Fig. A2. This curve showed that all feces microbiota samples from the A/J Min/+ mice reached satisfaction in terms of observed phylotypes (amplicon sequence variants [ASV]), and a sampling depth of 37838 was chosen for the alpha and beta diversity computing.

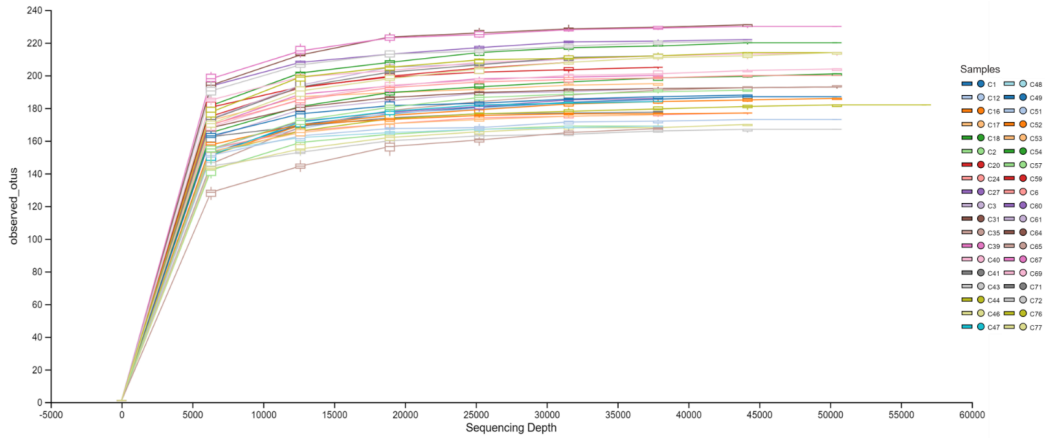

Figure A2. Rarefaction curves connecting the alpha diversity metric distribution (observed amplicon sequence variants) of each sample across the increasing sequencing depth. Bacterial 16S rRNA gene sequencing was conducted on feces from 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels (n = 14 and n = 24 in the Control and Exposed groups, respectively). Boxes extend from the 25th, through the median (50th), to the 75th percentile. The lower and upper whisker are at the 9th and 91st percentiles, respectively. Outliers are not shown.

S6. Statistical analysis

Size and location distribution graphs (Fig. A4) were made using Excel 2013 (Microsoft Office, Redmond, USA).

Results

S7. Chemical concentrations

Wet weight concentrations of PCBs, OCPs, BFRs and PFASs (ng/g) in 20-week-old A/J Min/+ mice are presented in Table A5.

Table A5. Median, mean, standard error (SE), minimum and maximum values of fat (%) and concentrations (ng/g wet weight) of PCBs, OPCs, BFRs and PFASs in 20-week-old A/J Min/+ mice. The mice were exposed maternally at concentrations approximate to 5000 times human estimated daily intake levels. PCBs, OCPs and BFRs were analyzed in abdominal fat while PFASs were analyzed in liver tissue. n = 13 (n = 14 for PFASs) and n = 24 for the Control and Exposed groups, respectively.

	Median		Mean		SE		Min		Max	
Compounds	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Fat%	81.5	68.5	74.2	69.1	5.9	2.4	14.9	50.6	92.1	90.7
Polychlorinated biphenyls (PCBs)										
PCB-28	0.308	0.208	0.291	0.290	0.035	0.059	0.036	0.153	0.510	1.609
PCB-52*							<LOD	<LOD	<LOD	<LOD
PCB-101	0.308	0.259	0.299	0.353	0.021	0.046	0.173	0.179	0.419	1.217
PCB-118	1.384	26.523	1.660	29.004	0.270	7.904	0.466	0.887	3.969	188.652
PCB-138	6.534	417.773	7.835	505.916	1.714	62.055	2.120	162.677	21.274	1636.696
PCB-153	7.021	424.317	8.522	516.092	1.759	60.499	2.597	204.662	22.951	1580.072
PCB-180	1.807	146.036	2.549	171.782	0.518	18.955	0.782	74.629	6.908	481.710
Σ 7PCBs	16.818	1014.634	21.186	1223.438	4.113	147.528	6.975	458.233	54.025	3889.957
Organochlorine pesticides (OCPs)										
p,p'-DDE	1.967	2.366	2.228	4.835	0.550	1.289	0.398	0.512	8.289	28.00
HCB	44.119	84.150	46.797	87.019	2.915	7.184	36.130	38.199	70.255	227.043
α-Chlordane*							<LOD	<LOD	0.026	<LOD
Oxy-chlordane	2.293	53.742	3.767	70.898	0.679	10.617	1.636	31.452	9.539	288.333
Trans-nonachlor	1.596	51.602	2.082	60.462	0.255	7.101	1.022	25.795	3.623	192.261
α-HCH	0.897	0.839	0.844	0.895	0.067	0.056	0.252	0.575	1.092	1.913
β-HCH	1.704	7.786	2.095	10.415	0.347	1.151	0.988	3.519	5.495	22.913
γ-HCH	0.111	0.120	0.116	0.139	0.005	0.013	0.095	0.093	0.142	0.391
Σ HCHs	2.644	8.706	3.054	11.449	0.367	1.194	1.561	4.225	6.570	25.217
Dieldrin	5.099	22.843	5.502	29.859	0.908	4.488	<LOD	6.989	14.414	85.248
Σ OCPs	57.883	232.631	63.429	264.521	4.681	28.023	46.164	112.041	104.869	819.857
Σ OCPs + 7PCBs	71.001	1220.849	84.615	1487.959	8.075	173.646	53.139	570.275	147.137	4709.813
Brominated flame retardants (BFRs)										
BDE-47	0.328	0.121	0.319	0.459	0.046	0.137	0.044	0.012	0.598	2.417
BDE-99	0.282	3.907	0.276	5.110	0.018	1.095	0.177	0.157	0.386	28.043
BDE-100	0.201	6.952	0.234	8.142	0.035	2.083	0.072	0.060	0.461	50.652
BDE-153	0.093	10.641	0.126	11.802	0.023	1.320	0.041	0.082	0.286	31.835
BDE-154	0.040	3.192	0.045	3.388	0.005	0.530	<LOD	<LOD	0.074	13.993
BDE-209	0.453	2.281	0.654	2.525	0.202	0.259	0.164	0.699	2.893	6.232
HBCD*							<LOD	<LOD	0.866	0.365
Σ BFRs	1.376	29.803	1.600	32.593	0.244	4.811	0.658	11.043	4.091	132.260
Perfluoroalkylated substances (PFASs)										
PFHxS	0.969	1.646	0.979	1.718	0.0741	0.11079	0.599	0.676	1.525	2.894
PFOS	4.276	62.620	5.359	58.993	0.66069	2.68653	3.093	31.852	10.575	77.429
PFOA	0.563	5.487	0.956	8.485	0.22128	1.09302	0.350	3.238	3.244	24.754
PFNA	0.961	47.378	1.713	55.795	0.31364	4.4478	0.636	29.437	3.893	105.595
PFDA	1.096	78.979	1.761	87.294	0.30322	6.09532	0.740	47.339	4.278	165.273
PFUnDA	0.510	22.163	0.659	25.844	0.09305	2.14293	0.278	12.658	1.308	54.136
Σ PFASs	8.334	217.618	11.426	238.130	1.427	13.482	6.370	153.665	22.176	409.969

*PCB-52 were not detected in any of the samples and replaced with 0 to be included in the Σ 7PCBs. α-Chlordane and HBCD were detected in 16% and 19% of the samples, respectively, and levels below LOD were replaced with 0 to be included in the Σ OCPs and Σ BFRs.

S8. Biometric measurements

Measurements including body weight, cecal and liver weight (relative to body weight), and length of the small intestine and colon in A/J Min/+ mice maternally exposed to the mixture of POPs are presented in Fig. A3. No significant differences were found between the Control and Exposed groups in any of the parameters.

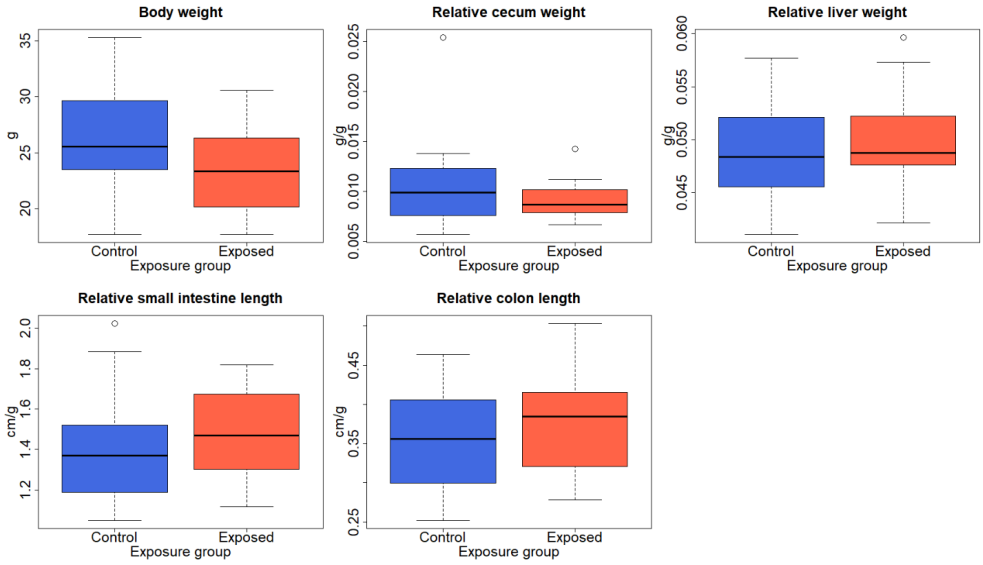

Figure A3. Biometric measurements of 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. Cecal and liver weights, and small intestine and colon length, were divided by the body weight to obtain the relative values. 25th, 50th (median) and 75th percentiles constitute the boxes. Whiskers extend to 1.5 interquartile range and outliers are displayed as open circles. n = 14 and n = 24 for Control and Exposed groups, respectively.

S9. Intestinal scoring

Figure A4 show the size and location distribution of the average number of lesions in the small intestine and colon of A/J Min/+ mice maternally exposed to a mixture of POPs. A slightly higher

number of small intestinal and colonic lesions were evident in the control mice. In addition, more lesions were seen in the distal parts of the small intestine and colon.

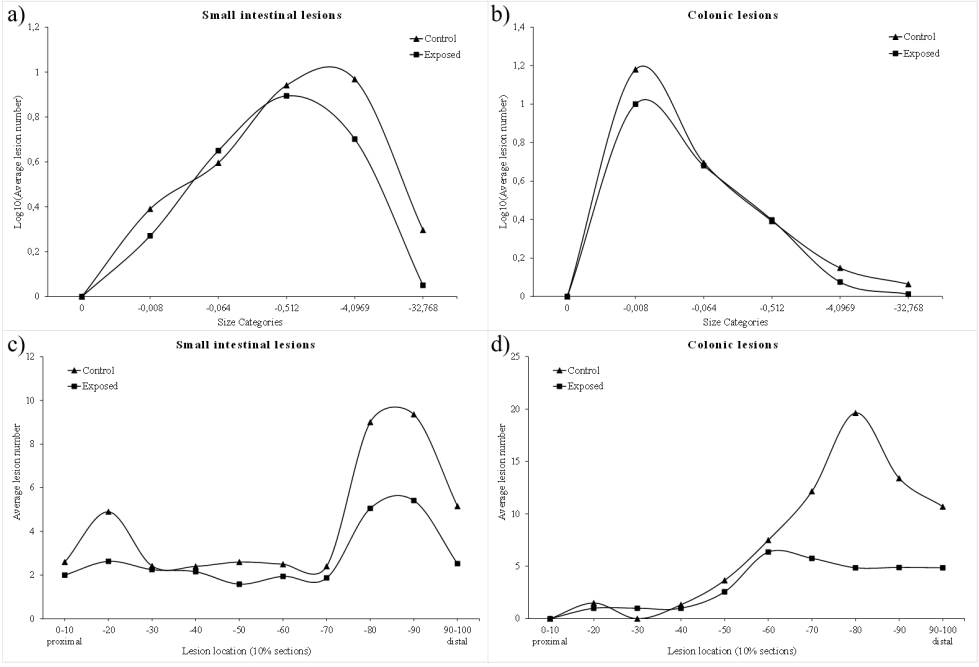

Figure A4. Size (a and b) and location (c and d) distribution of average small intestinal (a and c) and colonic (b and d) lesions in 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. Size categories are described in Sødring *et al.* (2016b). n = 14 and n = 24 for Control and Exposed groups, respectively.

S10. Metabolic profiles of cecal tissue and content

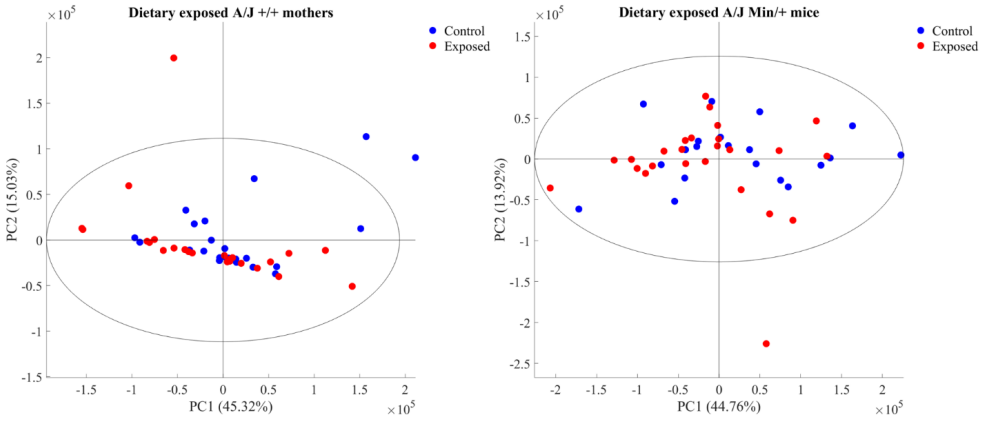

Figure A5. PCA scores plots of ^{1}H NMR spectra from cecal tissue extracts of control and dietary exposed A/J +/+ and A/J Min/+ mice. The mice were exposed through AIN-93G feed to a mixture of POPs at concentrations resembling 5000 times human estimated daily intake levels. A/J +/+ mice (n = 23 in both groups) were exposed for 24 weeks and went through three periods of pregnancy and lactation. A/J Min/+ mice (n = 20 and n = 25 for the Control and Exposed groups, respectively) were exposed for 10 weeks in the study by Hansen *et al.* (2018). The ellipse represents the Hotelling T^2 (multivariate generalization of the Student's t-test).

The PCA scores plots of metabolic profiles in dietary exposed A/J +/+ mothers (from the present study) and A/J Min/+ mice (from the study by Hansen *et al.* (2018)) are presented in Fig. A5 (cecal tissue extract) and Fig. A6 (cecal content). No categorization was visible between the Control and Exposed groups in any of the plots.

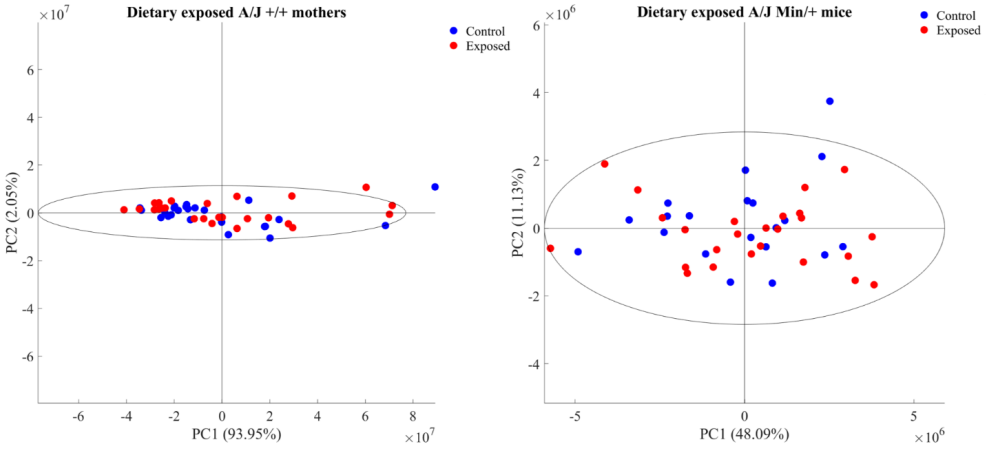

Figure A6. PCA scores plots of ^{1}H NMR spectra from cecal content of control and dietary exposed A/J +/+ and A/J Min/+ mice. The mice were exposed through AIN-93G feed to a mixture of POPs at concentrations resembling 5000 times human estimated daily intake levels. A/J +/+ mice (n = 26 and n = 25 for the Control and Exposed groups, respectively) were exposed for 24 weeks and went through three periods of pregnancy and lactation. A/J Min/+ mice (n = 20 and n = 25 for the Control and Exposed groups, respectively) were exposed for 10 weeks in the study by Hansen *et al.* (2018). The ellipse represents the Hotelling T^2 (multivariate generalization of the Student's t-test).

Furthermore, Fig. A7 present the scores plots from the PCA and OPLS-DA models of the metabolic profiles in cecal tissue samples from maternally exposed A/J Min/+ mice sampled 17 weeks after ended exposure. No strong categorization of the two groups was visible in the PCA scores plot and only a slight separation along the first component (describing the between group variation) was seen in the OPLS-DA cross-validated scores plot.

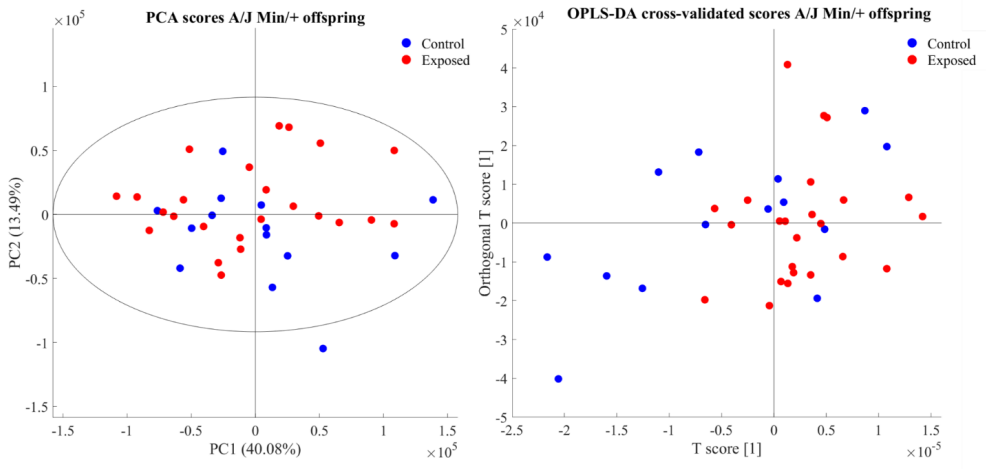

Figure A7. PCA scores plot (left) and OPLS-DA cross-validated scores plot (right) of ^{1}H NMR spectra from cecal tissue extracts of control and exposed 20-week-old A/J Min/+ mice. The mice were maternally exposed to a mixture of POPs at concentrations resembling 5000 times human estimated daily intake levels. The ellipse in the PCA scores plot represents the Hotelling T^2 (multivariate generalization of the Student's t-test). The OPLS-DA model giving the values for the cross-validates scores plot had a predictive performance (Q^2Y) of 0.1127 and p = 0.028 after 1000 permutation testing. n = 14 and n = 24 in the Control and Exposed groups, respectively.

A heatmap of metabolites significantly altered by maternal exposure to the mixture of POPs is presented in Fig. A8. 3-hydroxyisobutyrate, isovalerate, phosphorylcholine and propylene glycol were elevated, while ethanolamine, glycerol, lactate and S-adenosyl homocysteine were depressed, in 20-week-old maternally exposed A/J Min/+ mice. Furthermore, the multiplicities and chemical shifts of the metabolites are presented in Table A6.

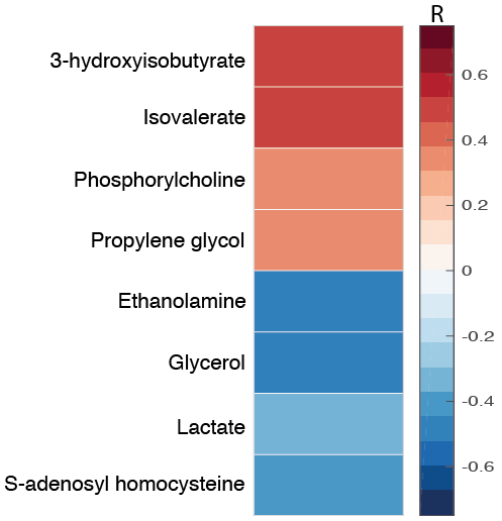

Figure A8. Heatmap of metabolites significantly ($p \leq 0.05$) altered by maternal exposure to a mixture of POPs at concentrations resembling 5000 times human estimated daily intake levels. Maximum correlation coefficients (R) were extracted from the significant OPLS-DA model constructed from ^{1}H NMR spectra of cecal tissue extracts from 20-week-old A/J Min/+ mice. n = 14 and n = 24 in the Control and Exposed groups, respectively.

Table A6. Multiplicity and chemical shift (δ) of metabolites in cecal tissue from 20-week-old A/J Min/+ mice that were significantly altered by maternal exposed to a mixture of POPs at concentrations approximate to 5000 times human estimated daily intake levels. Metabolites included were identified in the coefficient plot from the ($p \leq 0.05$) OPLS-DA (model predictive performance [Q2Y] = 0.1127 and p = 0.028 from 1000 permutations). n (total) = 38, n = 14 and 24 in the Control and Exposed groups, respectively.

Metabolite	Multiplicity	δ
Isovalerate	Doublet	0.92
Propylene glycol	Doublet	1.14
3-hydroxyisovalerate	Singlet	1.25
Lactate	Doublet	1.33
Ethanolamine	Triplet	3.15
Phosphorylcholine	Singlet	3.22
Glycerol	Doublet of doublets	3.58
S-adenosyl homocysteine	Singlet	8.27

No separation between the Control and Exposed groups was visible in the PCA scores plot of ^{1}H NMR spectra from cecal content in 20-week-old A/J Min/+ offspring maternally exposed to the mixture of POPs (Fig. A9).

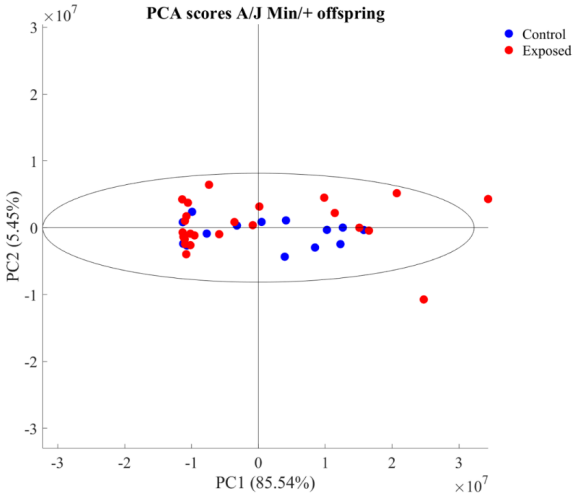

Figure A9. PCA scores plot of ^{1}H NMR spectra from cecal content of control and maternally exposed 20-week-old A/J Min/+ mice (offspring). The mice were maternally exposed to a mixture of POPs at concentrations resembling 5000 times human estimated daily intake levels. The ellipse in the PCA scores plot represents the Hotelling T^2 (multivariate generalization of the Student's t-test). n = 14 and n = 24 in the Control and Exposed groups, respectively.

S11. Microbial diversity and composition

All ASVs identified in fecal samples from 20-week-old maternally exposed A/J Min/+ mice are presented in Table A7. The table shows ASVs that were exclusive or shared between the Control and Exposed groups.

Table A7. Core amplicon sequence variants identified in the core microbiota analysis of fecal microbiota from 20-week-old A/J Min/+ mice maternally exposed to a mixture of POPs at concentrations resembling 5000 times human estimated daily intake levels. Features found in both, only the Control (n = 14) or only the Exposed (n = 24) groups are presented.

	Feature ID	Taxonomy
Core features found both in CONTROL and EXPOSED groups	acde4ca417d6579eb9ae519108c4fa59	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__
	61b8b812e012774b26f2cc438d55502c	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__; s__
	a423d39e55d929a9ec01b45c5e8da42c	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__; s__
	113a8eaca2c6c0bf755aeb11f6922f79	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	56ed34c092b72745a44d40a7eebba299	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	acaed66f38a8a35e3713608a56802310	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	3c85c7b9193e15dcb202277fbad56654	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
	fed9ad98a3240bafa2818e2fb6ae859a	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
	5a3910c1d965b1064ea4003c25c6259b	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	f9c8ac317ce0569424c4402df8ccc4af	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__
	1ca53a7bd4854e158ec19e5056047689	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	0e0c7ed6bfd2d156f3cd3ee546c60521	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
	7e8bfe047d1a698a8878004ae8ee2e09	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__
	40f97a1972cc12e3199e5d0dee0f49b3	k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Desulfovibrionales; f__Desulfovibrionaceae; g__Desulfovibrio; s__
	deb94462bf8466f08a62477d756ef2e5	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	544349ad01c94b2ee3da6971c4ff5098	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	647384be8fef33d612eb5ff0302dfa33	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	05f1992ceb34d275468fda5800e28e70	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__[Ruminococcus]; s__gnavus
	bb758ffff346a4ec0cec367033f9a54a	k__Bacteria; p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__Adlercreutzia; s__
	9c5c71b3392880c4351feb7cf9241995	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Parabacteroides; s__distasonis
	20b29dca91c75af85d0b98d2125d02e8	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	8d1350959f1951200bea146d75dbd40e	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__
	f0c97db43fc284ba460db897f73db191	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	90f711377cee18160056656b5522ff775	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	8c32f8e471e76649379e3bd3a8848c34	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	a4f39fc8baaa588b9908e9ea01323c87	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Parabacteroides; s__distasonis
	4bca99987f10c19f27db86e43185fb0e	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__
	64c2105f03b8c317f4bc9fceaedc0520	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
	f1576641318ac3a1244d33096d335278	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
Core features found only in CONTROL group	d5dadfef917fb5f2e0d365da82be40c7	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
	3a414966a1ecc488c668ef1418a87eb5	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	927058f511173b9be980782ca86db779	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	e1f6ccf969d447ed55cb1234832ad2e7	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	d1ec173f8dc14cf839adf5f53d6944c1	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	053ee5741ca0a5090fc8e3a3af21f8d0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	50226329febdcd6d78d627a62439fabd	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	4d3a2efd043e3f0ccae4c52eaac0143d	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	652c43e2ac9ba8e003805747357bed8f	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
	a2812a52910c5b7a8320f9b38a819770	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	a406186b1356e8df3a20ed59874af46c	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	252e5a6d9e7f090d55b14b136476e7ab	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	712a4f92e1cb8d7078175cc978866df1	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
	a377dd6a55492d46b98f1f5d35299d8f	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__
	b3ff84690ee2235c0ffa6108e684545e	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
Core features found only in EXPOSED group	b6cf6b21e1173260963ae23c35c71d21	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__
	b4c586d63bf06ded3b499634dcebc3fe	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
	36238d903b18d8517a53c1b53a1a8aca	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
	130026a7cee134ec54bd5c7ad7f67579	k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Desulfovibrionales; f__Desulfovibrionaceae; g__Bilophila; s__
	e568867aacb3422f2db4e72db0349c81	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	ea66c71ebff642aa7b0b72918a0ea07a	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
	c094f3fbcd13007966e524bc8364d1fa	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__
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	1e0c47eeb2920d508ba4e44092abea2c	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	fcc763f1be406185af34ff57853539c7	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	5b18b25e5a62eafec41082f08d161292	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
	4982b39648b579a313dd7b3a24a5dc0e	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Peptococcaceae; g__; s__
	da2da458d4e1672d5a9d66c19f7f3a29	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	aa87797523e26fb14c229907c6853d54	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	24fee79167b4d1dd6e0e43ec1c7cb4ca	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
	b776fbe642206fb6363e8506ca747aec	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__
	92596279e2ae7b46cabaec4688d04973	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
	339b7792798d6a6422e2eda159522387	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	9ce915d9cc17d47a97d433f6560b7d5e	k__Bacteria; p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__Adlercreutzia; s__
	3529269a68f52b1ab7d437c68ea8dac9	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__
	0dce37ac4051693cb2dc6bc0d7a010f9	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__[Mogibacteriaceae]; g__; s__
	208fbec6e805a6273bdcf5f3b0a4e856	k__Bacteria; p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__Adlercreutzia; s__
	caff057aac39d52629b9bc710dd1d19c4	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae
	146149b9133c1ef774f8d1226365ab49	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales

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Errataliste

Ph.d.-kandidat: Silje Modahl Johanson

Avhandling: Persistent organic pollutants and effects on developmental and functional processes in mice

Dato: 16.09.2020

Side	**Linje**	**Endret fra**	**Endret til**
22	4	…interaction may change with dose…	…interaction may change with the dose…
28	19	…2) when the mammary gland growth exponentially…	…2) when the mammary gland grows exponentially…
33	2	Structure of the small intestine (A)…	Structure of the small intestinal (A)…
38	4/5	…of Wnt (wingless-type MMTV integration site family)…	…of *Wnt* (*wingless-type MMTV integration site family*)…
38	5	…the oncogenes c-myc and cyclin D1…	…the oncogenes *c-myc* and *cyclin D1*…
38	17	…which leads to the truncated of…	…which leads to the truncation of…
40	5	(scale bar = 10 µm)	(scale bar = 100 µm)
49	3	…adapted to a 3 g mouse…	…adapted to a 25 g mouse…
50	8	…a reference diet was also created…	…a reference diet was created…
51	4	…produced large litters…	…produces large litters…
51	15	…National Institute of Public health…	…National Institute of Public Health…
58	25/26	…no a priori knowledge…	…no *a priori* knowledge…
61	2	A mixture of Persistent Organic Pollutants (POPs) and Azoxymethane (AOM)…	A mixture of persistent organic pollutants (POPs) and azoxymethane (AOM)…
62	4	S. M. Johanson, J. R. Swann, …	S. M. Johanson, J. R. Swann, …
70	7	…contribution of the Wnt/β-catenin pathway…	…contribution of the *Wnt*/β-catenin pathway…
71	26	…the mixture of POPs could have induced hepatic carcinogenesis.	…the mixture of POPs can induce hepatic carcinogenesis.
96	11	…polychlorinated biphenyls: A case-control study…	…polychlorinated biphenyls: a case-control study…
101	27/28	…, p,p'-DDT, and p,p'-DDE, …	…, *p,p'*-DDT, and *p,p'*-DDE, …
105	31	*PlOS ONE*	*PLOS ONE*
129	1	Appendix: Paper I-III	Appendix: Papers I-III
136	14	…focused on the use single compounds…	…focused on the use of single compounds…
151	7	Scalebar = 2000 µm.	Scale bar = 2000 µm.
151	7	Ln = Nymph node.	Ln = Lymph node.

ISBN: 978-82-575-1710-6
ISSN: 1894-6402

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