



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



Silje M. Johanson<sup>a, \*</sup>, Jonathan R. Swann<sup>b</sup>, Özgün C.O. Umu<sup>c</sup>, Mona Aleksandersen<sup>d</sup>, Mette H.B. Müller<sup>c</sup>, Hanne F. Berntsen<sup>a, e</sup>, Karin E. Zimmer<sup>d</sup>, Gunn C. Østby<sup>a</sup>, Jan E. Paulsen<sup>c</sup>, Erik Ropstad<sup>a</sup>

<sup>a</sup> Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, P.O. Box 369 Sentrum, NO-0102, Oslo, Norway

<sup>b</sup> Department of Metabolism, Digestion and Reproduction, Faculty of Medicine, Imperial College London, SW7 2AZ, United Kingdom

<sup>c</sup> Department of Paraclinical Sciences, Norwegian University of Life Sciences, P.O. Box 369 Sentrum, NO-0102, Oslo, Norway

<sup>d</sup> Department of Preclinical Sciences and Pathology, Norwegian University of Life Sciences, P.O. Box 369 Sentrum, NO-0102, Oslo, Norway

<sup>e</sup> National Institute of Occupational Health, P.O. Box 5330 Majorstuen, NO-0304, Oslo, Norway

### 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.

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### 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. <sup>1</sup>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.

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**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.

\* Corresponding author. NMBU-School of Veterinary Sciences, P.O. Box 369 Sentrum, NO-0102, Oslo, Norway.

**E-mail addresses:** [silje.modahl.johanson@nmbu.no](mailto:silje.modahl.johanson@nmbu.no) (S.M. Johanson), [j.swann@imperial.ac.uk](mailto:j.swann@imperial.ac.uk) (J.R. Swann), [ozgun.umu@nmbu.no](mailto:ozgun.umu@nmbu.no) (Ö.C.O. Umu), [mona.aleksandersen@nmbu.no](mailto:mona.aleksandersen@nmbu.no) (M. Aleksandersen), [mette.helen.bjorge.muller@nmbu.no](mailto:mette.helen.bjorge.muller@nmbu.no) (M.H.B. Müller), [Hanne.Berntsen@stami.no](mailto:Hanne.Berntsen@stami.no) (H.F. Berntsen), [karin.zimmer@nmbu.no](mailto:karin.zimmer@nmbu.no) (K.E. Zimmer), [gunn.ostby@nmbu.no](mailto:gunn.ostby@nmbu.no) (G.C. Østby), [jan.erik.paulsen@nmbu.no](mailto:jan.erik.paulsen@nmbu.no) (J.E. Paulsen), [erik.ropstad@nmbu.no](mailto:erik.ropstad@nmbu.no) (E. Ropstad).

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## 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 (Fearhead 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 Gıda San A.Ş., Manisa, Turkey). The solvent was evaporated under N<sub>2</sub>-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  $20 \pm 2$  °C, and  $45 \pm 5\%$  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. Cecae 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,  $\alpha$ -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 ( $\text{mm}^2$ ) 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 ( $\text{mm}^2$ ) 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 Ruitenbergh (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  $\mu\text{m}$  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  $\mu\text{m}$  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  $\mu\text{m}$  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  $-80\text{ }^{\circ}\text{C}$  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  $-80\text{ }^{\circ}\text{C}$  and shipped on dry ice to Imperial College, London (UK) for  $^1\text{H}$  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  $-20\text{ }^{\circ}\text{C}$  until sample preparation. Cecal tissue was rinsed in distilled water and 75–80 mg tissue was finely homogenized (6500 rpm  $2 \times 45\text{ s}$ ) in 300  $\mu\text{L}$  chloroform:methanol (2:1) using a Precellys 24 lysis machine (Bertin technologies, Montigny-le-Bretonneux, France). The homogenate was mixed with 300  $\mu\text{L}$  water and spun at 13 000 rpm for 10 min at  $4\text{ }^{\circ}\text{C}$  (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  $\mu\text{L}$  of water and 300  $\mu\text{L}$  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  $-20\text{ }^{\circ}\text{C}$  until NMR spectroscopy. On the day of analysis, the aqueous phase was reconstructed in 700  $\mu\text{L}$  phosphate buffer (pH 7.4) with 9:1  $\text{D}_2\text{O}:\text{H}_2\text{O}$  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 ( $4\text{ }^{\circ}\text{C}$ ), to pellet out any debris, before 600  $\mu\text{L}$  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  $\mu\text{L}$  phosphate buffer (pH 7.4) with 9:1  $\text{D}_2\text{O}:\text{H}_2\text{O}$  containing 1 mM TSP and centrifuged at 13 000 rpm for 15 min at  $4\text{ }^{\circ}\text{C}$ . A volume of 550  $\mu\text{L}$  supernatant was transferred to a 5 mm NMR tube.

### 2.9.2. $^1\text{H}$ NMR spectroscopy

$^1\text{H}$  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

$^1\text{H}$  NMR spectra were digitized into consecutive integrated spectral regions of equal width (0.00055 ppm). Spectral regions corresponding to TSP ( $\delta -0.2\text{--}0.2$ ) and water ( $\delta 4.7\text{--}4.9$ ) were removed from all spectra (both cecal tissue and content). The region  $\delta 3.345\text{--}3.375$  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'-CCAGGGTATC-TAATCC-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 (Kato 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 phylogenetic 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 <sup>1</sup>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 <sup>1</sup>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 <sup>1</sup>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<sup>2</sup>Y) 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 ≤ 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	<b>3.234 ± 1.199</b>	<b>44.631 ± 12.023</b>
PCB-138	<b>18.840 ± 10.417</b>	<b>769.930 ± 101.660</b>
PCB-153	<b>20.572 ± 11.210</b>	<b>784.766 ± 99.772</b>
PCB-180	<b>6.252 ± 3.384</b>	<b>261.949 ± 31.754</b>
Σ 7PCBs	<b>49.806 ± 26.284</b>	<b>1862.214 ± 242.270</b>
Organochlorine pesticides (OCPs)		
<i>p,p'</i> -DDE	3.697 ± 1.078	7.183 ± 1.918
HCB	<b>78.894 ± 18.569</b>	<b>12.957 ± 10.977</b>
Oxy-chlordane	<b>8.767 ± 4.627</b>	<b>107.116 ± 16.670</b>
<i>Trans</i> -nonachlor	<b>3.980 ± 1.405</b>	<b>90.630 ± 11.318</b>
α-HCH	1.171 ± 0.057	1.298 ± 0.074
β-HCH	<b>3.342 ± 0.785</b>	<b>14.869 ± 1.570</b>
γ-HCH (Lindane)	0.209 ± 0.062	0.207 ± 0.020
Σ HCHs	<b>4.722 ± 0.878</b>	<b>16.374 ± 1.623</b>
Dieldrin	<b>8.259 ± 1.679</b>	<b>42.525 ± 6.419</b>
Σ OCPs	<b>108.320 ± 27.292</b>	<b>391.786 ± 43.753</b>
Σ OCPs + 7PCBs	<b>158.127 ± 53.473</b>	<b>2254.000 ± 283.595</b>
Brominated flame retardants (BFRs)		
BDE-47	0.474 ± 0.097	0.682 ± 0.201
BDE-99	<b>0.480 ± 0.139</b>	<b>7.965 ± 1.634</b>
BDE-100	<b>0.460 ± 0.195</b>	<b>12.625 ± 3.160</b>
BDE-153	<b>0.227 ± 0.138</b>	<b>18.919 ± 2.104</b>
BDE-154	<b>0.048 ± 0.008</b>	<b>5.372 ± 0.794</b>
BDE-209	<b>2.077 ± 1.444</b>	<b>3.909 ± 0.393</b>
Σ BFRs	<b>3.816 ± 1.978</b>	<b>49.473 ± 7.569</b>
Perfluoroalkylated substances (PFASs)		
PFHxS	<b>0.979 ± 0.074</b>	<b>1.718 ± 0.111</b>
PFOS	<b>5.359 ± 0.661</b>	<b>58.993 ± 2.687</b>
PFOA	<b>0.956 ± 0.221</b>	<b>8.485 ± 1.093</b>
PFNA	<b>1.713 ± 0.314</b>	<b>55.795 ± 4.448</b>
PFDA	<b>1.761 ± 0.303</b>	<b>87.294 ± 6.095</b>
PFUnDA	<b>0.659 ± 0.093</b>	<b>25.844 ± 2.143</b>
Σ PFASs	<b>11.426 ± 1.427</b>	<b>238.130 ± 13.482</b>

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

chlordan,  $\alpha$ - and  $\gamma$ -HCH did not differ significantly between groups, and  $\alpha$ -chlordan 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

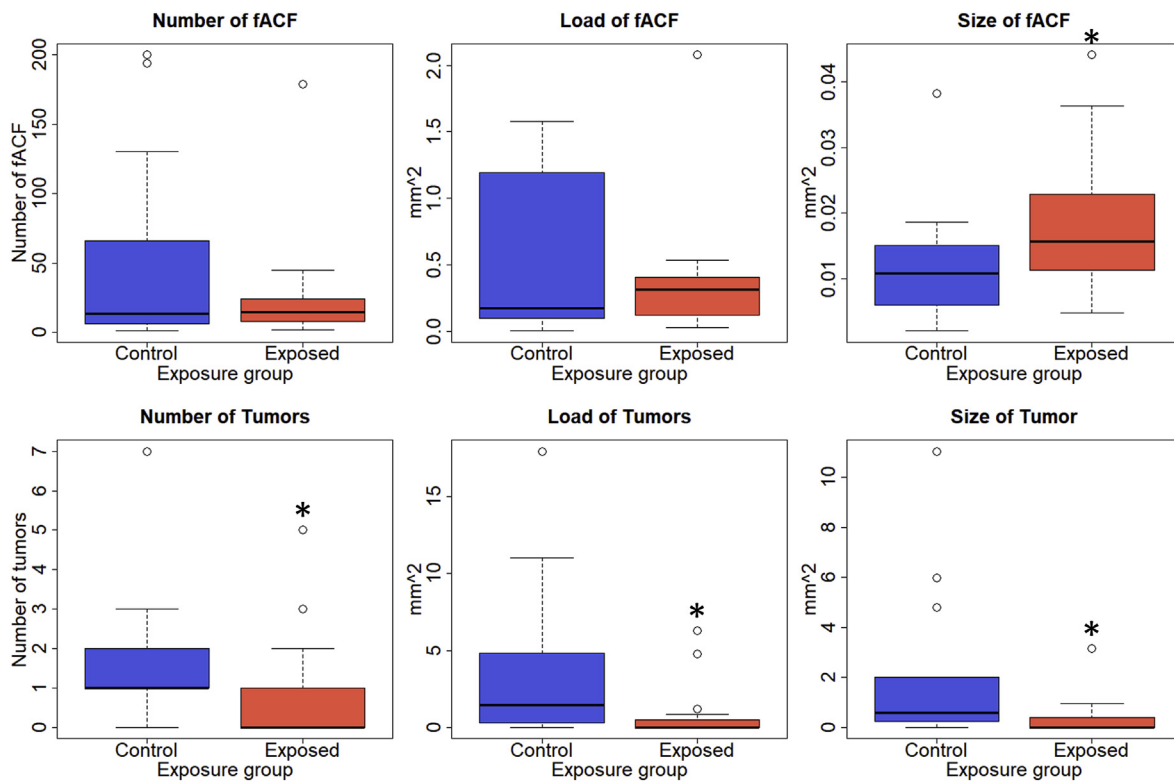
Swiss rolls were made from the intestines of A/J Min/+ mice and examined for hyperplastic/dysplastic lesions, adenomas and carcinomas. Results are presented in [Table 2](#). The maternal POP exposure decreased the number of carcinomas in the small intestine ( $p < 0.01$ ) and adenomas in the colon ( $p = 0.04$ ). 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  $\pm$  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 $\pm$ 2.53	5.52 $\pm$ 0.94	2.43 $\pm$ 1.34	1.16 $\pm$ 0.62
Adenoma	12.43 $\pm$ 4.88	3.79 $\pm$ 1.07	<b>0.71 <math>\pm</math> 0.38</b>	0.08 $\pm$ 0.08
Carcinoma	<b>1.79 <math>\pm</math> 0.94</b>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 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 shown 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  $\delta$  3.46 and  $\delta$  3.48 and a doublet at  $\delta$  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.

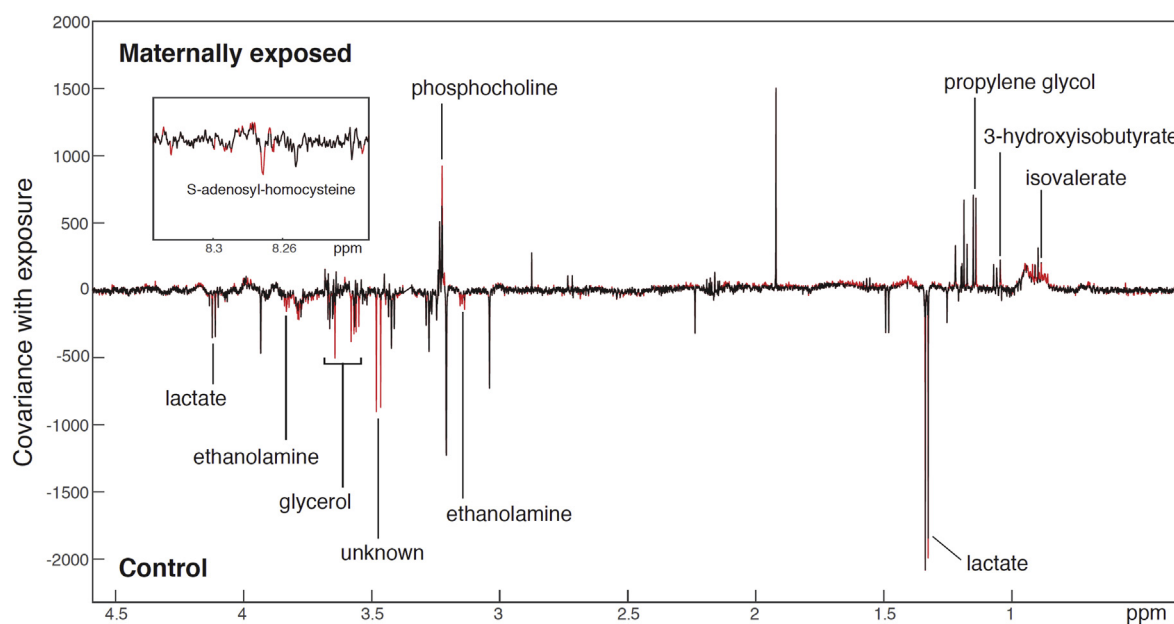
Maternal exposure did not affect the metabolite composition in cecal content (PCA plot shown in Supplementary Fig. A9).

### 3.6. Microbial diversity and composition

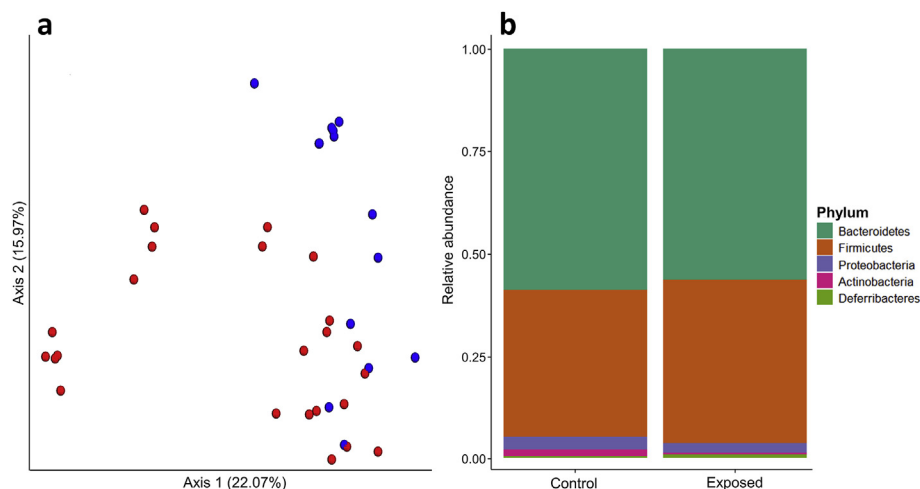
Maternal exposure to the mixture of POPs did not change the microbial diversity (i.e. alpha diversity) in fecal samples from A/J Min/+ mice. However, there was a significant difference in fecal bacterial composition (i.e. beta diversity) between the Control and Exposed groups ( $p < 0.01$ ), which is 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  $94.8\% \pm 1.3\%$  and  $96.4\% \pm 0.4\%$  (mean  $\pm$  standard error) of the total fecal microbiome in the Control and Exposed groups, respectively. The third most represented phylum was the *Proteobacteria*, with  $3.1\% \pm 0.7\%$  and  $2.4\% \pm 0.3\%$  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 were 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  $^1\text{H}$  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  $^1\text{H}$  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  $\delta$  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  $^1\text{H}$  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  $\delta$  0.88,  $p < 0.01$ ), acetate (singlet at  $\delta$  1.91,  $p = 0.05$ ) and trimethylamine (TMA, singlet at  $\delta$  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 needed. 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/ $\beta$ -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  $\beta$ -HCH (Liu et al., 2017). Moreover, PFOS has been shown to cause dose-dependent alterations in the abundance and composition of intestinal bacteria in ER $\beta$  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. **Özgül 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.

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## 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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