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Developmental Exposure to Mixtures of Persistent Environmental Pollutants with Focus on Bone and Retinoid System Modulations

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To the memory of my father

To my mother, sister and brothers

To my husband Mohamed and my son Awab

ABSTRACT

Environmental pollution of Arctic regions has evoked a scientific as well as public concern. Arctic Inuit inhabitants consume large amounts of fatty fish and marine mammals and are therefore exposed to levels of environmental toxicants that are suspected to cause adverse health effects. Exposure to mixtures of environmental pollutants affects a wide range of clinical and biochemical parameters. Developing fetuses and infants are the most vulnerable groups to environmental contaminants. Experimental studies demonstrate that alterations of bone tissue and modulations in the retinoid system are considered as sensitive toxicological end-points. The present thesis is based on a one-generation toxicity study performed according to standard guidelines with enhancement to accommodate bone and retinoid system endpoints. Rats were exposed during gestation and lactation to two chemical mixtures: the Northern Contaminants Mixture (NCM) or the commercial mixture Aroclor 1254. The overall aim of the current work was to generate new experimental data, which will contribute new knowledge to improve the health risk assessment associated with the exposure to chemical mixtures with a focus on the postnatal consequences of the exposure during fetal life, using the situation of the Canadian Arctic populations as an example.

The body and organ weights, as well as serum levels of thyroid hormones and cholesterol were altered in young offspring at post-natal day (PND) 35 by perinatal exposure to the NCM or Aroclor 1254. In addition, levels of hepatic retinoids were decreased in the dams, as well as in their offspring at all post-natal follow-up time points following perinatal exposure to NCM or Aroclor 1254. Based on these findings, it was decided to perform both bone studies and more detailed retinoid studies.

Multiple bone parameters, including geometrical and biomechanical parameters, were clearly affected in the offspring at PND35 and partly affected at PND77, while no bone changes were detected at PND350 following perinatal exposure to NCM or Aroclor 1254. Affected parameters included reduced bone length, cross-sectional area, thickness and strength. None of these bone changes were observed in the dams after NCM or Aroclor 1254 exposure.

Results of detailed retinoid analysis demonstrated that retinol levels in liver were reduced in the dams and the offspring at PND35 and that, hepatic levels of the active metabolite of the retinoic acid were markedly reduced in the dams and the PND35 offspring after perinatal exposure to the NCM or Aroclor 1254. Reduction of retinyl palmitate levels in liver, as well as increase of retinol levels in kidney was observed in dams and their offspring at all time-points up to PND350 after perinatal exposure to NCM or Aroclor 1254. Results based on partial least square (PLS) analyses indicated that changes of different retinoid forms in livers induced by exposure to NCM or Aroclor 1254 were strongly associated with changes in body and liver weights, with alterations in levels of thyroid hormones, as well as with induction of liver cytochrome P450 activities.

Obtained results of alterations in bone and retinoid parameters are of relevance to dietary exposure situations, and this data may contribute to human health risk assessment by providing useful information for hazard characterization and exposure assessment. The generated data will be relevant not only for the Canadian Arctic population, but also for other populations with similar exposure profiles.

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LIST OF PUBLICATIONS

The present thesis is based on the five papers listed below, and they will be referred to in the text by their roman numerals. The published articles are printed with permission of the respective publishers.

I. Ih Chu, Wayne J. Bowers, Don Caldwell, Jamie Nakai, Mike G. Wade, Al Yagminas, Nanqin Li, David Moir, **Lubna El Abbas**, Helen Håkansson, Santokh Gill, Rudi Mueller, Olga Pulido. Toxicological Effects of In Utero and Lactational Exposure of Rats to a Mixture of Environmental Contaminants Detected in Canadian Arctic Human Populations. Journal of Toxicology and Environmental Health, Part A, 71 (2008) 93-108.

II. **Lubna E. Elabbas**, Mikko A. Finnilä, Maria Herlin, Natalia Stern, Christina Trossvik, Wayne J. Bowers, Jamie Nakai, Juha Tuukkanen, Rachel A. Heimeier, Agneta Åkesson, Helen Håkansson. Perinatal Exposure to Environmental Contaminants Detected in Canadian Arctic Human Populations Changes Bone Geometry and Biomechanical Properties in Rat Offspring. Journal of Toxicology and Environmental Health, Part A, 74 (2011) 1304-1318.

III. **Lubna E. Elabbas**[*], Maria Herlin[*], Mikko A. Finnilä, Filip Rendel, Natalia Stern, Christina Trossvik, Wayne J. Bowers, Jamie Nakai, Juha Tuukkanen, Matti Viluksela, Rachel A. Heimeier, Agneta Åkesson, Helen Håkansson. In Utero and Lactational Exposure to Aroclor 1254 Affects Bone Geometry, Mineral Density and Biomechanical Properties of Rat Offspring. Toxicology Letters 207 (2011) 82-88.

IV. **Lubna E. Elabbas**, Daniel Borg, Javier Esteban, Xavier Barber, Wayne J. Bowers, Jamie Nakai, Gerd Hamscher, Heinz Nau, Agneta Åkesson, Helen Håkansson. Gestational and Lactational Exposure to Environmental Contaminants Detected in Canadian Arctic Human Populations Alters Retinoid Homeostasis in Rat Offspring *(Manuscript).*

V. Javier Esteban[*], **Lubna E. Elabbas**[*], Xavier Barber, Wayne J. Bowers, Jamie Nakai, Gerd Hamscher, Heinz Nau, Agneta Åkesson, Helen Håkansson. Perinatal Exposure to the Technical Polychlorinated Biphenyls (PCBs) Mixture Aroclor 1254 Modulates Retinoid Homoeostasis in Rat Offspring *(Manuscript).*

[*] These authors contributed equally to this study.

CONTENTS

LIST OF ABBREVIATIONS

9c4o13,14dh-RA	9*cis*-4*oxo*-13,14dihydro-retinoic acid
9*c* RA	9 *cis* retinoic acid
A	Age
AhR	Aryl hydrocarbon receptor
At RA	All *trans* retinoic acid
BMD	Benchmark dose
BROD	7-benzyloxyresorufin O-deethylase
bw	Body weight
CAR	Constitutive androstane receptor
CV	Coefficient of variation
CYP	Cytochrome P450
DXA	Dual energy X-ray aborptiometry
EROD	7-ethoxyresorufin-O-deethylase
ER	Estrogen receptor
GC	Gas chromatography
GD	Gestation day
GS-MS	Gas chromatography-mass spectrometry
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
Hg	Mercury
HPLC	High pressure liquid chromatography
LOD	Limit of detection
LRAT	Lecithin:retinol acyltransferase
MeHg	Methyl mercury
NCM	Northern contaminant mixture
NCP	Northern Contaminants Program
OC	Organochlorine
p,p´-DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene
p,p´-DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzop- dioxin
PCDF	Polychlorinated dibenzofuran
PLS	Partial least square
PMI	Polar moment of inertia
PND	Postnatal day
pQCT	Peripheral quantitative computed tomography
PROD	7-pentoxyresorufin O-deethylase
PXR	Pregnane X receptor
RARE	Retinoic acid response element
RAR	Retinoic acid receptor
RIA	Radioimmunoassay
RXR	Retinoid X receptor
S	Sex

SSIp	Polar strength strain index
T	Treatment
T_3	Triiodothyronine
T_4	Thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEQ	Toxic equivalents
TSH	Thyroid stimulating hormone

1 INTRODUCTION

Individuals from the general population are exposed to low-dose levels of complex mixtures of chemicals throughout their entire life (De Rosa et al., 2004; Van Oostdam et al., 2005). These environmental chemical pollutants include different categories of compounds. Some of these compounds are persistent, bio-accumulating and toxic compounds that can bio-magnify in the food chain. It is suspected that human health is potentially affected in the general population and not only in high exposure groups such as people of Faroe Island, Baltic Sea and Arctic regions.

The Canadian Arctic Inuit inhabitants constitute one example of a high-risk group regarding dietary exposure to environmental chemical pollutants. The high exposure is mainly due to their unique culture, traditions and dietary habits. Inuit people consume large amount of fish and marine mammals, which are major vectors of many toxic contaminants. It was reported that some Canadian Arctic groups are exposed to concentrations which are higher than the established tolerable daily/weekly intake levels of some contaminants like polychlorinated biphenyls (PCBs) and pesticides. These exposure levels could have the potential for adverse health effects, particularly in the context of early human development (Van Oostdam et al., 2005). During the last decade most research efforts in Arctic Canada were concentrated on the characterization of exposure of northern population. Few studies have analyzed diseases or exposure outcomes. However, conducting epidemiological studies in the Arctic regions is difficult due to many factors like complexity of contaminant mixtures, small size of population, genetic variability, nutrient-contaminant interactions and non-dietary confounding factors (AMAP, 2009a).

It is well known that most, if not all, environmental chemical pollutants can cross the blood-placenta barrier, and can thereby reach the developing fetus, which is highly sensitive to the effects of persistent environmental pollutants. It is also well known that the early postnatal life-stage experiences a considerably higher exposure level of contaminants via lactation than otherwise throughout life and this is serious since the developmental programming of vital organs is still ongoing. Nevertheless, few experimental or human studies have investigated the impact of perinatal exposure to chemical mixtures.

In experimental studies, it has been established that maternal exposure to individual chemicals at environmental levels modulates a wide range of biochemical parameters in their offspring, including multiple components of classical endocrine pathways, as well as several additional nuclear receptor and other cell signaling pathways. Based on these and other related findings it is suspected that gestational exposure to environmental levels of chemicals can alter fetal programming and modulate developmental maturation processes so that structural and/or functional tissue changes become permanent and sometimes visible first in adult stages of life. Thus, as a consequence of developmental exposure to chemicals, it is hypothesized that any organ or tissue might be affected and that susceptibility to diseases either during post-natal stages or later in life increases.

For these reasons, there is an increasing public and scientific concern that efforts should be oriented towards studying the toxicology of chemical mixtures (Mumtaz et al., 2011; Silins and Högberg, 2011); and to assess their potential health risks at doses which are similar to those which humans experience (US EPA, 2000; Groten et al., 2001). Furthermore, in modern chemicals legislation it is now emphasized that there is an urgent need to develop and establish relevant experimental models and effect biomarkers to be able to identify and assess individual chemicals as well as mixtures of those, which can modulate endocrine pathways so that adverse health consequences occur.

For the current investigation, a combined fetal and lactational study design was chosen since, infants, being exposed both *in utero* and via breast milk, constitutes the most vulnerable population group to the toxic effects of persistent environmental pollutants (Stefanidou et al., 2009). This study was designed to investigate the impacts of maternal exposure to environmental levels of two chemical mixtures on bone tissue and the retinoid system in the developing rat from early postnatal life and into adulthood i.e. at postnatal days 35, 77, or 350. One of the mixtures was composed of contaminants similar to those which the Canadian Arctic population experience, here referred to as the Northern Contaminants Mixture (NCM), while the other one was a commercial mixture of PCBs, i.e. Aroclor 1254.

Recent research has identified alterations in bone tissue and the retinoid system as two novel and sensitive endocrine system-related endpoints of potential relevance to human health risk assessment. It is clear, both from human and animal studies that exposure to environmental levels of chemicals can alter bone tissue composition and function (Côté et al., 2006; Ramajayam et al., 2007; Hodgson et al., 2008; Cocchi et al. 2009). Furthermore, experimental studies demonstrated that exposure to individual persistent environmental pollutants at low dose-levels interfere with retinoid signaling and metabolism in a dose-dependent manner and induce a toxicity profile which is compatible with health consequences of abnormalities in the retinoid system. However, little is known about perinatal exposure situation.

2 BACKGROUND

2.1 ENVIRONMENTAL POLLUTION IN THE CANADIAN ARCTIC REGIONS

Environmental pollution of Canadian Arctic regions is of scientific and public concern. The alarming contaminant levels are either of local origin via different activities, or reach these areas through long-range transport via water and atmosphere from more distant sources, altogether leading to the accumulation of different toxicants in Arctic environment and biota (Evans et al., 2005). Inhabitants of Canadian Arctic consume large amounts of the so called "traditional food". This traditional food consists mainly of fish and land and marine mammals; which are considered as more highly contaminated. These food items are from the higher trophic levels of the food chain, which considerably bioaccumulate and biomagnify persistent environmental contaminants (AMAP, 2009b). Consumers of traditional food exceed their tolerable daily intake of many contaminants. Dietary habits of many northern Canadian Arctic communities constitute the main exposure source to environmental pollutants such as organochlorines (OCs) or metals, resulting in high body burdens of these contaminants compared to southern populations (Van Oostdam et al., 1999). Chemical structures of some examples of environmental pollutants in the Canadian Arctic regions are shown in **Figure 1**.

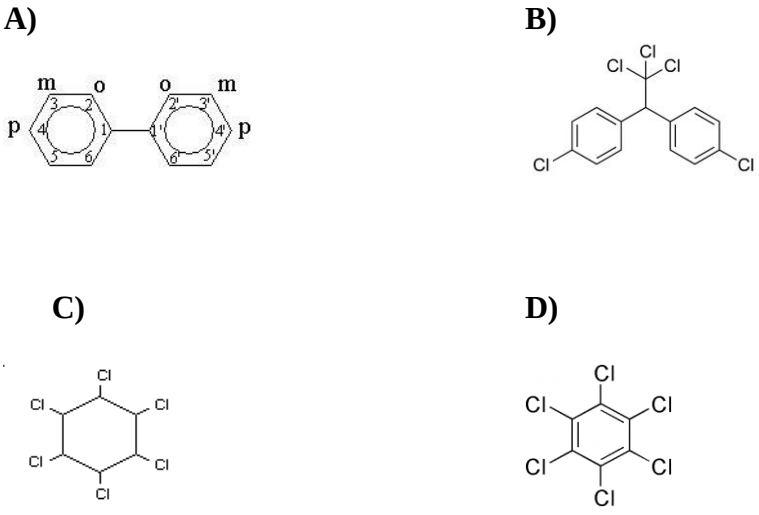


Figure 1. Examples of some environmental pollutants present in the Canadian Arctic regions. **A)** Structures of PCBs showing the possible positions of the chlorine atoms at ortho (o), meta (m) and para (p) positions. **B)** Structure of p,p´-DDT. **C)** Structure of HCH. **D)** Structure of HCB.

Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants that have damaging effects on both ecosystem and human health. PCBs are members of the group of halogenated aromatic hydrocarbons and consist of 209 congeners with different number and positions of chlorine atoms substituted on the biphenyl moiety (Aken et al., 2010; El-Shahawi et al., 2010). PCBs are synthetic products that were widely used in industry (Diamond et al., 2010). Although their production has been banned since 1970s, PCBs are still present, commonly in form of mixtures, in the environment due to their high chemical stability. It has been estimated that approximately 10% of the PCBs ever produced are still environmentally available (Schmidt, 2010). Exposure to PCBs is known to evoke various adverse effects in experimental studies including body weight loss, thymic atrophy, hepatotoxicity, carcinogenicity and neurtoxicity (Safe, 1990, 1994; Tilson and Kodavanti, 1998). For organochlorine pesticides, previous studies have shown that toxaphene, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (p,p´-DDT) and chlordane were the predominant OCs in Canadian Arctic and sub-Arctic regions (Evans et al., 2005). 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p´-DDE), hexachlorocyclohexane (HCH) and hexachlorobenzene (HCB) are examples of other contaminants in the Canadian Arctic regions. Mercury (Hg) is a highly toxic element which is found naturally, or as an introduced contaminant in the environment through human activities. Hg is transported to the Arctic via the air currents, ocean currents and by the rivers. Hg presents in different chemical forms, and its toxic effects depend on its chemical composition and the route of exposure. Methylmercury (MeHg) is considered as the most toxic form. MeHg affects the immune system, alters genetic and enzyme systems, and damages the nervous system (AMAP, 2011).

2.1.1 Exposure to organochlorines and mercury

For the high-exposure groups, e.g. indigenous people of the Arctic regions of the United States, Canada, Greenland, and the Russian Federation, they have higher tissue levels of environmental pollutants. For example, levels of contaminants, such as 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p´-DDE), polychlorinated biphenyls (PCBs), mirex, and chlordane are 2 to 10 fold higher in milk of Inuit women from Arctic Canada than women from southern Canada. Furthermore, it was shown that levels of various OCs in Inuit newborn cord blood tend to be 2-4-fold higher than those reported for the newborns from the general population, or from southern Canada (AMAP, 2009a). Nevertheless, results of bio-monitoring studies suggest that concentrations of contaminants such as p,p´-DDE, PCBs, oxychlordane, and MeHg are decreasing in many Arctic populations. This reduction may be related to lower concentrations of OC chemicals in the environment as well as to dietary changes. However, emerging compounds such as brominated flame retardants and fluorinated compounds receive increasing attention, because they are present in Arctic people and biota and their levels globally have increased over the past 15 years (AMAP, 2009a).

Consumption of contaminated fish and marine mammals constitutes the major MeHg exposure source for the general population. Unlike other contaminants which concentrate in the skin and fat, MeHg concentrates in the muscle tissue of fish. The proportion of Arctic people who exceed blood MeHg guidelines has declined during recent years. However, a significant proportion of populations from eastern Canadian Arctic still exceed those guidelines (AMAP, 2011). As previously demonstrated, Inuit

women of child-bearing age who consume marine mammals have MeHg concentrations that are three- to ten-fold higher than in other populations in the Arctic who consume market foods (AMAP, 1998, 2003). A recent study on Inuit pre-school children in Arctic Canada found that 59% of the children exceeded the tolerable weekly intake level for children of MeHg (Tian et al., 2011).

2.1.1.1 Early life exposure

Fetuses and newborns are the two sub-populations of particular concern in the Arctic (Van Oostdam et al., 2005). Fetuses are sensitive to many pollutants that cross the placenta, since they are in stages of rapid development of all body systems. In addition, newborns are also exposed to higher levels of mixtures of pollutants via their mothers' breast milk (Stefanidou et al., 2009). Levels of contaminants in maternal blood during pregnancy give an indication of the potential risk to the developing fetus. Of particular concern are long-term, subtle effects, for example, adverse reproductive and pregnancy outcomes, reduced childhood defense against diseases (immune system impacts), and impairment of children's mental development (AMAP, 2009a).

There is a lack of data on association between contaminants and pregnancy outcomes in the Arctic. However, preliminary findings from a large Russian Arctic cohort confirmed the observations that higher levels of maternal blood serum PCBs and HCB might be associated with more frequent occurrences of low birth weight, premature births, stillbirths, and menstrual irregularities (AMAP, 2004). Furthermore, the relative risk of spontaneous abortion increased with rising total Hg in maternal blood (AMAP, 2004). Though, these possible adverse reproductive health effects of environmental pollutants require more detailed evaluation. Based on these facts, further experimental studies are required to explore the effects of maternal exposure to mixtures of environmental pollutants on the developing fetuses and newborns.

2.2 IMPACT OF EXPOSURE TO ENVIRONMENTAL POLLUTANTS

Many environmental pollutants including PCBs, polychlorinated dibenzop- dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and pesticides can perform their action in similar ways on the endocrine system. As potential endocrine disrupters, they are suspected to contribute to increasing the risk of cancer, birth defects and reproductive and neuro-immune disorders in humans (Carpenter et al., 1998; Bonefeld-Jorgensen and Ayotte, 2003; Bonefeld-Jorgensen, 2004). However, no clear indication for adverse endocrine-related health effects of environmental pollutants has been obtained at the human individual or population level. Nevertheless, records from studies on wildlife species, laboratory animals and biomarker effects in vitro have strengthened the need for further research to address the potential impacts of endocrine disruptors on human populations (AMAP 2009a).

Experimental studies deliver essential data to establish the biological plausibility of associations between exposure to toxicants and adverse health effects described in epidemiological studies. Findings of most experimental studies support and confirm observations reported in some epidemiological studies of infants and children. On the

other hand, *in vitro* studies are conducted in order to investigate the mechanisms of action for different compounds.

2.2.1 Effects on humans

In individual cohort studies from United States, the Netherlands and Nunavik (Canada); associations between fish consumption (reflecting exposure to environmental pollutants mainly PCBs) and some effects like lower head circumference, birth weight, duration of pregnancy and infant growth have been reported (Fein et al., 1984; Jacobson et al., 1990; Patandin et al., 1998; Muckle et al., 2004). However, in the two most highly PCB-exposed cohort studies conducted in the Faroe Islands and West Greenland, fetal growth and duration of gestation were not related to prenatal exposure to PCBs (Grandjean et al., 2001). Results from the PCB studies performed in the Faroe Islands and Nunavik suggested that prenatal exposure to PCBs is related to a relatively specific profile of cognitive and behavioral impairments in children. Among the endpoints assessed, impaired effects have been most clearly shown for executive functions, speed of information processing and visual recognition memory (Jacobson et al., 1992; Grandjean et al., 2001; Muckle et al., 2001a, 2001b). These negative effects have also been observed in studies from United States and the Netherlands (Jacobson and Jacobson, 2003; Vreugdenhil et al., 2004). Several recent studies in Arctic Canada confirmed and supported the relationship between contaminant exposure (like PCBs, p,p´-DDE, and Hg) and depressed immunity, which is in turn, associated with a greater susceptibility to infectious diseases (Dallaire et al., 2004, 2006a, 2006b).

Results of a large international cohort study performed in European and Inuit populations, the INUENDO study, indicated some links between exposure to environmental pollutants and biomarkers of male reproductive function. It has been suggested that abnormalities of male genital organs, poor sperm counts and testicular cancer can be attributed to, among other factors, exposure to exogenous endocrine disrupters (Virtanen et al., 2005). Furthermore, exposure to the environmental pollutants may contribute to the development of metabolic syndrome, which precedes the onset of type II diabetes, as well as the development and progression of atherosclerosis and other cardiovascular diseases (Zimmet et al., 2001). It was also reported that environmental contaminant mixtures might affect bone mineral density. One study has shown that exposure to p,p´-DDT reduced bone mineral density in women through an effect on bone cells (Beard et al., 2000). Negative associations between plasma levels of PCBs and bone mineral density have been reported in a group of peri- and post-menopausal Inuit women from Greenland, and in human populations near the Baltic coast (Côté et al., 2006; Hodgson et al., 2008). However, this association disappeared after adjustment for age and body mass index (Côté et al., 2006).

Exposure to MeHg can have serious effects on human health, such as effects on brain development, as well as effects on reproductive, immune and cardiovascular systems (AMAP, 2011). In the Faroe Islands, several associations have been observed between hair Hg or cord blood Hg concentrations and neurobehavioral outcomes (Grandjean et al., 1997, 1998; Murata et al., 1999a, 1999b). Moreover, recent studies in the Faroe Islands, Greenland and Nunavik revealed that MeHg can affect circulatory parameters, blood pressure, hypertension and atherosclerosis (Sorensen et al., 1999; Pedersen et al.,

2005; Valera et al., 2008). Prenatal exposures to MeHg may also affect the development of cardiovascular homeostasis (Sorensen et al., 1999).

2.2.2 Effects on animals

Findings from recent toxicological studies which investigated the impact of perinatal exposure to some chemical mixtures are reported in this section. A complex mixture of PCBs and pesticides (referred to as mixture A) was composed by Bilrha et al., (2004). Mixture A approximate the combination of environmental contaminants found in seal blubber in Arctic Canada. Feeding mixture A to female pigs pre-mating, through pregnancy and until weaning of their first litter induced a variety of developmental and immunological alterations. Results revealed a reduction in length-at-birth for female piglets and decreases in testis weight and sperm motility. Moreover immunosuppression, expressed primarily as a reduction in antibody response, was observed in piglets exposed prenatally to mixture A (Bilrha et al., 2004). *In utero* and lactational exposure of male rats to mixture A adversely affected the development and function of their reproductive system, suggesting a possible reproductive health hazard for humans and other species (Anas et al., 2005). The results showed reduction in the number of pups per litter, the percentage of live offspring and the pup weight. Further the weight of genital organs was reduced, sperm motility was impaired and the testicular and epididymal morphology was severely altered, with these changes persisted until adulthood (Anas et al., 2005).

A second series of studies was performed using another mixture (referred to as mixture B). Mixture B was composed of PCBs, pesticides and MeHg. The mixture B was designed to simulate the contaminants composition that was found in plasma samples from Inuit mothers. Following perinatal exposure to mixture B neurobehavioral disturbances, including decreased neuromuscular development, hyperactivity as well as effects on learning and memory were observed in offspring (AMAP, 2009a). Pelletier and co-workers (2009) examined the potential toxicological impact of individual components of the mixture B. Their findings indicated that the observed increase in pup mortality could be ascribed to the MeHg component (Pelletier et al., 2009). Using mixture B, Padhi et al., (2008) investigated the modifications in the expression of genes involved in nerve cell differentiation, migration, myelination and synaptic transmission in the cerebellum of young pups. It was found that the full mixture B produced minimal changes in gene expression, whereas the PCB, MeHg and pesticide components all altered the expression of a number of genes (Padhi et al., 2008). Regarding bone effects, developmental exposure of rats to a PCB mixture composed of PCBs 138, 153, 180 and 126 caused long-lasting bone alterations in male offspring. These bone effects, which persisted into adulthood, included a significant decrease of bone mineral content and cortical bone thickness of tibiae (Cocchi et al., 2009). In a separate study, exposure of rats to the commercial PCB mixture (Aroclor 1254) induced a reduction in femur length, increased bone resorption and increased bone fragility (Ramajayam et al., 2007). These findings could be significant for humans, as it confirmed and supported the finding of epidemiological studies (Côté et al., 2006; Hodgson et al., 2008).

2.2.3 Effects *on in vitro* work

Mixture A (described above) was tested *in vitro* for its ability to affect oocyte maturation, fertilization, and subsequent embryo development. The results suggested

that exposing porcine oocytes and sperm to an environmentally-relevant mixture *in vitro* adversely affected oocyte development, sperm fertility, and further embryonic development (Campagna et al., 2001). These findings supported recent concerns that such pollutants can harm the reproductive system in humans and other species. For the bone tissue, most of the *in vitro* studies were performed using single chemicals, e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). To our knowledge, no *in vitro* bone work was performed using chemical mixtures. A recent study, which was conducted using a proteomic approach, indicated that TCDD exposure induced significant changes in a number of proteins that are involved in the osteoblast differentiation process (Carpi et al., 2009). Further, TCDD exposure of human osteosarcoma cell line modulated the osteogenesis process and inhibited the proliferation of osteoblasts (Guo et al., 2007). Exposure of the osteoblastic cell line (UMR-106) to TCDD down-regulated the transcription of osteopontin which is one of the predominant proteins in bone tissue (Wejheden et al., 2006).

2.3 ENDOCRINE SYSTEM - RELATED ENDPOINTS

During recent years, some of toxicological studies are performed according to standard guidelines of Organization for Economic Co-operation and Development (OECD) with enhancement to detect endocrine system-related endpoints. The enhancement is introduced due to the particular concern that endocrine system modulations during early stages of life might induce increased susceptibility to disease development or impaired life quality. In this thesis work, bone tissue was analyzed as a potential target tissue for two mixtures of endocrine system modulating chemicals. In addition, the thesis analyzed the impact of the same mixtures on the developing retinoid system.

2.3.1 Bone

Bone is a highly specialized form of connective tissue, which is under strict endocrine control (Takeda et al., 2003). Bone has various functions which include structural support and movement, protection of vital organs, and maintenance of mineral homeostasis. Morphologically, there are two forms of bone: cortical bone which is compact; and trabecular bone which is spongy. Cortical bone provides mechanical and protective functions; while trabecular bone provides metabolic functions. Anatomically; bones can be classified as flat bones (e.g. skull and scapula), long bones (e.g. femur and tibia), short bones (e.g. carpal bones) and irregular bones (e.g. vertebrae). Bone is composed of organic and ingorganic bone matrix as well as of four different cell types, i.e. osteoblasts, osteoclasts, osteocytes, and bone-lining cells. The organic matrix is composed mostly of type I collagen, while the inorganic matrix is composed primarily of calcium and phosphate in the form of hydroxyapatite (Sandy et al., 2002). Bone is composed of

Bones are shaped and reshaped by independent actions of osteoblasts and osteoclasts, and this process is called bone modeling. Bone modeling occurs during growth as well as in adult stages (Dempster, 2006). Bone tissue undergoes breakdown and renewal through a process termed remodeling, which begins *in utero* and continues throughout the whole life (Dempster, 2006). Remodeling is achieved by sequential actions of osteoclasts and osteoblasts and the whole process is regulated by osteocytes. Bone remodeling is composed of two processes, bone growth and bone resorption that occur

simultaneously (**Figure 2**). Deviation from balanced bone formation and resorption cause several bone disorders such as osteoporosis. Bone remodeling has two principal functions: the maintenance of mechanical strength as well as mineral homeostasis (Dempster, 2006). The modeling and remodeling processes are regulated by multiple systemic hormones as well as various nutritional factors. Examples of hormones which are essential for bone tissue development are thyroid hormones, parathyroid hormone (PTH) and estrogen. Thyroid hormones, mainly triiodothyronine (T3), are required for skeletal development, establishment of peak bone mass, as well as for maintaining optimal bone strength (Bassett and Williams, 2008). PTH keeps a physiological balance of calcium and phosphate concentrations in bone tissue (Jüppner et al., 1991). Estrogen contributes to the strength of bone tissue by maintaining bone density. It is well established that estrogens are implicated in the regulation of bone metabolism, particularly in women, as illustrated by the considerable loss of bone mass and increased risk of fracture in the first few years after natural menopause (Riggs et al., 1998).

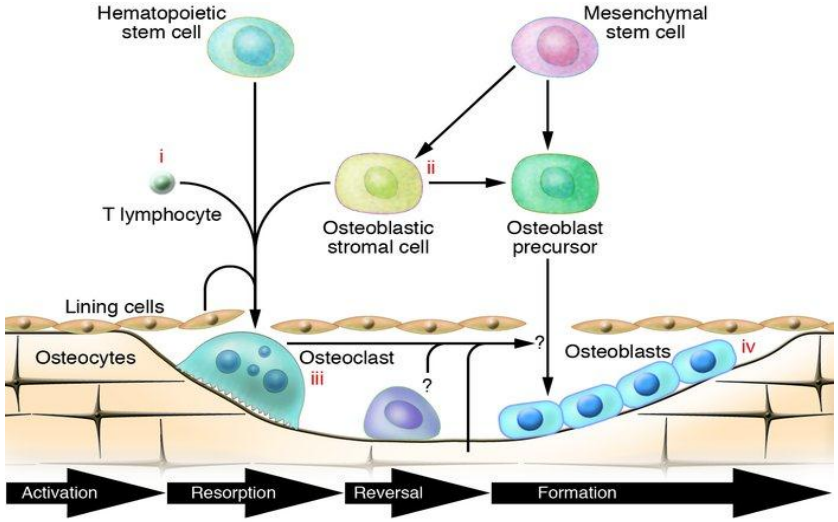


Figure 2. Bone modeling and remodeling. With permission from the American Society for Clinical Investigation; Raisz, (2005).

Examples of nutritional factors which act in a similar manner as classical hormones and are essential for bone development and maintenance are vitamins D and A. Vitamin D status plays a very important role in bone health; and has a key role in mineral ion absorption, particularly calcium, and skeletal growth and mineralization. Vitamin D can be classified as a hormone due to its action as a ligand for the vitamin D receptor (VDR), which exerts its effects via the formation of a complex with retinoid X receptor (RXR). The VDR-RXR heterodimer then interacts with DNA response elements on target genes (MacDonald et al., 2001). Vitamin D deficiency leads to decreased intestinal calcium absorption, hypophosphatemia and increased bone turnover (Holick, 2007; Viljakainen et al., 2009). All these alterations in mineral metabolism will result in lower bone mineral density and an increased risk of bone loss or fracture (Bischoff-Ferrari et al., 2004, 2009; Cauley et al., 2008, 2010; Ensrud et al., 2009). Traditional

manifestations of severe vitamin D deficiency are known as rickets or osteomalacia. Retinoids effects bone both during embryonic development and in adult stage, and are essential in the bone remodeling process. Retinoid deficiency in animals leads to morphological changes in bone and increases bone thickness (Palacios, 2006; See et al., 2008). On the other hand, prolonged high intake of retinoids induces toxic effects, which are related to the time and duration of exposure as well as to the exposure concentration. Chronic poisoning of retinoids is characterized by the presence of painful tender swellings on the long bones. Experimental studies have also indicated that excess retinoids in form of retinoic acid suppresses osteoblastic activity, stimulates osteoclast formation, and decreases the ability of vitamin D to sustain proper serum calcium concentrations (Barker and Blumsohn, 2003; Polizopoulou et al., 2005; Rothenberg et al., 2007). Further, several observational studies have suggested that similar effects of consuming large amounts of retinoids may also be important in humans; since it will result in increased fracture risk (Michaëlsson et al., 2003; Genaro Pde and Martini, 2004; Opotowsky and Bilezikian, 2004; Rothenberg et al., 2007). The action of retinoids in bone is cell-specific; and is regulated on two levels (Harada et al., 1995). One is regulation of gene expression through the actions of nuclear retinoid receptors. The other is the intracellular retinoid transport.

Bone tissue has proven to be sensitive to many chemicals and contaminants. In the mid-1980s, it was first reported that high-level accidental dietary exposure to the fungicide hexachlorobenzene (HCB) resulted in severe osteoporosis (Cripps et al., 1984; Gocmen et al., 1989), and infants exposed *in utero* to high concentrations of PCBs and PCDFs developed irregular calcification of skull bones (Miller, 1985). Since then epidemiological studies have shown that exposure to food-derived environmental contaminants alters bone tissue properties (Beard et al., 2000; Glynn et al., 2000). Exposure to PCBs in human populations near the Baltic coast resulted in sex-specific changes in bone quality, as assessed by altered bone mineral density, with a greater effect in males (Hodgson et al., 2008). Increased vertebral fractures and incidences of osteoporosis were found in fishermen and their wives exposed to environmental pollutants through dietary intake of fatty fish from the Baltic (Alveblom et al., 2003), while three independent studies have found no statistically-significant association between bone mineral density and OC exposure after adjustment for age and body mass index (Wallin et al., 2005; Côté et al. 2006; Rignell-Hydbom et al., 2009). Alterations in bone quality have also been described for wildlife from organohalogen-contaminated environments (Lind et al., 2003, 2004; Sonne et al., 2004; Rodriguez-Navarro et al., 2006; Fox et al., 2008; Johnson et al., 2009; Roos et al., 2010). Consistent with these observations, experimental studies have revealed that single chemical exposure to organohalogens modulate bone modelling and remodelling in both adult animals (Andrews et al., 1990; Lind et al., 2000a, 2000b; Jämsä et al., 2001; Van Der Ven et al., 2006; Badraoui et al., 2007; Alvarez-Lloret et al., 2009; Herlin et al., 2010) and in the offspring following maternal exposure (Hermsen et al., 2008; Van Der Ven et al., 2009). The most detailed toxicological bone studies have been performed with TCDD, which exerts most of its toxic effects through activation of the aryl hydrocarbon receptor (AhR) (Jämsä et al., 2001; Denison and Nagy, 2003; Herlin et al., 2010). *In utero* and lactational exposure to TCDD alters bone geometry, mineral density and biomechanical strength in rat offspring (Miettinen et al., 2005; Finnilä et al., 2010), and retards bone maturation patterns at the bone matrix level (Finnilä et al., 2010). **Table 1** summarizes some bone effects observed in experimental animals following developmental as well as adult exposure to environmental pollutants.

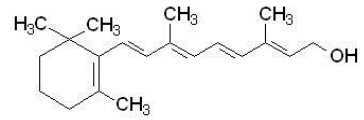
Table 1. Bone changes induced by exposure to environmental pollutants

	Compound	Animal (sex)	Effects	References
Perinatal exposure	TCDD	Rat (♂,♀)	Reduced bone mineral density, length, cross-sectional area and strength	Miettinen et al., 2005
		Rat, S-D (♀)	Reduced bone mineral density, length, thickness, cross-sectional area, mineralization and strength	Finnilä et al., 2010
		Mice (♂)	Reduced bone mineral density, thickness, mineralization and osteoblast activity	Nishimura et al., 2009
	PCB mixture: (126,138,153,180)	Rat, S-D (♂♀)	Reduced bone thickness, cross-sectional area and strength	Cocchi et al., 2009
	HBCD	Wistar rat (♂♀)	Reduced bone mineral density, thickness, length and cross-sectional area	Van Der Ven et al., 2009
	MeHg chloride	Fischer rat fetuses	Reduced bone ossification	Lee and Han, 1995
	Cadmium	MF1 mice (fetuses)	Reduced bone ossification	Padmanabhan and Hameed, 1990
Adult exposure	TCDD	Rat, L-E & H/W (♀)	Reduced bone length, cross-sectional area and strength. Bone mineral density was reduced in LE strain, while increased in HW strain	Herlin et al., 2010
	PCB 126	Rat, S-D (♀)	Increased bone mineral density and reduced mineralization	Alvarez-Lloret et al., 2009
		Rat, S-D (♀)	Reduced bone length, cross-sectional area and strength	Lind et al., 2000a
		Rat, S-D (♀)	Reduced bone length, cross-sectional area, strength and osteoblast activity. Increased bone mineral density and thickness	Lind et al., 2000b
	Aroclor 1254	Wistar rat (♂)	Reduced osteoblast activity and bone formation; and increased bone resorption	Ramajayam et al., 2007
	PCB mixture: (138,153, 163, 164, 170, 180, 190)	Deer mice (♂,♀)	Reduced bone thickness and strength; and increased fracture risk	Johnson et al., 2009
	HBCD	Wistar rat (♀)	Increased bone mineral density and cross-sectional area	Van Der Ven et al., 2006
	HCB	Fischer rat (♂)	Increased bone mineral density, cross-sectional area and strength	Andrews et al., 1990
	HCB	Fischer rat (♂)	Increased bone mineral density, reduced osteoblast activity and bone formation	Andrews et al., 1989
	MeHg chloride	Rats	Reduced bone length	Yonaga et al., 1985
	Cadmium	Wistar rat (♀)	Reduced bone mineral density, cross-sectional area and strength	Brzóska, 2011
		Wistar rat (♂)	Reduced bone mineral density, length, strength, osteoblast activity and bone formation; increased bone resorption	Brzóska et al., 2007
		Mice (♀)	Reduced bone mineral density and bone formation; increased bone resorption	Imai, 1995

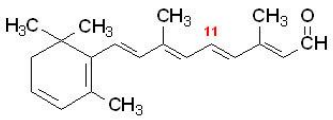
♂= male. ♀= female. S-D = Sprague-Dawley, L-E = long-Evans and H/W = Han/Wistar strains. HBCD = hexabromocyclododecane. HCB = hexachlorobenzene.

2.3.2 Retinoid system

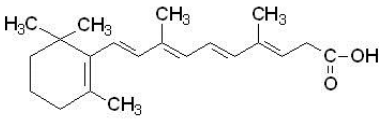
Natural retinoids are introduced into the body via food items of animal origin, particularly liver and dairy products, and food items of plant origin, e.g. orange and leafy green plant foods. Both clinical reports and experimental findings have revealed that retinoids have a wide variety of effects on vertebrate embryonic body shaping and organogenesis, tissue homeostasis, cell proliferation, differentiation and apoptosis (Blomhoff, 1994; Morriss-Kay and Ward, 1999; Mark et al., 2006). The functions of retinoids in the embryo beginning soon after conception and continuing throughout life in all vertebrates (Napoli, 1999). In adults, retinoids are essential for the functionality of several organs, including liver, lungs, nervous system, and immune system. Retinoids have also crucial roles on integrity of membranes, growth, vision, bone tissue growth and gene expression (Ribaya-Mercado and Blumberg, 2007; Duester, 2008). The basic structure of the retinoid molecule consists of a cyclic end group, a polyene side chain and a polar end group (Trapasso et al., 2009). Alternation of side chains and end groups creates the various classes of retinoids as shown in **Figure 3**.



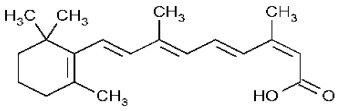
All-*trans* retinol



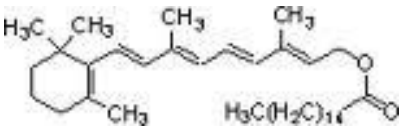
All-*trans* retinal



All-*trans* retinoic acid



13-*Cis* retinoic acid



Retinyl palmitate

Figure 3. Selected retinoid structures

Most functions of retinoids are mediated through retinoic acid (RA), which acts as a regulator of hundreds of genes, through its active metabolites *at* RA and 9-cis retinoic acid (9*c* RA) which bind to the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) respectively (**Figure 4**). Retinoid receptors are ligand-activated, DNA-binding, *trans*-acting, transcription-modulating proteins (Chambon, 1996). *At* RA binds only to RAR, while 9*c* RA binds to the RXR and RAR receptors with similar affinities (Ziouzenkova and Plutzky, 2008). RARs can form heterodimers with RXRs, and this complex bind to specific DNA sequence-retinoic acid response elements (RAREs). Thereby, the RAR/RXR heterodimers are able to modulate the

transcription of target genes (Blomhoff and Blomhoff, 2006). RA is obtained from dietary-derived retinyl esters through a complex process controlled by a large number of enzymes and binding proteins (**Figure 4**).

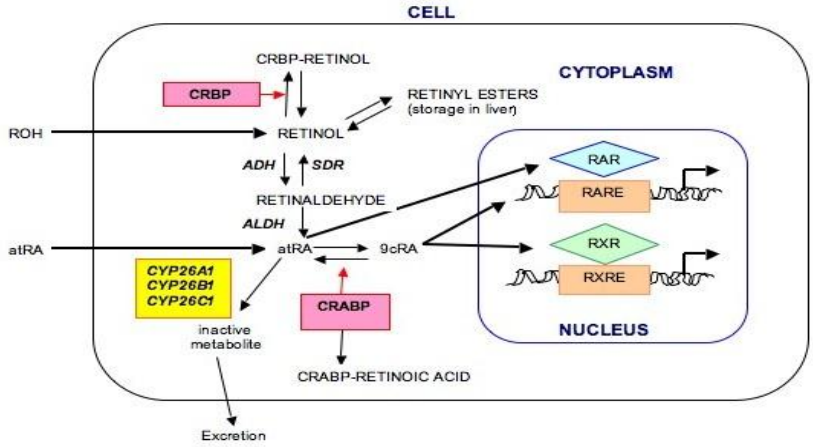


Figure 4. Retinoid metabolism and mechanism of action. With permission from the American Chemical Society; Yee et al., (2005).

Human studies have shown that tissue retinoid levels are modulated by exposure to environmental pollutants. Retinoid status of newborns from Inuit Arctic and from the general population was assessed by measuring plasma retinol concentration. It was shown that retinol concentrations were markedly lower in Inuit newborns compared to newborns from the general populations (Dallaire et al., 2003). This finding could be linked to the consumption of contaminated food in the Inuit Arctic region. A recent human study demonstrated that plasma retinol of Thai women showed highly significant positive correlations with individual DDT compounds, particularly with p,p′-DDT (Stuetz et al., 2006). This finding could be explained by the notion that DDT can induce an increase of plasma retinol by depletion of the liver retinoid reserves. Experimental studies also revealed that reduction of hepatic retinoids is one of the most sensitive parameters following long-term low-dose exposure to AhR ligands, such as dioxins and PCBs, in several species (Brunström et al., 1991; Van Birgelen et al., 1995; Murk et al., 1998; Fattore et al., 2000; Fletcher et al., 2001). Also, a single dose of TCDD has been shown to alter RA concentrations in liver, serum and kidney of Sprague-Dawley rats (Nilsson et al., 2000; Hoegberg et al., 2003; Schmidt et al., 2003a). Studies in wildlife have shown decreased plasma retinoid levels in response to increased concentrations of PCBs in species such as in polar bears (Skaare et al., 2001) and grey seals (Jenssen et al., 2003). **Table 2** summarizes some retinoid changes observed in experimental animals following developmental as well as adult exposure to environmental pollutants. All of the perinatal as well as the adult exposure studies examined the alterations in the storage and circulatory retinoid forms, i.e. retinyl palmitate and retinol respectively. However, only a few the adult exposure studies but not the perinatal exposure studies investigated the outcome on the signaling retinoid form, i.e. *at* RA or its metabolites (**Table 2**).

Table 2. Retinoid changes induced by exposure to environmental pollutants

	Compound	Animal (sex)	Effects	References
Perinatal exposure	TCDD	Rat, S-D (♂,♀)	Reduced hepatic total retinoids, increased renal total retinoids	Håkansson et al., 1987
		Holtzman rat (♂,♀)	Reduced hepatic retinol and retinyl esters, increased renal retinol and retinyl esters	Kransler et al., 2007
	Aroclor 1254	Wistar rat (♂♀)	Reduced hepatic retinol and retinyl esters, reduced serum retinol	Morse and Brouwer, 1995
	HBCD	Wistar rat (♂♀)	Reduced hepatic retinol and retinyl esters.	Van Der Ven et al., 2009
Adult exposure	TCDD	Rat, S-D (♂)	Reduced hepatic retinol and retinyl esters, increased renal retinol and retinyl esters, increased serum retinol and retinoic acid (RA)	Nilsson et al., 2000
		Rat, S-D (♂)	Reduced hepatic retinol, retinyl esters and RA metabolite; increased hepatic RA; increased renal RA and serum RA	Schmidt et al., 2003a
		Rat, L-E & H/W (♀)	Reduced hepatic retinol, retinyl esters, RA and RA metabolite; increased retinol, retinyl esters, RA and RA metabolite; increased serum retinol and RA	Fletcher et al., 2005
		Rat, S-D (♀)	Reduced hepatic total retinoids, increased renal and serum total retinoids	Håkansson et al., 1987
		Holtzman rat (♀)	Reduced hepatic retinol, retinyl esters and total retinoids; increased renal retinol and retinyl esters	Kransler et al., 2007
	Aroclor 1254	Wistar rat (♀)	Reduced hepatic retinol and retinyl esters; reduced serum retinol	Morse and Brouwer, 1995
	PCBs 77 or 118	Rat, S-D (♂,♀)	Reduced hepatic total retinoids, increased renal total retinoids	Chu et al., 1995
	PCB 126	Rat, S-D (♂,♀)	Reduced hepatic total retinoids, increased renal total retinoids	Chu et al., 1994
	PCB 156, TCDD, PCB 156 + TCDD	Rat, S-D (♀)	Reduced hepatic retinol and retinyl esters; increased serum retinol	Van Birgelen et al., 1994a
	PCB 156, TCDD, PCB 156 + TCDD	Rat, S-D (♀)	Reduced hepatic retinol and retinyl esters; increased serum retinol	Van Birgelen et al., 1994b
	PCBs 52, 77 or 153; or p,p´-DDT	Rat, S-D (♂)	Reduced hepatic retinyl esters and total retinoids, and increased renal retinol and retinyl esters after exposure to PCB 77. Reduced serum retinol after exposure to PCBs 77 or 52.	Azais et al., 1987
	HBCD	Wistar rat (♂,♀)	Reduced hepatic retinol and retinyl esters in females only	Van Der Ven et al., 2006

♂= male. ♀= female. S-D = Sprague-Dawley, L-E = long-Evans and H/W = Han/Wistar strains. HBCD = hexabromocyclododecane. HCB = hexachlorobenzene.

As shown in **Tables 1** and **2**, most of the experimental studies were performed using single chemicals. Further, most of the studies investigated the effects of exposure to environmental pollutants in adults, and only few studies were performed during early stages of life. There is also need for studies which follow-up the animals for longer periods to test for reversibility of effects observed in young offspring, or to examine effects which appear later in life following exposure during fetal stages. More close quantitative evaluation of changes in bone and retinoid parameters using methods such as benchmark dose is also required. Retinoids are essential for the almost every tissue in the body, and alterations in its levels might have severe consequences during perinatal as well as for adult stages.

3 THE PRESENT INVESTIGATION

3.1 AIMS

The overall objective of the current project was to generate new experimental data, which will contribute new knowledge to improve the health risk assessment associated with the exposure to human-relevant chemical mixtures with a focus on the postnatal consequences of the exposure during fetal life and lactation, using the situation of the Canadian Arctic populations as an example.

Following *in utero* and lactational exposure to NCM or Aroclor 1254, the specific project objectives were to:

- Evaluate changes in bone geometry, mineral density and biomechanical properties to establish both quantitative and qualitative toxicity characteristics (papers II and III).
- Assess changes in retinoid system parameters to establish both quantitative and qualitative toxicity characteristics (papers I, IV and V).
- Clarify if any observed effects on bone tissue or the retinoid system could be ascribed to the dioxin-like components in the respective mixtures or not, by using the toxic equivalent (TEQ) calculation approach (papers III and V).
- Examine if there were any differences in response between male and female rat offspring (papers II, III, IV and V).

3.2 MATERIALS AND METHODS

3.2.1 Experimental animals (papers I-V)

Rat is a commonly used animal model; and is considered as a good model for toxicological studies that aims to assess human health risks. Rats in current study were maintained and used in accordance with the Canadian Council Animal Care guidelines and Health Canada's Institutional Animal Care Committee. Sprague-Dawley rats; 77 females weighing 200-230 g, and 38 males weighing 320-350 g were obtained from Charles River laboratories, St Constant, Quebec, Canada. Upon arrival, females were housed two per cage while males were housed individually. The animals were housed in polycarbonate hanging cages measuring 35 cm (L) x 30 (W) x 16.5 (H) with shaved wood bedding in rooms maintained at 22 ± 2°C and 50 ± 10% humidity. One day after arrival in the housing facilities, the animals were gradually switched to a reverse light/dark cycle; and then were maintained on this 12 hours reverse cycle thereafter. One week after arrival, permanent identification chips (Model IMI-1000, Bio Medic Data Systems, Seaford, DE) were implanted in the females under light isoflurane anesthesia. After acclimatization period of three weeks, breeding was conducted by placing two females into each male cage. Females were monitored two times daily for vaginal plugs. Once a vaginal plug was detected, the female was removed from the male cage and housed individually in a standard rodent cage. The day of detection of vaginal plug was denoted gestation day 0 (GD 0). Beginning on GD18, dams were monitored 3 times daily for parturition. The day of birth was denoted as postnatal day 0 (PND 0). Litters were culled to 8 pups (4 males and 4 females) on PND4. Blood, bones,

and tissue materials (livers and kidneys) of dams were collected seven to ten days after weaning; while blood, bones, livers and kidneys from one male and one female rat offspring from each litter were collected at PND 35, 77 and 350, respectively.

3.2.2 Chemicals

All of the test chemicals were of analytical grade (≥99%), procured commercially from Cerilliant, Round Rock, TX, with the exception of toxaphene (also from Cerilliant, Round Rock, TX) and Aroclor 1254 (AccuStandard, New Haven, CT), which were of technical grade. Oxychlordane was a generous gift from Julie Fillion of the Pest Management Regulatory Agency (Ottawa, ON, Canada). The sources of test chemicals not already indicated are as follows: polychlorinated biphenyl (PCB) 28 and PCB 183 (AccuStandard, New Haven, CT); methylmercury chloride (Aldrich Chemical Co, Milwaukee, WI), p,p′-DDE (Sigma-Aldrich, St. Louis, MO), p,p′-DDT (Riedel-de Haën, Sigma-Aldrich Laborchemikalien, Seelze, Germany), and hexachlorobenzene (Fluka, Steinhein, Switzerland).

3.2.2.1 NCM preparation (papers I, II and IV)

The composition of the NCM is shown in **Table 3**. NCM was based on the profile of chemicals found in maternal blood of Canadian Inuit populations (Van Oostdam et al., 1999; Butler Walker et al., 2003) as part of the Northern Contaminants Program (NCP) studies. The final mass of each chemical in the final mixture represented the proportionate mass of each chemical found in human blood in μg/L. The dosing solutions were prepared by dissolving individual chemicals in di-ethyl ether, mixing ethereal solutions with corn oil, and evaporating the ether under a gentle stream of nitrogen. The dosing solutions were analyzed by the gas chromatographic (GC) method (paper I). Doses of NCM were based on results of a pilot study which was conducted to determine the maximum dose tolerated by pregnant rats. Since 5.0 mg/kg/day produced no obvious alterations in offspring mortality and little effect on maternal weight gain, this dose was selected as the highest dose. Lower doses selected were 0.05 and 0.5 mg/kg/day. It was shown that the dose level of 0.05 mg/kg bw of the mixture is comparable to human exposure in the Arctic region (paper I). Total toxic equivalents (TEQ) concentrations for dioxin-like congeners in the high dose of the NCM and Aroclor 1254, estimated according to (Van den Berg et al., 2006), were calculated to be 0.153 μg/kg body weight (bw) (data not shown) and 5.289 μg/kg bw (paper III) respectively.

3.2.2.2 Aroclor 1254 preparation (papers I, III and V)

PCB and PCDF congeners of Aroclor 1254 are shown on Table 1, paper 3. In the present study, we are investigating how perinatal exposure to Aroclor 1254 affect bone tissue and retinoid system in offspring. The dosing solution of Aroclor 1254 was prepared by dissolving Aroclor 1254 in corn oil. The dose of Aroclor 1254 (15 mg/kg bw/day) used in this study was chosen based on previous studies (Goldey et.al., 1995; (Herr et al., 1996; Roegge et al., 2000; Geller et al., 2001; Widholm et al., 2001; Zahalka et al., 2001).

3.2.3 Diet (papers I-V)

One cookie (Nabisco Ltd, Toronto, Canada) per day was supplied to each dam. The dosing solution (1 µl mixture/g bw) was administered onto the cookies and provided as such to the dams. This dosing approach has many advantages. It permitted administration of controlled doses of the dosing solutions that were adjusted daily on the basis of the body weight. This dosing method to pregnant rats permitted precise control over dosing during pregnancy and lactation where food intake (and thus dosing) can vary significantly. Furthermore, this dosing method avoided the stressfulness of oral gavage in pregnant rats.

Table 3. Concentrations of chemical constituents of the NCM (PCBs, OC pesticides and MeHg). The dose of each chemical for the 5.0 mg/kg body weight (bw) dose group is shown below[a]**.**

PCB components	mg	Non PCB components	mg
28	0.0072	Aldrin	0.0049
52	0.0154	β-HCH	0.0746
99	0.0973	Cis-nonachlor	0.0525
101	0.0145	p,p´-DDE	0.9187
105	0.0165	p,p´-DDT	0.0569
118	0.0727	Dieldrin	0.0223
128	0.0071	Hexchlorobenzene	0.02961
138	0.2146	Heptachlor epoxide	0.0232
153	0.3177	Mirex	0.0291
156	0.029	Oxychlordane	0.1359
170	0.0562	Toxaphene	0.0699
180	0.1522	Trans-nonachlor	0.2203
183	0.0193	Methylmercury Chloride	1.9965
187	0.0795		
Sum PCBs	1.0992	Sum Non-PCBs	3.9009
% PCBs	22.0%	% Non-PCBs	78.0%
Total Dose			5.0001

[a] NCM dose levels used were 0.05, 0.5 and 5.0 mg/kg bw. The dose of each chemical is based on the relative amounts of each chemical found in maternal blood samples from Canadian Arctic populations. The concentration of each chemical in the mixture was determined by gas chromatography-mass spectrometry (GC-MS). Animals were dosed at 1 ml/kg body weight.

3.2.4 Body and organ weights (papers I, IV and V)

In toxicity studies, organ weights, serum biochemical data, hematological values and histological observations are the most important toxicological parameters for evaluating organ damage. In the current study dams were individually weighed on daily basis throughout the study. Pups were also individually weighed daily starting from PND4. Organ weights were presented as absolute weight or relative weight (i.e. the ratio of organ weight to body weight).

3.2.5 Bone quality measurements (papers II and III)

The right femur and tibia were dissected, cleaned of muscle and soft tissue, and stored in Ringer solution (1 liter contains 0.3 g Tris, 0.24 g $CaCl_2(H_20)_2$, 0.4 g KCl, and 2.05 ml 1M HCl, pH 7.4) and stored at -20° C until analysis. On the day of analysis, bones were thawed at room temperature and stored moistened in closed plastic tubes until examination.

3.2.5.1 Bone length

The total length (mm) of femur and tibia was measured using an electronic sliding caliper (IP65, Sylvac SA, Crissier, Switzerland) to the nearest 0.01 mm. The femoral bone length was measured from the top of the caput femoris to the distal point of the condylus medialis. The tibial bone length was measured from the proximal point of the medial tibial condyle to the medial malleolus.

3.2.5.2 Bone geometry and mineral density

Bone geometrical and densitometrical parameters were evaluated using peripheral quantitative computed tomography (pQCT). The pQCT method measures bone mineral density and cross sectional bone dimensions at peripheral skeletal sites. Comparing pQCT to other radiological imaging methods like dual energy X-ray aborptiometry (DXA), pQCT has more advantages. pQCT allows separate measurement of cortical and trabecular bone (Rico et al., 1994). This is beneficial because these two types of bone are heterogeneous (Ninomiya et al., 1990) and have distinct temporal remodelling characteristics (Eriksen et.al., 1993). Furthermore, a pQCT scan is able to measure volumetric bone mineral density (3D, mg/cm^3), plus other measures such as the geometry of the bone. However, DXA is only able to provide the areal bone mineral density (2D, mg/cm^2). pQCT, being used in humans as well as in experimental studies, is regarded as sensitive, reproducible, non-invasive tool that allows for shorter duration of experiments. pQCT measurements provide a lot of information regarding the parameters which might be altered by specific treatment. However, it cannot determine how bone strength is affected. Hence, this method needs to be complemented by specific bone biomechanical testing.

In **papers II** and **III**; the excised femur and tibia were scanned using pQCT system (Stratec XCT 960A with software version 5.50 Norland Stratec Medizintechnik, GmbH, Birkenfeld, Germany) as previously described (Herlin et al., 2010). The bones were inserted into a plastic tube filled with Ringer solution to position the samples for the measurements. Calibration of the machine was performed with a validation phantom provided by the manufacturer. To evaluate the reproducibility of the pQCT measurements, the coefficient of variation (CV) was calculated from 10 repeated measurements with one sample repositioned before each measurement. The CV values of parameters measured were below 3 %. Cortical bone parameters were acquired from femoral and tibial diaphyseal scans, performed at a site distanced 50% of the length from the end of the bone (**Figure 5**). A threshold of 710 mg/cm^3 was used to define the cortical bone. Trabecular bone parameters were acquired from tibial metaphyseal scans, performed at sites distanced 10% of the length from the proximal end of tibia. Thresholds of 400 mg/cm^3 as an upper limit and 200 mg/cm^3 as a lower limit were used to define the trabecular bone. Image processing and the calculation of numerical values were performed using the manufacturer's software package.

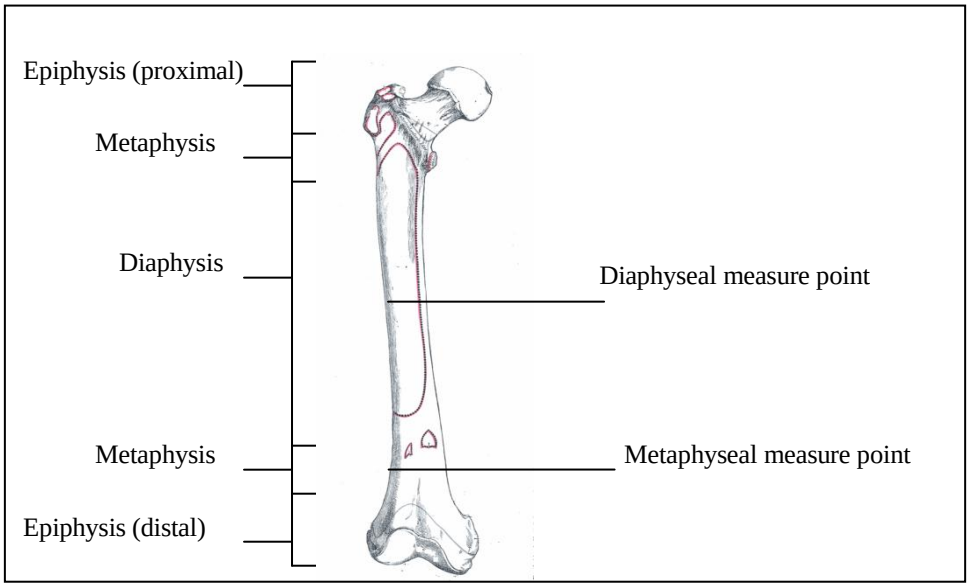


Figure 5. A representative diagram illustrating the measuring points of rat femur in the peripheral quantitative computed tomography (pQCT).

3.2.5.3 Bone biomechanical testing

Biomechanical testing of bone can be considered as an important tool to investigate the functional changes in bone material and architecture (Broulik et al., 2007). Biomechanical tests of intact animal bones provide an objective method for evaluating the effects of age, sex, nutrition, contaminants, and environment on the physical condition of the animal. The four basic configurations that are commonly used to assess the bone strength and biomechanical properties are bending, torsion, compression and tension. For long bones, bending until failure using three- or four-point method is the most commonly used configuration. The three-point bending tests should be used only when the bone is straight, has a symmetrical cross section, and has a support length to diameter ratio greater than 10. The main advantage of a three point bending test is the ease of the specimen preparation and testing. However, this method has also some disadvantages; the results of the testing method are sensitive to specimen and loading geometry and strain rate. Determination of the bending properties of animal bone requires the development of a bending force-deformation (displacement) curve (**Figure 6**). From a bending force-displacement curve, ultimate bending force, stiffness and fracture energy can be obtained.

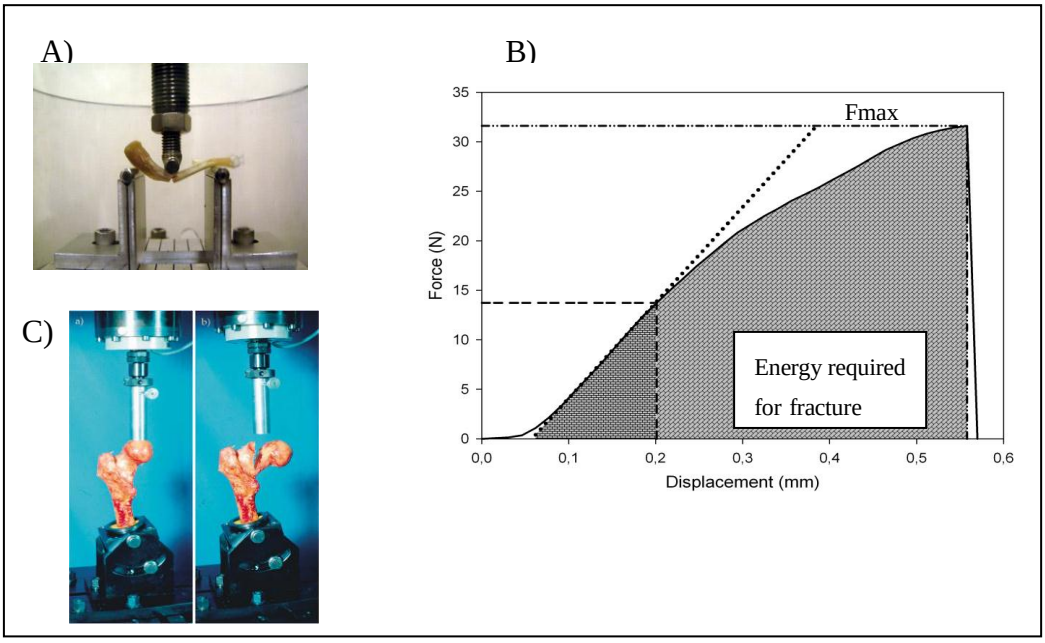


Figure 6. Biomechanical testing of long bones. **A)** Three-point bending test , and **B)** A force-displacement curve obtained from three-point bending test in the current study; where the height of the curve (y axis) represents the breaking force (bone strength), the width of the curve (x axis) represents the ultimate displacement, the slope of the curve represents the stiffness, and the area under the curve describes the energy to break the bone. **C)** Axial loading of the femoral neck.

The bones were subjected to biomechanical testing after pQCT measurements. In **papers II** and **III**; biomechanical properties of femur were investigated using three point bending test for femoral diaphysis and the axial loading for femoral neck. Biomechanical properties of distal femur (by three point bending test) were analyzed as described by Jämsä et al., (1998). The axial loading of femoral neck was performed as presented by Peng et al., (1994). Biomechanical testing was performed with the universal mechanical testing machine (Model 3366, Instron Corp., Canton, MA) controlled by the computer software program Bluehill ver 2.6 (Instron Corp.), which collected load deformation data at a sampling rate of 10 Hz until failure. Distal femur was set horizontally on a holder with span length of 12-20 mm depending on the bone length. A load was applied at a speed of 0.155 mm/s vertically to the anterior surface of femur mid-diaphysis at the same site where the pQCT scan was performed.

3.2.6 Biochemical analyses

3.2.6.1 Retinoid analyses (papers I, IV and V)

In **papers I**, **IV** and **V**, different forms of retinoids were analyzed in homogenates of liver and kidney of rat offspring and dams using high pressure liquid chromatography (HPLC). Retinol and retinyl palmitate were analyzed in dams and in both sexes of offspring; while retinoic acid and its metabolite were only analyzed in dams and female offspring.

Retinol and retinyl palmitate in liver and kidney were analyzed by HPLC as described in (Nilsson et al., 2000). Briefly, retinoids were extracted using diisopropyl ether and

separated on a Nucleosil C18 5-µm HPLC column (Macherey-Nagel, GmbH, Germany) using an ethanol:water mobile phase and retinol, retinyl acetate (internal standard) and retinyl palmitate were detected with a JASCO821-FP fluorescence detector (λex = 325 nm, λem = 475 nm). Retinoic acid (RA) and its metabolite 9c4o13,14dh-RA in liver and kidney were analyzed by HPLC as described by (Schmidt et al., 2003b). In brief, retinoids were extracted using isopropanol and RA and its metabolite were separated from retinol and retinyl palmitate by solid-phase-extraction using an aminopropyl phase. RA and its metabolite were then separated on a Spherisorb ODS2 column (2.1×150mm, 3 µm particle size, Waters, Eschborn, Germany) using a binary gradient, and RA and its metabolite were detected with a UV detector at 340 nm.

3.2.6.2 Cytochrome P450 (CYP) activities (papers I, IV and V)

Hepatic CYP1A, CYP2B and CYP3A activities were assessed as EROD, PROD and BROD activities according to (Lubet et al., 1985). Resorufin formation was measured in quartz cuvettes using an Aminco-Bowman spectrophotofluorimeter. The dealkylation of the alkoxyresorufin compounds was monitored by recording the increase in relative fluorescence of the product (resorufin) as a function of time (Lubet et al., 1985). Assessment of renal CYP1A activity as EROD activity was carried out in 96-well plates, and the reaction product (resorufin) was measured with a fluorescence plate reader (GENios Pro, Tecan Ltd.) as previously described (Kennedy et al., 1993). Separate 96-well plates were used for determining total protein concentration using the fluorescamine assay which was adapted for use with a fluorescence plate reader (Lorenzen and Kennedy, 1993). Analysis of renal EROD activity was performed for dams and female offspring.

3.2.6.3 Thyroid hormones (paper I)

Serum thyroxine (T4), triiodothyronine (T3), and triiodothyronine uptake were analyzed in a Bayer instrument (ACS 180 Plus), while serum thyroid-stimulating hormone (TSH) was assayed using a commercial radioimmunoassay (RIA) kit.

3.2.7 Mathematical data evaluation

3.2.7.1 General statistics (paper I-V)

- *One way analysis of variance (ANOVA)*

All data are expressed as mean ± standard deviation (SD). Homogeneity of variances was verified using Levene's test. One-way ANOVA followed by Tukey's *post-hoc* test when variances were homogeneous or with Dunnett's T3 test when the variances were not homogeneous, was used to compare different dose groups against corresponding controls. The level of significance was set at $P < 0.05$. Statistical analyses were performed using SPSS 18.0 Statistical Software (SPSS Inc. Chicago, USA).

- *Two- and three-way repeated-measures ANOVA*

Data were analyzed by two- and three-way repeated-measures ANOVA to assess the effect of treatment (T) over age (A) considering the impact of sex (S) and possible

interactions AxT, AxS, TxS, and AxTxS. Homogeneity of variances was verified using sphericity test; and all reported results were corrected by the Greenhouse–Geisser procedure where appropriate. Significant T effects were subsequently identified by Tukey's HSD or Dunnett's T3 post hoc test when variances were homogeneous or not, respectively.

- *Two-tailed independent sample T-test*

All data are expressed as mean ± SD. Two-tailed independent-samples T test was used to compare Aroclor 1254-exposed rats against corresponding control group. The level of significance was set at $P < 0.05$.

3.2.7.2 Dose-response modeling (papers II and IV)

A dose-response modeling approach was applied to establish benchmark dose (BMD) for the individual parameters tested following NCM exposure using a family of exponential models (PROAST version 18.2 in R software version 2.9.2). The BMD value was defined as the dose causing a 5% (or 100% for CYP enzymes) change in response relative to background. The likelihood ratio test was used as formal criteria to select a member from this family of models (Slob, 2002). The same model was then applied to combined data of both sexes in order to describe the dose-response relationship by using the minimum number of parameters. Background, sensitivity and residual variation were compared by sex as a covariate.

3.2.7.3 Calculations of TCDD equivalents (papers III and V)

The biological potency of Aroclor 1254, relative to TCDD, to induce changes in bone tissue and retinoid parameters was estimated by quantitatively comparing the bone and retinoid effects induced by Aroclor 1254 to those induced by TCDD. Using a family of exponential models (PROAST version 20.2 in R software), dose response curves were fitted to individual bone parameters derived from a TCDD dose–response exposure study (unpublished data; Finnilä et al., 2010) with similar design as the present study. The bone and retinoid parameters selected were those which were affected both by Aroclor 1254 and TCDD. The likelihood ratio test was used as formal criteria to select a member from a family of nested models, and the selected model was then applied to both sexes simultaneously in order to describe the dose–response relationship by using the minimum number of parameters (Slob, 2002). BMDL, i.e. benchmark dose (BMD) at the lower bound of the 95% confidence interval, and the BMD/BMDL ratio, which is a measure of the statistical uncertainty where a ratio greater than 10 is not acceptable, were determined; the latter was used as a criterion to select parameters for inclusion. The effect sizes of the Aroclor 1254- induced bone and retinoid effects, which were calculated as the mean percentage change in the Aroclor 1254 exposed group compared to the control group, were used as the benchmark response levels (Table 4, paper III; Table 2, paper V). The TCDD-benchmark dose corresponding to these Aroclor 1254 effect sizes were set as the TCDD equivalents for the various bone and retinoid parameters, and were compared to the theoretical TEQ calculated from the chemical composition of the Aroclor dosing solution (Tables 1 and 4, paper III; Table 2, paper V).

3.2.7.4 Partial least-squares (PLS) analysis (papers IV and V)

A multivariate regression between NCM/Aroclor 1254 treatment, body and liver weight, hepatic EROD, PROD and BROD activities (explanatory variables) and hepatic retinoid levels (9c4o13,14dh-RA, retinol, and retinyl palmitate as result variables) was performed by PLS analysis (Höskuldsson, 1988; Wold et al., 2001; Bastien et al., 2005; Eriksson et al., 2006) using R software version 2.9.2 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). The same PLS method was applied for female rats at PNDs 35, 77 and 350 and dams. Variables within components were entered in a PLS model using a backward method The PLS-2 multivariate response model allowed high explained variances of both explanatory and result variables as determined by R^2X and R^2Y fractions, respectively. A model with both R^2X and R^2Y values > 0.6 was considered to be acceptable in order to study associations between variables. Variables with variable importance in the projection (VIP) values $\geq$ 0.8 in, at least, one component and time point were considered significant variables and entered in the PLS model (Chong and Jun, 2005). Explanatory (X) and result variables (Y) were visualized on correlation circles using their correlations with PLS components.

3.2.8 Ethical approvals

The animal exposure part of this thesis work (papers I-V) was carried in Canada, and was approved by the Animal care committee, Health Canada, Ottowa (assigned number is ACC 01045). Moreover, the animal exposure part of paper III was conducted in Finland, and was approved by the the animal experiment committee of the University of Kuopio, Finland (ethical permit number 03-04).

3.3 RESULTS AND DISCUSSION

3.3.1 Toxicological evaluation (papers I, IV and V)

The dose range used in the present project is considered as low-dose range. Therefore, the observed toxicological symptoms following exposure to this dose levels were relatively small and transient being only measureable in young offspring. The purpose of using such a low-dose range was to mimic the *in utero* and lactational human exposure situation, and also to examine the outcome of these levels on different endpoints.

In **paper I**, perinatal exposure to NCM or Aroclor 1254 resulted in growth suppression, decreased weight of thymus, kidney and spleen (Table 1, paper I). In addition, serum levels of thyroid hormones and cholesterol were altered, liver microsomal enzyme activities were increased, and hepatic retinoid levels (analyzed as retinol and retinyl esters) were decreased (Table 3, paper I). All these effects were pronounced in offspring at PND35, less marked at PND77 and returned to normal values by PND350. The interesting finding in this study was the reductions of hepatic retinoid levels which were observed in the dams, as well as in their male and female offspring at all post-natal follow-up time points after perinatal exposure to NCM or Aroclor 1254.

Following NCM exposure (**paper IV**), body weight and absolute kidney weight were decreased in both sexes of the offspring at PNDs 35 and 77 (Figure 2, paper IV). Relative liver weight was increased in both sexes at PND35 (Figure 2, paper IV). Associated BMD values of these effects were in the range of 1.06 - 2.16 mg/kg bw/day (Table 2, paper IV). For the dams, an increase in absolute and relative kidney weight was observed (Figure 2, paper IV).

Following Aroclor 1254 exposure (**paper V**), reductions in body weight and absolute kidney weight, as well as increase in absolute and relative liver weight were observed in female and male offspring at PND35 (Figure 4, paper V). For the dams, no treatment-related changes were observed for body or organ weights.

Reduction in body weight and increased liver weight are considered as common markers of exposure to environmental pollutants. Current results of reduced body weight and increased liver weight of offspring were clearer in juvenile, but not in adult, offspring. These findings are consistent with previous studies (Pohjanvirta and Tuomisto, 1994; Van Birgelen et al., 1995; Fletcher et al., 2001; Alsharif and Hassoun, 2004). In contrary to a previous study (Van Birgelen et al., 1995), our findings in paper IV revealed a reduction in kidney weight of both sexes of PND35 and PND77 offspring.

3.3.2 Bone status (papers II and III)

3.3.2.1 Bone geometry and mineral density following perinatal NCM or Aroclor 1254 exposure

Multiple bone parameters, including geometrical and mineral density parameters, were clearly affected in the offspring at PND35; partly affected at PND77, while no bone changes were detected at PND350 following perinatal exposure to NCM (**paper II**). Affected parameters included reduction in femur length, cross-sectional area, thickness and total area of diaphysis (Table 1B, paper II). For offspring at PND35 and following NCM exposure, the calculated benchmark dose (BMD) of the above mentioned parameters revealed values of 3.2, 1.6, 2.2 and 2.4 mg/kg bw/day, respectively (Table 2A, paper II). The pQCT-derived indicators for bone strength, polar moment of inertia (PMI) and polar strength strain index (SSIp), were also reduced in PND35 offspring following NCM treatment (Table 1B, paper II). Associated BMD values of PMI and SSIp for PND35 offspring were 0.9 and 1.1 mg/kg bw/day respectively (Table 2A, paper II).
A similar pattern of changes in bone geometrical parameters, including PMI and SSIp, was observed in the offspring after perinatal exposure to Aroclor 1254 (Table 2, paper III). However, bone mineral density was only affected by perinatal exposure to Aroclor 1254, and not by NCM (papers II and III). The TCDD equvivalents for different bone changes induced by perinatal exposure to Aroclor 1254 were calculated using the TEQ approach (Table 4, paper III). When the estimated TCDD equivalents were compared to the theoretical TEQ calculated for the Aroclor 1254 dosing solution, the estimated TCDD equivalents were in the range of 16% to 23% of the theoretical TEQ, indicating

that both dioxin-like as well as non dioxin-like components contributed to the observed bone changes (Table 4, paper III).

For the offspring, changes of bone geometrical parameters induced by exposure to NCM (Table 1A, paper II) or to Aroclor 1254 (**Table 4**) showed a similar response between males and females. Furthermore, bone changes were recovered for offspring at adult stages as exposure was discontinued. For the dams, no changes were observed in bone geometrical or mineral density parameters after NCM or Aroclor 1254 exposure.

Perinatal exposure of rats to NCM or Aroclor 1254 induced reductions in femoral and tibial bone geometrical parameters. These changes in bone geometry were marked in young offspring. Observations also showed that some bone changes, but to a lesser extent, were still noticed in offspring at PND77. However, all bone defects observed in young offspring were recovered by the age of one year. These findings are in agreement with previous reports (Andrews, 1989; Lind et al., 1999, 2000a; Miettinen et al., 2005;). Findings from current study demonstrate that perinatal treatment with NCM or Aroclor 1254 produced no maternal toxicity (paper I), indicating that bone effects seen in the offspring are due to a direct effect of the contaminants rather than a secondary effect of maternal toxicity. The results also suggested that bone remodeling was not impaired on a permanent basis, as bone properties of adult offspring were restored when the exposure was discontinued. It is also possible to speculate that observed bone changes in young offspring form the current study could be explained by altered thyroid hormone in these animals, i.e. reduced T4, since balanced status of thyroid hormones during early stages of development is required for optimal bone growth and ossification in adulthood (Bassett and Williams, 2008).

Table 4. Mean square values from three-way repeated measures ANOVA for femoral and tibial pQCT parameters of perinatally Aroclor 1254-exposed offspring at PNDs 35, 77 and 350.

	Source	df[a]	Length	Total area of diaphysis	Periosteal circumference	Endosteal circumference	Cortical bone mineral density	Cortical area	Cortical thickness	PMI	SSIp
Femur	Age (A)	2	0.401^{***}	0.700^{***}	0.175^{***}	0.012^{***}	$0.287^{*** b}$	$3.311^{*** b}$	$2.418^{*** b}$	7.246^{***}	$5.719^{*** b}$
	Treatment (T)	1	0.004^{**}	0.041^{***}	0.010^{***}	0.012^{***}	$3x10^{-4*}$	0.050^{***}	0.014^{*}	0.213^{***}	0.115^{***}
	Sex (S)	1	0.034^{***}	0.299^{***}	0.075^{***}	0.096^{***}	$3x10^{-4*}$	0.221^{***}	0.033^{***}	1.103^{***}	0.542^{***}
	A x T	2	0.002^{***}	0.004	0.001	$2x10^{-4}$	$2x10^{-4}$	$0.018^{* b}$	$0.012^{** b}$	0.037^{*}	$0.024^{* b}$
	T x S	1	$4x10^{-4}$	0.001	$3x10^{-4}$	0.001	$3x10^{-5}$	0.003	0.001	0.006	0.004
	A x T x S	2	$4x10^{-4}$	0.001	$3x10^{-4}$	0.002^{*}	$1x10^{-4}$	0.001	0.005	$3x10^{-4}$	$1x10^{-4}$
Tibia	Age (A)	2	0.452^{***b}	1.021^{***b}	0.255^{***b}	0.047^{***}	0.093^{***b}	$2.11^{*** b}$	0.801^{***}	7.47^{***b}	$3.82^{*** b}$
	Treatment (T)	1	0.007^{***}	0.030^{**}	0.007^{**}	0.008^{**}	0.001^{**}	0.027^{**}	0.005^{*}	0.18^{***}	0.058^{**}
	Sex (S)	1	0.043^{***}	0.262^{***}	0.065^{***}	0.079^{***}	$2x10^{-5}$	0.239^{***}	0.050^{***}	1.17^{***}	0.578^{***}
	A x T	2	0.005^{**b}	0.011^{**b}	0.003^{**}	0.003^{*}	0.001^{**b}	$0.010^{** b}$	0.001	0.062^{**}	$0.041^{** b}$
	T x S	1	0.001	0.003	0.001	$1x10^{-4}$	$4x10^{-5}$	0.007	0.004	0.020	0.010
	A x T x S	2	$3x10^{-4}$	$2x10^{-4}$	$4x10^{-5}$	$1x10^{-5}$	$2x10^{-4}$	0.001	$3x10^{-4}$	0.004	0.002

[a] df, degrees of freedom. Statistically significant *F*-values are indicated by $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p < 0.05$. [b] Greenhouse-Geisser's test was used. PMI = polar moment of inertia. SSIp = polar strength strain index.

3.3.2.2 Bone biomechanics following perinatal NCM or Aroclor 1254 exposure

To further elucidate if bone strength is compromised in the offspring perinatally exposed to NCM or Aroclor 1254, bone biomechanical testing was performed on femoral diaphysis and femoral neck. The biomechanical properties of both sexes showed similar pattern of response following perinatal exposure to NCM (Table 3A, paper II) or to Aroclor 1254 (**Table 5**), as indicated by non-significant treatment by sex interaction. Results of NCM-exposed offspring at PND35 showed a significant reduction in the maximum force of femoral neck (Table 3B, paper II), with an associated BMD value of 1.4 mg/kg bw/day (Table 2A, paper II). Stiffness of femoral neck was significantly reduced at PND35 following exposure to the high dose level of NCM (Table 3B, paper II). Stiffness of femoral neck was not markedly affected at PND77 or PND350 (Table 3B, paper II). Further analysis with post-hoc comparisons indicated a significant increase of maximum force of femoral diaphysis in males at mid (PND77) and low (PND350) dose of NCM relative to the corresponding controls (Figure 1, paper II).

Maximum force, maximum energy absorption and stiffness of femoral diaphysis were decreased by 12%, 7%, and 13% respectively in Aroclor1254-exposed offspring at PND35, but this reduction was not statistically significant (Table 3, paper III). These parameters were not affected in offspring at PND77 or PND350. For the femoral neck, maximum force, maximum energy absorption and stiffness were significantly decreased by 15%, 24% and 22% respectively after Aroclor 1254 treatment in PND35 offspring (Table 3, paper III). Again, no significant effects were observed at PND77 or PND350.

The observed reductions in bone strength and biomechanical properties in offspring at PND35 following exposure to NCM or Aroclor 1254 could be related to the changes of their bone geometrical parameters; and this is in line with former findings by (Jämsä et al., 1998). At PNDs 77 and 350, offspring showed no changes on biomechanical parameters following either treatment, and bone biomechanical properties seem to be restored. (Miettinen et al., 2005) observed similar results of reduced bone biomechanical properties in juvenile rats perinatally exposed to low doses of TCDD; and that the effects were reversible in old offspring. It was reported that the technique of mechanical testing of femoral neck requires a larger sample size than do the mechanical testing of diaphyseal sites (Leppänen et al., 2008), and it is likely that the sample size in this study is not large enough to get a precise result of femoral neck biomechanics and to avoid uncertainties.

Table 5. Mean square values from three-way repeated measures ANOVA for femoral biomechanical parameters of perinatally Aroclor 1254-exposed offspring at PNDs 35, 77 and 350.

	Source	df[a]	Max. force	Max. energy absorption	Stiffness
Femoral diaphysis	Age (A)	2	5.037^{***}	$5.158^{***\ b}$	$8.139^{***\ b}$
	Treatment (T)	1	$3x10^{-4}$	0.005	0.001
	Sex (S)	1	0.027	0.001	0.009
	A x T	2	0.029^{**}	0.017	0.024
	T x S	1	0.004	0.006	0.020
	A x T x S	2	0.011	0.119	0.001
Femoral neck	Age (A)	2	2.074^{***}	1.397^{***}	3.966^{***}
	Treatment (T)	1	0.029	0.148	0.010
	Sex (S)	1	0.090^{**}	0.356^{**}	0.013
	A x T	2	0.006	0.018	0.049^{*}
	T x S	1	$3x10^{-5}$	0.004	$3x10^{-4}$
	A x T x S	2	0.004	0.090	0.001

[a] df, degrees of freedom. Statistically significant *F*-values are indicated by $^{***}p \leq 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. [b] Greenhouse-Geisser's test was used.

3.3.3 Retinoid levels (papers I, IV and V)

3.3.3.1 Hepatic retinoids

To further characterize the alterations in retinoid system parameters observed in **paper I**, more detailed retinoid analysis was conducted in **papers IV** and **V**. Obtained results demonstrated that retinol levels in livers were reduced in the high-dose groups of dams and both sexes of the offspring at PND35 following perinatal NCM exposure (Table 1, paper IV). At PND77, hepatic retinol levels were increased in low- and mid-dose groups of NCM-exposed male and female offspring, while no changes in hepatic retinol levels could be observed for PND350 offspring (Table 1, paper IV). Calculated BMD value for the hepatic retinol levels of the offspring at PND35 was 0.42 mg/kg bw/day (Table 2, paper IV). The levels of hepatic retinyl palmitate were reduced in dams as well as in both sexes of the offspring at PNDs 35, 77 and 350; with associated BMD values of 0.74, 0.44, 1.4 and 0.35 mg/kg bw/day, respectively (Tables 1 and 2, paper IV). Liver levels of 9c4o13,14dh-RA, a more recently discovered active metabolite of retinoic acid, was markedly reduced in the dams and the PND35 offspring after perinatal exposure to NCM; with the lowest BMD value in this study (0.1 mg/kg bw/day) being calculated for the reduction of hepatic 9c4o13,14dh-RA (Table I, paper IV).

Hepatic retinol concentrations were decreased in Aroclor 1254-exposed dams and female and male offspring at PND35, whereas no significant changes were observed at PNDs 77 or 350 (Table 1, paper V). Retinyl palmitate concentrations in the liver were significantly reduced by Aroclor 1254 exposure in dams and offspring at all time-points (Table 1, paper V). A life stage-specific change in hepatic *at* RA levels was observed following Aroclor 1254 exposure; and this was proved by significant reduction for the dams and significant increase for the PND35 females (Table 1, paper V). Hepatic 9c4o13,14dh-RA concentration in female offspring at PND35 was significantly reduced by perinatal Aroclor 1254 exposure, but that reduction did not remain at PNDs 77 or 350 (Table 1, paper V). The TCDD equvivalents for changes in retinoid parameters induced by perinatal exposure to Aroclor 1254 were calculated using the TEQ approach (Table 2, paper V). When the estimated TCDD equivalents were compared to the theoretical TEQs calculated based on the chemical composition of Aroclor 1254, the TCDD equivalents of hepatic retinyl palmitate were found to be 62% and 58% of the theoretical TEQ for PND35 females and males respectively (Table 2, paper V). This indicates that both dioxin-like as well as non dioxin-like components contributed to the observed retinoid changes.

Results based on PLS analyses indicated that changes of different retinoid forms in livers induced by exposure to NCM (Figure 4, paper IV) or Aroclor 1254 (Figure 8, paper V) were strongly associated with changes in body and liver weights, with alterations in levels of thyroid hormones, as well as with induction of liver CYP activities, such as CYP1A (EROD), CYP2B (PROD), and CYP3A (BROD). Moreover, it was shown that hepatic retinoid parameters of male and female offspring were affected in a similar manner following perinatal NCM (Supplementary Table 1, paper IV) or Aroclor 1254 (Supplementary Table S1, paper V) exposure.

Findings from the current study indicated pronounced reduction in hepatic retinoid levels of young offspring following perinatal NCM or Aroclor 1254 exposure; with this reduction still observed in adult offspring. Previous studies revealed that alterations of retinoids status is evident following *in-utero* and/or lactational exposure to TCDD or mixture of PCBs in rats (Morse and Brouwer, 1995; Kransler et al., 2007), or after maternal and *in-ovo* exposure in quail (Boily et al., 2003). Severe reduction of hepatic retinoids was also seen after exposure to individual PCBs (Chen et al., 1992; Van Birgelen et al., 1994a, 1994b). The profile of the modulations in the retinoid system parameters induced by perinatal exposure to NCM or Aroclor 1254 was also comparable to the profile observed in other developmental studies with exposure to different compounds. Rat offspring treated orally with hexabromocyclododecane (HBCD) during gestation and lactation, and rat dams and their offspring given diet contaminated with organochlorine chemicals showed reduced stores of vitamin A in the livers (Iverson et al., 1998; Van Der Ven et al., 2009). Decreased retinoids in liver could be due to both inhibited storage of newly administered vitamin A and increase endogenous vitamin A mobilization from liver reserves (Håkansson and Ahlborg, 1985; Håkansson et.al., 1988; Zile, 1992; Kelley et al., 1998). Increased mobilization of hepatic retinoids might result from a direct effect on hepatic enzyme activities or by an up-regulation of a signal controlling release of vitamin A stores into circulation (Zile, 1992). TCDD was found to severely decrease the lecithin:retinol acyltransferase (LRAT) activity, an enzyme involved in the conversion of retinol into retinyl esters in the hepatic stellate cells (Nilsson et al., 1996). Aroclor 1254 and/or some components

of the NCM used in the present study might have the similar effect as that of TCDD. Other factors that can contribute to reduced liver retinoids could be related to decreased-retinol uptake into the liver cells from plasma. It was shown that DDE, the metabolite of the pesticide DDT, disturbs retinoid homeostasis by reducing the activity of retinol-metabolizing enzymes (Leiva-Presa et al., 2006). Other pesticides, e.g. aldrin, dieldrin, chlordane and toxaphene, were found to affect the retinoid system by interacting with the retinoid receptors RAR (Lemaire et al., 2005). Findings from this study showed that treatment with NCM produced no effects on *at* RA levels, while markedly decreased levels of the metabolite 9c4o13,14dh-RA in the liver; indicating the sensitivity of the metabolite. The observed reduction of the metabolite levels could be explained by decreased hepatic retinol, leading to insufficient availability of retinol required for RA synthesis. Thus the effects of environmental contaminants on retinoid signaling seems to be mediated directly by affecting activities of retinoid-metabolizing enzymes or interacting with retinoid receptors, or indirectly via crosstalk with other receptors like AhR and estrogen receptors (ERs).

3.3.3.2 Renal retinoids

Following perinatal exposure to NCM (**paper IV**), renal retinol levels were significantly increased in dams as well as in offspring at PND35 and PND350 (Table 1, paper IV). The calculated BMD revealed values of 0.57, 1.2 and 0.68 mg/kg bw/day (Table 2, paper IV). Renal levels of retinyl palmitate and 9c4o13,14dh-RA in the dams and offspring were close to or below the limit of detection (LOD).

Renal retinol concentrations were significantly increased in Aroclor 1254-exposed female and male offspring at all time-points, i.e. at PNDs 35, 77 and 350 (Table 1, paper V). In Aroclor 1254-exposed female offspring, renal *at* RA concentrations were increased at PND35, while it was not changed at PNDs 77 or 350 (Table 1, paper V). Results also showed that renal retinoids of male and female offspring were influenced in a similar pattern following perinatal NCM (Supplementary Table 1, paper IV) or Aroclor 1254 (Supplementary Table S1, paper V) exposure.

Observations from the present investigation showed increased retinoid levels in kidney of the offspring. These findings are in agreement with previous studies that have shown marked and persistent increases in kidney vitamin A levels following dosing with TCDD; suggesting that there is an attempt to increase renal retinoid stores (Brouwer et al., 1989; Pohjanvirta et al., 1990). A causal relationship between the increased LRAT activity and the increased retinyl ester levels in the kidneys was suggested, based on the elevated LRAT activity and retinyl ester levels in kidneys from male rats treated with TCDD (Nilsson et al., 1996; Nilsson et al., 2000). The observed reduced kidney weight (see organ weights section above (under *"Toxicological evaluation"*), together with increased renal retinoids levels and pronounced induction of renal EROD (see renal EROD activity section below) activity; all these effects could propose nephrotoxicity, although histopathological results from current study showed no marked changes in the kidney (paper I).

3.3.4 Cytochrome P450 (CYP) activities

3.3.4.1 Hepatic CYP activities (papers I, IV and V)

For NCM-exposed rats (**paper IV**), hepatic EROD activity was induced in dams and both sexes of offspring at PND35, while only females at PND77 showed a significant induction of the hepatic EROD activity (Figure 1, paper IV). Corresponding BMD values were 0.52, 0.41 and 4.01 mg/kg bw/day respectively (Table 2, paper IV). The hepatic BROD activity was increased in dams and female and male offspring at PND35 (Figure 1, paper IV), with associated BMDs of 1.84, 1.60 and 2.70 mg/kg bw/day, respectively (Table 2, paper IV). The hepatic PROD activity was induced in dams as well as in offspring at PND35 (Figure 1, paper IV), with BMD values of 1.72 and 2.78 mg/kg bw/day respectively (Table 2, paper IV).

Following perinatal exposure to Aroclor 1254 (**paper V**), hepatic EROD activity of male and female offspring was markedly induced at PND35 and slightly induced at PND77 (Figure 2A, paper V). Similarly, hepatic PROD activity was induced in Aroclor 1254-exposed female, but not male, offspring at PNDs 35 and 77 (Figure 2B, paper V). Hepatic BROD activity in female offspring was markedly induced at PND35 and slightly induced at PND77. (Figure 2C, paper V). For the dams, hepatic EROD, PROD and BROD were significantly induced after Aroclor 1254-exposure (Figure 3, paper V).

3.3.4.2 Renal EROD activity (papers IV and V)

The renal EROD activity was significantly increased in NCM-exposed female offspring at PNDs 35 and 77, while no significant changes were observed at PND350 nor in the dams (Figure 1, paper IV). At PNDs 35 and 77, the associated BMD revealed values of 0.22 and 4.05 mg/kg bw/day, respectively (Table 2, paper IV). For Aroclor 1254-exposed rats, the renal EROD activity was induced in dams as well as in female offspring at PNDs 35 and 77 (Figures 2D and 3, paper V). Results also showed that hepatic and renal CYP activities of male and female offspring were induced in a similar manner following perinatal NCM (Supplementary Table 1, paper IV) or Aroclor 1254 (Supplementary Table S1, paper V) exposure.

Results from the current study illustrated significant induction of CYP enzymes both in liver and kidney of offspring at PND35, with the effects becoming less at PND77. The three assessed hepatic enzyme systems; CYP1A, CYP2B, and CYP3A, showed increased activities i.e. EROD, PROD and BROD respectively in response to NCM or Aroclor 1254 exposure. Regarding renal CYP1A activity (EROD), the induction was marked at PND35, while it was less induced at the age of PND77. Our observations are consistent with previous reports (Brunström et al., 1991; Hallgren et al., 2001). Observed induction of hepatic and renal CYP enzymes suggested activation by NCM or Aroclor 1254 of the corresponding receptor pathways, i.e. AhR (for CYP1A), constitutive androstane receptor (CAR) (for CYP2B), and pregnane X receptor (PXR) (for CYP3A). Induction of CYP enzymes, by itself, is not regarded as an adverse effect; however it could be associated with other adverse effects, like effects on circulating hormones and on tumor promotion (Stiborova et al., 2008; Tomaszewski et al., 2008). In this way, and considering its sensitivity based on BMD values mentioned

above; induction of CYP enzymes may be considered as a marker of other adverse effects.

3.3.5 Thyroid hormones (papers I, IV and V)

Following perinatal NCM exposure, serum T4 levels were significantly reduced in females at PND35 (Table 3, paper I), with a BMD of 0.98 mg/kg bw/day (Table 2, paper IV). Effects on serum T4 were restored to normal at PNDs 77 and 350. Serum levels of T3 and TSH showed no significant changes (paper I). Results from the same study revealed that the Aroclor 1254-treated groups showed an increase in serum TSH, and reductions in T3 and T4 in both sexes at PND35 (Table 3, paper I).

Reduced serum levels of T4 could be due to increased activation of CYP enzymes. In spite of reduced T4 levels, T3 levels remained unchanged. These observations are in line with previous studies examined relation between PCB exposure and thyroid hormones in rats and mice (Hallgren et al., 2001; Meerts et al., 2002). Meerts and co-workers showed that treatment of pregnant rats with a PCB metabolite produced decreased levels of T4 in fetal plasma.

3.4 CONCLUSIONS

The overall conclusion of this thesis is that bone and retinoid system parameters are of relevance to be assessed in human-relevant exposure situations to chemical mixtures. Using the status of the Canadian Arctic populations as an example, the generated data has contributed new knowledge to improve the health risk assessment associated with the exposure to complex mixtures of environmental pollutants. The generated data is relevant not only for the Canadian Arctic population, but also for other populations with similar exposure profiles.

More specifically, perinatal exposure to the chemical mixtures NCM (papers I, II, IV) or Aroclor 1254 (papers I, III, V) resulted in:

- shorter and thinner bones with reduced strength in young rat offspring. However, no treatment-related bone changes were observed in offspring at later time-points when exposure was discontinued. BMD calculations for changes in bone parameters of young offspring induced by perinatal exposure to NCM revealed values in the range of 0.8 - 4.8 mg/kg bw/day; with the most sensitive bone change being the reduction in stiffness of femoral diaphysis. Male and female rat offspring responded in a similar manner regarding alterations in bone parameters. The results indicate that the observed bone effects are mainly driven by the dioxin-like congeners; however, the contribution of the non-dioxin-like congeners to the exposure outcome cannot be excluded.

- persistent reductions of hepatic retinyl palmitate levels, persistent increase in renal retinol levels, and transient alterations in hepatic levels of retinol, retinoic acid and its metabolite 9c4o13,14dh-RA. For the modulations in retinoid system

parameters of young offspring following perinatal NCM exposure; calculated BMD values were in the range of 0.1 - 1.2 mg/kg bw/day, with the most sensitive retinoid system parameter being the reduction of hepatic 9c4o13,14dh-RA. Retinoid system parameters of male and female rat offspring responded in a similar pattern. A life-stage specific change in the retinoid system at the retinoic acid level by Aroclor 1254 exposure was demonstrated. Female offspring at PND35 and their dams exposed to Aroclor 1254 during gestation and lactation showed completely different hepatic retinoid profiles, i.e. all-*trans* RA concentration in liver was increased in female offspring at PND 35 and reduced in dams. Although, the contribution of non-dioxin-like components cannot be excluded, dioxin-like congeners of NCM and Aroclor 1254 treatment modulated retinoid concentrations and CYP activities in tissue- and life-stage specific manners.

3.5 FUTURE PERSPECTIVES

It is clear from studies of chemical mixtures that data derived from single chemical experiments cannot be used to predict the risk resulting from exposure to complex mixtures of environmental pollutants. It is also clear that exposure to environmental pollutants can adversely affect prenatal and postnatal development in human populations. So there is a need to develop models in order to study in-depth the outcome of exposure to complex mixtures of environmental pollutants.

This study investigated the impact of combined gestational and lactational exposure to NCM or Aroclor 1254. In the future it will be interesting to compare the outcome of this combined exposure versus only gestational or lactational exposure. By doing so, it will be possible to determine which is the most critical time-period.

This study has shown that perinatal exposure to two mixtures of environmental pollutants induced modulation in bone development as well as in homoeostasis of the retinoid system. However, the mechanism of action remained unclear. Conduction of cell work would facilitate the investigation of mechanism of action of NCM or Aroclor 1254 on bone tissue and retinoid system. Results of this study also showed that alterations in retinoid parameters following perinatal exposure to NCM or Aroclor 1254 are associated with changes in body and organ weights, alterations in levels of serum thyroid hormones, as well as with induction of CYP enzymes. It would be interesting to examine the association of modulations in development of bone tissue with other endpoints analyzed in the same study.

Our findings revealed that exposure during gestation and lactation to NCM or Aroclor 1254 modulates bone quality and strength. However, the detailed and specific pattern of bone effects for NCM and Aroclor 1254 are not yet well-described. Hence more thorough bone tissue analyses at the microscopic and nanoscopic levels are required in order to draw firm conclusions on how NCM or Aroclor 1254 influence the bone architectural and material properties.

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