From the National Institute of Environmental Medicine, Division of Biochemical Toxicology and Experimental Cancer Research

PERSISTENT ORGANIC POLLUTANTS AND BONE TISSUE – STUDIES IN WILD AND IN EXPERIMENTAL ANIMALS

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Cover photo: Matthew Milnes, University of Florida (alligator) and Mats Block, Uppsala University (bone).
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To my family

Life is what happens to you while you're busy making other plans.
John Lennon, "Beautiful Boy"
(1940 – 1980)

ABSTRACT

Since the mid 1900s, there has been a dramatic increase in the number of osteoporotic-related fractures in the industrialised world. The reason for this is unknown, but there could be a link between the increased use and production of chemicals and effects on bone. Many chemicals possess endocrine-disrupting properties, affecting reproductive tissues and other endocrine-regulated tissues, such as bone tissue. In this thesis, studies have been performed on wild (alligator and herring gull) as well as experimental animals (goat and rat) with the aim to investigate potential bone effects of different persistent organic pollutants (POPs), such as dicofol, DDT and its metabolites, polychlorinated biphenyls (PCBs) and dioxins.

The methods used include peripheral quantitative computed tomography (pQCT), biomechanics (three-point bending test), mineralisation analysis (bone chemical composition), and measurements of the bone markers alkaline phosphatase (ALP) (bone formation) and carboxyterminal telopeptide of type I collagen (CTX) (bone resorption).

The results in this thesis show that free-ranging female alligators residing in a pesticide-polluted lake (dicofol, DDT and its metabolites) in Florida, USA show increases in trabecular bone mineral density (BMD), total BMD and bone mineral content, compared to females from a control lake. The bone effects indicate exposure to an estrogenic environment, suggesting the mainly anti-androgenic *p,p'*-DDE and/or the mainly estrogenic dicofol to elicit estrogenic actions on bone tissue homeostasis. Bone effects have been observed in free-ranging herring gulls residing in the Great Lakes, a lake system in North America polluted with POPs and metals and situated near many large cities and industries. Alterations include decreases in bone length, periosteal circumference, total cross-sectional area (CSA) and an increase in displacement at failure, compared to herring gulls from a freshwater reference site. The pQCT results indicate disrupted estrogen-signalling in bone tissue and in addition, the biomechanical results suggest disruption of the mineralisation process. This thesis also presents bone effects observed in experimental animals. Female goat offspring exposed to an environmentally relevant dose of the non dioxin-like putative estrogen PCB 153 *in utero* and through mother's milk had bone changes indicating estrogenic bone effects, such as increased trabecular BMD in the metaphysis and decreases of the total CSA, moment of resistance and marrow cavity in the diaphysis. However, the dioxin-like anti-estrogenic PCB 126 did not produce any developmental effects on goat bone. Interestingly, male rats exposed to a single high dose (LD_{50}) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCCD) for only five days (short-term exposure) showed anti-estrogenic bone effects, such as decreased trabecular area, an increase of CTX, a decrease of ALP, and an altered bone chemical composition, resembling more mature bones.

In conclusion, bone tissue is a likely target for endocrine-disrupting chemicals, hence, bone effects were observed in a various number of species exposed to POPs. The observed effects suggest POPs to exert anti-estrogenic or estrogenic actions on bone composition, dimensions and strength, or to disrupt the mineralisation process. However, further studies are needed in order to elucidate by what mechanisms pesticides, PCBs and dioxins exert their potential effect on bone tissue.

Keywords: Bone, POPs, dioxin, DDT, PCB, pQCT, biomechanics, ALP, CTX

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-V).

I. P. Monica Lind, Matthew R. Milnes, **Rebecca Lundberg**, Dieldrich Bermudez, Jan Örberg, and Louis J. Guillette, Jr. Abnormal Bone Composition in Female Juvenile American Alligators from a Pesticide-Polluted Lake (Lake Apopka, Florida). *Environmental Health Perspectives, 2004, Volume 112, Number 3, s 359-362*

II. **Rebecca Lundberg**, Jan L. Lyche, Erik Ropstad, Mona Aleksandersen, Monika Rönn, Janneche U. Skaare, Sune Larsson, Jan Örberg, P. Monica Lind. Perinatal exposure to PCB 153, but not PCB 126, alters bone tissue composition in female goat offspring. *Toxicology. 2006 Nov 10, volume 228(1), s 33-40*

III. Carolina Wejheden, **Rebecca Lundberg**, Pedro Alvarez-Lloret, Alejandro B. Rodriguez-Navarro, Sune Larsson, Fedor Moncek, Agneta Rannug, and P. Monica Lind. Short-term exposure to dioxin impairs bone tissue composition in male Sprague-Dawley rats. *Submitted*

IV. **Rebecca Lundberg**, Glen A. Fox, Carolina Wejheden, Lars Lind, Sune Larsson, Jan Örberg, P. Monica Lind. Altered Bone Properties in Herring Gulls (*Larus Argentatus*) Residing in the Great Lakes in the Early 1990s - Caused by Environmental Contaminants? *Submitted*

Additional publications not included in the thesis:

Nick Fletcher, David Wahlström, **Rebecca Lundberg**, Charlotte B. Nilsson, Kerstin C. Nilsson, Kenneth Stockling, Heike Hellmold and Helen Håkansson. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis, and bile transport in rat liver: a microarray study. Toxicol Appl Pharmacol. 2005 Aug 22;207(1):1-24.

Rebecca Lundberg, Björn Munro Jenssen, Àngels Leiva-Presa, Monika Rönn, Carolina Hernhag, Carolina Wejheden, Sune Larsson, Jan Örberg, P. Monica Lind. Effects of Short-term Exposure to the DDT Metabolite *p,p*'-DDE on Bone Tissue in Male Common Frog (*Rana temporaria*). J Toxicol Environ Health A. 2007 Apr 1;70(7):614-9

Rebecca Lundberg, Brytting M, Dahlgren L, Kanter-Lewensohn L, Schloss L, Dalianis T, Ragnarsson-Olding B. Human herpes virus DNA is rarely detected in non-UV light-associated primary malignant melanomas of mucous membranes. Anticancer Res. 2006 Sep-Oct;26(5B):3627-31.

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LIST OF ABBREVIATIONS

AhR	aryl hydrocarbon receptor
ALP	alkaline phosphatase
ANCOVA	analysis of covariance
ANOVA	one-way analysis of variance
AR	androgen receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH-PAS	basic helix-loop-helix-PAS
BMC	bone mineral content
BMD	bone mineral density
Cd	cadmium
CSA	cross-sectional area
CTX	carboxyterminal telopeptide of type I collagen
DDT	1,1,1-trichloro-2,2-*bis*-(4-chlorophenyl)ethane
p,*p*´-DDD	dichlorodiphenyl-dichloro-ethane
p,*p*´-DDE	dichlorodiphenyldichloro-ethylene
DEXA	dual energy X-ray absorptiometry
DRE	dioxin-responsive elements
ELISA	enzyme-linked immuno-sorbent assay
ER	estrogen receptor
ERE	estrogen responsive elements
FTIR	fourier transform infrared spectrometry
HCB	hexachlorobenzene
Hg	mercury
IARC	International Agency for Research on Cancer
ICP-OES	optical emission spectroscopy
i.p	intraperitoneal
LH	luteinising hormone
LOAEL	lowest observed adverse effect level
Pb	lead
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-*p*-dioxins
PCDF	polychlorinated dibenzofurans
p-NPP	para-nitrophenylphosphate
POP	persistent organic pollutant
pQCT	peripheral quantitative computed tomography
PTH	parathyroid hormone
SERM	selective estrogen receptor modulator
SHP	short heterodimer partner orphan nuclear receptor
T	testosterone
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCP	trichlorophenol herbicide
TEF	toxic equivalence factors
TEQ	toxic equivalent
3MC	3-methylcholanthrene

INTRODUCTION

Since the mid 1900s, there has been a dramatic increase in the number of osteoporotic-related fractures in the industrialised world. The reason for this is unknown, but there could be a link between the increased use and production of chemicals and effects on bone. Many chemicals possess endocrine-disrupting properties, affecting reproductive tissues and other endocrine-regulated tissues, such as bone tissue.

The chemical compounds of interest in this thesis, dioxins, PCBs, DDT and its metabolites, are suggested to possess these endocrine-disrupting properties due to *e.g.* their structural similarity to estrogens or to their potential ability to reduce the estrogen level. Their chemical properties, such as lipophilicity and resistance to degradation, make them persist in nature as well as in adipose tissue of humans and animals, thereof the name: persistent organic pollutants (POPs). During the last decades, there has been an increased awareness of the harmful effects of certain POPs, hence their production and use have been reduced, but they are long-lived and are therefore still present in air, soil, and water. However, the main route of exposure to POPs is through our diet, especially through products with a high fat content, such as fish, meat, egg and dairy products.

The link between POPs and altered bone tissue homeostasis is poorly investigated, but I hope to add some information to the area through my four studies presented in this thesis.

BONE

STRUCTURE

Bone has three major functions: 1) provides support to extremities and body cavities containing vital organs, 2) important for locomotion, since the muscles are attached to different sites of the skeleton and 3) provides a large reservoir of ions, such as calcium, phosphorus, magnesium and sodium (Seibel 1999).

Except for dentine and enamel, bone is the hardest tissue in the body. About 40% of the bone consists of collagen fibres and water, while the remaining 60 % are hydroxyapatite crystals. The proportion between the organic matrix and the mineral component vary with age and location within the skeleton. These two main components have different functions in the bone, the collagen makes the bone resistant to pulling forces, and the crystals make the bone resistant against compression. The hard extracellular matrix of bone contains channels and cavities, which are filled with living cells and are essential for the process of bone remodelling (break-down and renewal of bone tissue) (Seibel 1999). This process in the interior of the bone is continuous, and the bone is constantly renewed throughout our life.

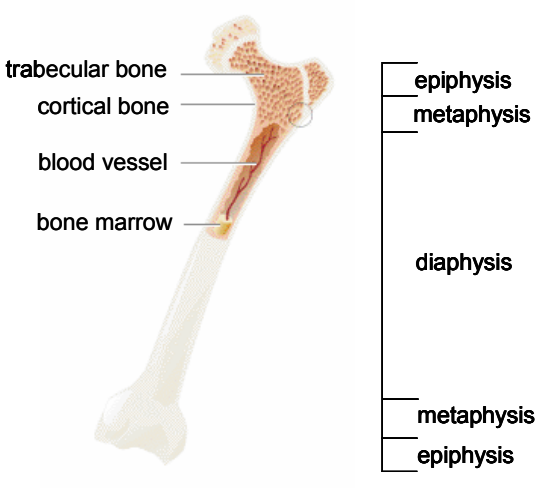


Figure 1. Schematic picture of a long bone, showing the internal and external parts of the bone. Modified from *Microsoft® Encarta® Online Encyclopedia 2007*

The skeleton consists of two different types of bone, cortical (80%) and trabecular (20%). Both types are found in all parts of the skeleton and the cortical bone always surrounds the trabecular bone. The cortical bone predominates in the diaphysis of the long bones of the extremities and the trabecular bone predominates at the ends of the long bones and in the vertebrae and pelvis (Fig.1). The difference between the two types is their appearance. The trabecular bone is spongy, like a loosely knit three-dimensional network, in contrast to the cortical bone, which is more compact and homogenous without any cavities (Seibel 1999). The mid-part of a long bone is called the diaphysis, while the metaphysis is located close to the outer most part, the epiphysis. The growth plate is located in the epiphyseal part of the bone. At the end of adolescence, the growth plate closes due to increased estrogen levels, and hence no more increase in height is achieved.

REMODELLING AND MINERALISATION

Remodelling consists of two processes; bone formation and bone resorption, where osteoblasts form bone tissue and osteoclasts destroy bone tissue (Fig 2). The four overlapping stages involved in remodelling include activation, resorption, reversal and formation. During activation, osteoclasts are recruited to the site and enzymatically digest bone tissue (resorption). Thereafter, reversal takes place, when the osteoblasts stop digesting bone tissue, and the cavities are filled with proteins secreted by the osteoblasts. Formation is when the deposited matrix hardens and becomes mineralised (Manolagas and Jilka 1995). In a human adult, remodelling replaces 3% of the cortical bone and 25% of the trabecular bone every year.

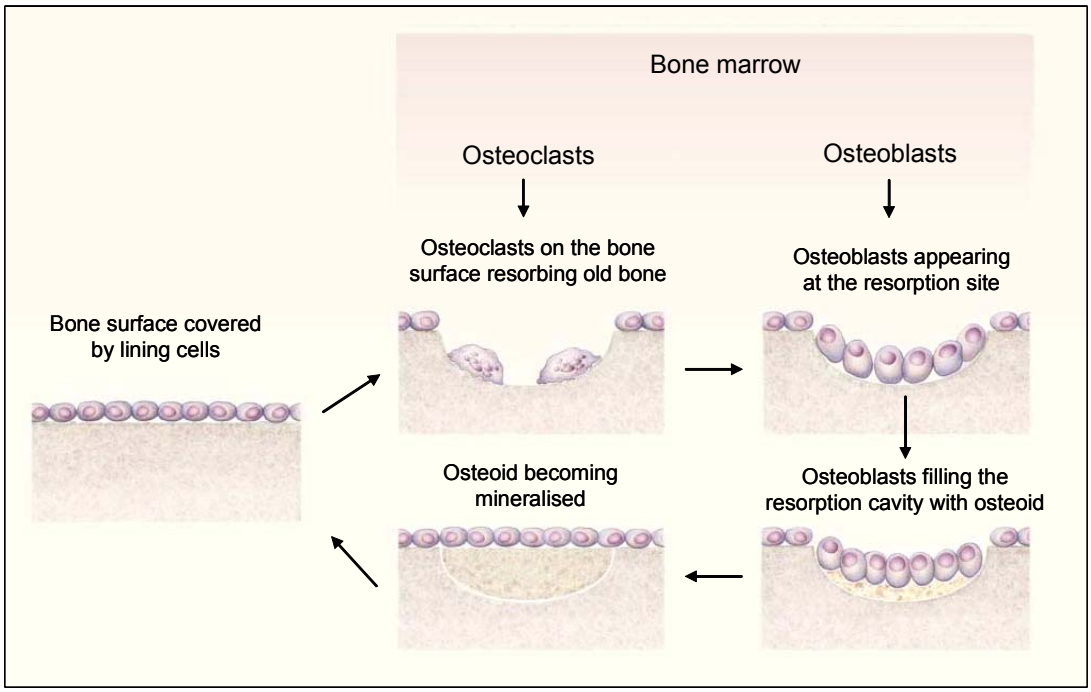


Figure 2. The remodelling process; osteoblasts originating from stromal stem cells, and osteoclasts, originating from hemopoietic stem cells in the bone marrow form and resorb bone tissue in a regulated manner under normal physiological conditions. (From Manolagas SC & Jilka RL. Bone marrow, cytokines, and bone remodelling. Emerging insights into the pathophysiology of osteoporosis. N Engl J Med. 1995 Feb 2;332(5):305-11. Copyright © *[1995] Massachusetts Medical Society. All rights reserved.*

The process of bone mineralisation occurs in two phases. Primary mineralisation involves deposition of bone mineral during bone remodelling, while secondary mineralisation is the process of further mineralisation after the remodelling cycle has ended. The degree of secondary mineralisation is dependent on the rate of bone turnover, where slow turnover allows more time for mineralisation, and fast turnover results in newly formed bone being removed more quickly, hence reducing the time for secondary mineralisation (Compston 2006).

During bone mineralisation, osteoblasts produce an organic matrix consisting of proteins, *e.g* type I collagen, osteonectin and osteocalcin, but about 90% of the matrix consists of collagen type I. The organic matrix is converted into hard mineralised bone tissue by deposition of hydroxyapatite crystals ($Ca_5(PO_4)_3(OH)$).

BIOCHEMICAL MARKERS OF BONE TURNOVER

Biochemical markers, such as the bone resorption marker carboxyterminal telopeptide of type I collagen (CTX) and the bone formation marker alkaline phosphatase (ALP), can be used when investigating the rate of bone turnover. CTX and ALP can be measured *in vitro* (Rissanen et al. 2006) as well as *in vivo*, *e.g.* in mouse (Bonnet et al. 2006) and in rat (Farrell et al. 2006). CTX is mainly expressed in bone and ALP is found attached to the extracellular surface of cell membranes in bone, liver, intestine and kidney. ALP is specific for bone formation only in the absence of liver or biliary disease (Bilezikian 1996).

HORMONAL AND NUTRITIONAL REGULATION

Hormonal regulation

Steroid hormones are produced by specific tissues in the body and are divided into sex hormones, glucocorticoids and mineralocorticoids. Following steroid biosynthesis of cholesterol, the steroids exert their effect on target tissues, such as the reproductive system and the skeleton. The actions in the target tissues are elicited by binding of the hormone to its intracellular receptor, resulting in a receptor-ligand complex that binds to the promotor region of the DNA (Boelsterli 2003).

Estrogen is an important factor in bone tissue homeostasis and there are two types of estrogen receptors (ER), ER-α and ER-β. Both receptor types are found in osteoblasts as well as in osteoclasts. (Eriksen et al. 1988; Krassas and Papadopoulou 2001; Onoe et al. 1997). Bone tissue is dependent on estrogen in order to maintain the equilibrium between the anabolic and catabolic processes in the bone. An increased level of estrogen induces bone formation, while a decreased level of estrogen decreases bone formation. During the years following menopause, women may develop osteoporosis due to a rapid reduction of circulating levels of estrogen (Ljunggren 2006; Riggs et al. 1998). The estrogen level is important also in men, where a decreased level can result in osteoporosis (Carlsen et al. 2000). However, decreased bone mineral density is not as common in men as in women, due to a slower age-related decrease in estrogen (no menopause) (Khosla et al. 1999). The lower fracture rate in men is due to differences in skeletal size, determined by genetic factors. During adolescence, androgens and growth factors induce periosteal bone formation (outer circumference), while estrogens inhibit periosteal bone formation, and instead promote endosteal bone formation (inner circumference). This result in thinner bones in females compared to the bones of men, and thus an increased risk of fractures (Åkesson 2006).

Androgens, *e.g.* testosterone (T), are also important for regulation of bone tissue. Bone mineral density can decrease in men with decreased T level (hypogonadal), which may contribute to the increased fracture rate in the elderly. In these men, testosterone therapy can improve bone mineral density and bone architecture by increasing bone formation and decreasing bone resorption (Kohn 2006).

Nutritional regulation

The nutritional factors influencing bone include vitamins and minerals. Vitamin A is a group of fat-soluble nutrients, which are essential for cell proliferation and differentiation in many different tissues. They are involved in physiological functions concerning development, the immune system, reproduction, vision, bone and maintenance of skin and mucous membranes (Tortora 1996). In the diet, two forms of vitamin A are found, retinol (preformed vitamin A) and carotenoids (provitamin A). The physiological role of vitamin A in bone is to regulate proliferation and differentiation of osteoblasts and osteoclasts. Vitamin A intoxication (overdose) has shown to result in fragile bone and increased fracture risk in rats due to stimulated bone resorption and inhibited bone formation (Johansson et al. 2002), however, the bone mineral density was not affected (Lind et al. 2006). The increased fracture risk seen is caused by alterations in geometrical properties of the bone, *e.g* decreased cross-sectional area of the diaphysis, hence a thinner bone. Contrary to intoxication, vitamin A deficiency in animals leads to morphological changes in bone by increasing bone thickness (Palacios 2006).

Vitamin C has many diverse physiological roles, including interaction with antibodies, promote wound healing, function as an antioxidant and promote protein metabolism, *e.g.* laying down of collagen in the formation of connective tissue (Tortora 1996). Thus, vitamin C deficiency results in decreased collagen production, which inhibits bone growth and delays fracture repair.

Another important vitamin is vitamin D, with the main task of increasing the absorption of calcium and phosphate in the intestine. It is also important for mineralisation of the skeleton (Palacios 2006; Waern 2006). Vitamin D is absorbed through the intestine and is also produced in the skin following exposure of the precursor 7-dehydrocholesterol to UV-B light. This leads to a photochemical reaction that produces previtamin D3, which quickly is transformed into vitamin D3. Following metabolism in the liver and kidney, 1,25-dihydroxivitamin D (calcitriol) is formed, the active form of vitamin D (Holick 2007). About 70-80% of the daily need of vitamin D is produced in the skin. Vitamin D deficiency leads to hypocalcemi, followed by increased level of parathyroid hormone (PTH), which gives rise to increased bone resorption and potentially osteoporosis. Increased PTH causes increased tubular resorption of calcium in the kidney, and when the systemic calcium level is too low, calcium is taken from the storage; the skeleton (Waern 2006). This compensatory action decreases the bone mass. Worth mentioning is that intense and prolonged exposure to sunlight does not give rise to a vitamin D intoxication, since the excess vitamin D formed is destroyed by sunlight (Holick 2007).

There is also a role for vitamin K in bone tissue homeostasis, since vitamin K-dependent proteins are important in osteocalcin metabolism. Osteocalcin is a non-collagenous protein thought to play a role in mineralisation and calcium homeostasis. Vitamin K deficiency has been detected in osteoporotic patients (Szulc et al. 1996). Others physiological functions of vitamin K involve blood coagulation, vascular calcification, cell growth and apoptosis (Cranenburg et al. 2007).

Calcium (Ca) is one of the main bone-forming minerals, and bone functions as the major reservoir of calcium in the body, storing more than 99% of the total calcium (Tortora 1996). The role of calcium in bone formation is controversial, some studies suggest bone forming potential (Johnston et al. 1992; Lloyd et al. 1993; Reid et al. 1995), while others see no effect (Grant et al. 2005; Porthouse et al. 2005). However, recent evidence seems to point towards the former suggestion; that calcium supplementation has a positive effect on bone formation (Prince 2007; Tang et al. 2007).

Phosphorous (P) is an essential element part of the mineralisation, and a decrease results in impaired mineralisation (Palacios 2006). However, that is not a major concern in healthy individuals. Instead, bone effects could result from a diet high in phosphorous and low in calcium due to increased PTH level (Calvo et al. 1990). There is also a concern regarding the high phosphorous level (and low calcium level) in soft drinks *e.g.* Coca Cola, which has been associated with an increase in fracture rates and lower bone mass in adults (Wyshak et al. 1989). Another report has a softer approach towards the carbonated drinks, concluding that the extraction of calcium from the bone is minimal and the bone effects that might appear are rather due to the substitution of milk to soft drinks (Heaney and Rafferty 2001).

OSTEOPOROSIS AND OTHER EXAMPLES OF IMBALANCES OF BONE TISSUE HOMEOSTASIS

Osteoporosis is the result of an imbalance in the remodelling process in favour of bone resorption, resulting in decreased bone mass (Fact box 1). Until age 20-30, bone formation slightly exceeds bone resorption and gradually reaches peak bone mass. Individual factors such as heredity, diet and exercise determine when peak bone mass is reached. The hip, spine and forearm are typical sites for osteoporotic fractures, with hip fractures being the most detrimental by leading to functional impairment and increased mortality for the individual. The Scandinavian countries are in top regarding osteoporotic-related fractures, with approximately 70 000 fractures in Sweden each year, out of which 20 000 are hip fractures. The life-time fracture risk is 50% for women and 25% for men and in view of health economics, osteoporosis alone is a large part of the medical care cost, needing around 1.1 % of the medical budget. The cost for a hip fracture has been estimated to approximately 150 000 SEK.

Fact box 1. Osteoporosis

Osteoporosis is a reduction in bone mass and a change in microarchitecture. There are several risk factors involved in obtaining osteoporosis (Tortora 1996), including:

Risk factors:

- low weight
- smoking
- physical inactivity
- vitamin D deficiency
- certain drugs (alcohol, some diuretics, cortisone)
- family history of osteoporosis
- menopause
- diabetes mellitus type I
- anorexia nervosa

Osteoporosis is responsible for shrinkage of the backbone (vertebrae), height loss, hunched backs, bone fractures and pain (Bjertness 2003). Postmenopausal osteoporosis is defined as a loss of bone tissue reaching more than 2.5 standard deviations compared to young women in the same population. (Ljunggren 2006).

Treatment of osteoporosis includes different pharmacological alternatives, such as bisphosphonates, estrogen, selective estrogen receptor modulators (SERM, *e.g.* Raloxifene) or parathyroid hormone (PTH), sometimes with the addition of vitamin D and/or calcium. Several factors are under consideration when deciding what type of drug should be used and a risk assessment is performed for each patient, determining the risk of a new fracture occurring. For patients with a high risk of attracting a new fracture, the following points are evaluated: result of bone density measurement, any previous low energy fracture, and any previous vertebral fracture. Interestingly, a recent study suggested smoking to exert a direct negative effect on bone mass at the most common sites of bone fracture (hip, spine and forearm) (Wong et al. 2007). The observed bone loss might be partly reversible, as observed in ex-smokers. The mechanism by which smoking exert bone reducing effects is unknown, but could involve decreases in estrogen (due to aromatase inhibition) and vitamin D levels, production of estrogen metabolites with low affinity for the ER, and impaired calcium absorption (Wong et al. 2007).

Other diseases resulting from altered remodelling are osteopetrosis, osteopenia and osteomalacia. Osteopetrosis is a rare inherited disorder of bone metabolism and involve increased bone mass due to the inability of the osteoclast to resorb bone tissue (Strickland and Berry 2005). There are three different human subforms of osteopetrosis and the classification is based upon on inheritance, age of onset, severity, and secondary clinical features (Balemans et al. 2005). Osteopenia is a milder form of osteoporosis, where the loss of bone mass is not as severe as in osteoporosis (Schimmer et al. 2000). Osteomalacia is characterised by soft and demineralised bones and potentially caused by a low level of calcium and phosphorous. However, osteomalacia may also be due to vitamin D-deficiency (Shoback 2007).

BONE TOXIC PERSISTENT ORGANIC POLLUTANTS

DDT

DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) can still be found in the environment, even though the production and use were banned decades ago in many Western countries (in Sweden, 1969) (Fig. 3). However, it is still used in some countries for malaria control. In living organisms, DDT is metabolised to *p,p´*-DDE (dichlorodiphenyldichloro-ethylene) and *p,p´*-DDD (dichlorodiphenyl-dichloro-ethane), which are more persistent than the parent compound (Hayes 1991). The persistence and lipophilicity of DDT and its metabolites make them accumulate in food chains and in temperate soils, the half-life of DDT is long, about 58 years (Cooke and Stringer 1982). The level of *p,p´*-DDE in Swedish breast milk from the Stockholm region was reported to be 129 ng/g lipid at the end of the 1990s (Noren and Meironyte 2000) and the level of *p,p´*-DDE found in maternal blood from Kiruna, Sweden was 0.84µg/L plasma, according to a 1994-1997 survey (Van Oostdam et al. 2004). The International Agency for Research on Cancer (IARC) has classified DDT into group 2B (possibly carcinogenic to humans).

DDT and dicofol

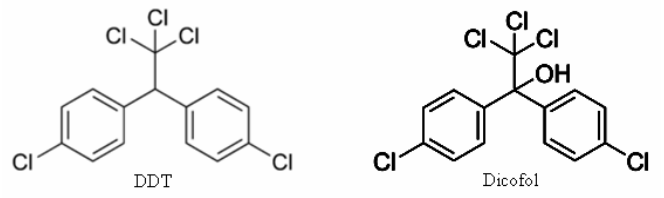


Figure 3. Chemical structure of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) and 1,1-bis(*p*-chloro-phenyl)-2,2,2-trichloroethanol (dicofol).

Effects on endocrine-regulated tissues

Studies in wildlife and laboratory animals have demonstrated potent anti-androgenic, anti-estrogenic, estrogenic, androgenic and enzyme-inducing properties of organochlorine insecticides, interfering directly or indirectly with reproduction and fertility (Baldwin et al. 1997; Bitman and Cecil 1970; Clark et al. 1998; Crain et al. 1997; Guillette et al. 1996). The DDT-metabolite *p,p´*-DDE has been reported to display mainly anti-androgenic properties. Previous studies conducted on free-ranging American alligators *(Alligator mississippiensis)* from the DDT- and dicofol-polluted Lake Apopka, Florida show effects on endocrine-regulated tissues that suggest exposure to anti-androgenic compounds. During embryonic development and postnatal growth in vertebrates, the development of the male external genitalia and reproductive ducts are dependant on androgens. In male juvenile alligators from the polluted lake, smaller phallus size and decreased plasma testosterone were observed (Guillette et al. 1996) and female alligators from Lake Apopka exhibit abnormalities of ovarian morphology, plasma estradiol concentrations, ovarian aromatase activity, and ovarian

steroidogenesis (Crain et al. 1997; Guillette et al. 1995; Guillette et al. 1994). Several experimental studies have shown the anti-androgenic properties of *p,p´*-DDE; Kelce *et al* suggested *p,p´*-DDE to be a potent androgen receptor (AR) antagonist, since *p,p´*-DDE inhibited several actions, including androgen binding to the ARs, androgen-induced transcriptional activity, and androgen action in developing, pubertal and adult male rats (Kelce et al. 1995). In addition, fetal tissue concentration of 10-20 ppm *p,p´*-DDE in male rat offspring was correlated with reproductive abnormalities, such as cryptorchidism (undescended testes) (Gray et al. 2001). However, studies in reptiles have shown that *p,p´*-DDE can exhibit estrogenic effects or induce no effect, *e.g.* in red-eared slider turtles (*Trachemys scripta elegans*), *in ovo* exposure to *p,p´*-DDE resulted in sex reversal from male to female (Willingham and Crews 1999) and in green sea turtles (*Chelonia mydas*), *p,p´*-DDE failed to influence sexual differentiation (Podreka et al. 1998).

Studies elucidating the DDT-metabolite *o,p´*-DDT have reported estrogenic actions, including bind to the ER (Leanos-Castaneda et al. 2007), exert estrogenic effects in females reproductive organs (Bitman and Cecil 1970) and induce plasma vitellogenin (biomarker for estrogenic xenobiotics) (Palmer and Palmer 1995).

Effects on egg-shell

Residues of environmental contaminants, especially *p,p´*-DDE, can reduce reproductive success in carnivorous birds. Contaminated birds lay eggs with abnormally thin shell, causing increased egg breakage and embryonic death, which results in a decrease in the bird population (Hickey and Anderson 1968; Lundholm 1997; Lundholm and Bartonek 1992; Vos et al. 2000). Prostaglandin is important in egg shell formation, and *p,p´*-DDE has been suggested to inhibit the prostaglandin formation in the egg shell gland (Lundholm and Bartonek 1992). Also, exposed birds have shown reduced medullary bone formation (bone formed in endosteal cavities of long bones). Estrogens stimulate deposition of calcium in the medullary bone, where it serves as a calcium source for egg shell formation.

Dicofol

Dicofol (1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol) was introduced as a commercial pesticide in 1955 to replace DDT, and it is structurally similar to DDT (Fig. 3). It is banned in Sweden, but is still in use in other countries (Belgium, France, Ireland, Luxemburg, and USA, for example). Dicofol is produced from DDT, and the final product may be contaminated with the parent compound. It is degradable in both water and soil and the rate of degradation is pH-dependent. In alkaline water, it is quickly (hours) hydrolysed to its metabolite and its persistence in soil is 60 days. Regarding its ability to induce cancer, IARC has classified dicofol into group 3 (not classifiable for human carcinogenicity).

Effects on endocrine-regulated tissues

Dicofol has been implicated to be a "potential endocrine disrupting compound" (Guillette et al. 1994; Vinggaard et al. 2000) and is considered to be estrogenic, since it has shown to interact with the human estrogen receptor (Hoekstra et al. 2006). However, in a cell proliferation assay, dicofol could bind to both the ER and the AR (Okubo et al. 2004). Studies conducted have shown that dicofol may disturb the reproductive system of rats. Decreases in testis weight, spermatogonia and spermatocytes were seen in albino rats treated with doses below the acute LD_{50} level of intoxication (Jadaramkunti and Kaliwal 2002). Studies in fish showed that dicofol might interfere with sex steroid synthesis and metabolism by inhibition or activation of important enzymes, such as 5α-reductase and 20ß-hydroxysteroid dehydrogenase respectively, (Thibaut and Porte 2004). The interference with these enzymes might lead to alterations in sexual differentiation (5α-reductase) and maturation of oocyte and spermatozoa (20ß-hydroxysteroid dehydrogenase).

PCB

Polychlorinated biphenyls (PCBs) are a group of chemicals belonging to the broader class of compounds known as organochlorines (Fig 4). PCBs were manufactured for industrial use starting in the 1930s and ending in most Western countries in the late 1970s. These compounds are widespread in the environment due to their resistance to degradation and they can be found in most biota from all environmental compartments, as well as in adipose tissue of humans. PCBs and a number of other organochlorines are known to bioaccumulate in fat tissue of exposed organisms and biomagnify through the food chain. This predispose top predators to suffer serious negative health effects (Skaare et al. 2000; Verreault et al. 2007; Verreault et al. 2005). Humans are exposed to PCBs mainly through contaminated food of animal origin, including meat, liver, fish and dairy products (La Rocca 2006). The most commonly found PCB congener in breast milk from Stockholm, Sweden at the end of the 1990s was PCB 153, with the ΣPCBs being in the concentration of 324 ng/g lipid (Noren and Meironyte 2000). IARC has classified PCBs into group 2A (probable human carcinogen).

There are 209 different PCB congeners, differing in the position of chlorine atoms and the degree of chlorination. These differences affect their physicochemical properties and biological activities. The PCB congeners included in paper II, PCB 153 (2,2',4,4',5,5'-PCB) and PCB 126 (3,3',4,4',5-PCB), display different toxicological and chemical properties and may be regarded as model substances for the different structural classes of PCBs. The di-*ortho* substituted putative estrogenic PCB 153 has low affinity to the aryl hydrocarbon receptor (AhR), low acute toxicity and is the most widely distributed PCB congener in breast milk, animal and human tissue (Foster 1995; Newsome et al. 1995). In contrast, the co-planar anti-estrogenic PCB 126 has high affinity to the AhR and high acute toxicity.

Polychlorinated Biphenyls (PCBs)

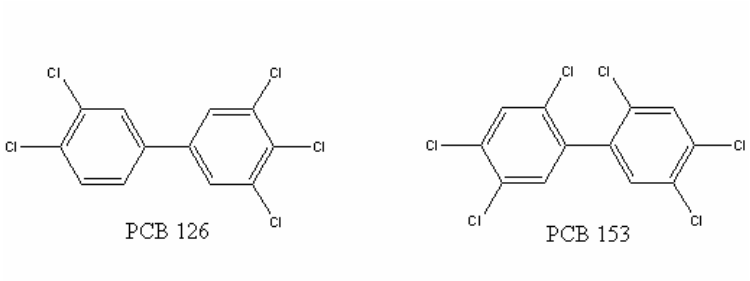


Figure 4. Chemical structures of the dioxin-like anti-estrogenic PCB 126 and the non dioxin-like putative estrogenic PCB 153.

Effects on endocrine-regulated tissue

PCBs have shown to interfere with the reproductive system in various species, such as rat (Hsu et al. 2003; Hsu et al. 2007; Shirota et al. 2006), mink (Beckett et al. 2007), mouse (Oskam et al. 2004) and goat (Lyche et al. 2004; Oskam et al. 2005). For example, the epididymis is responsible for the sustainance, protection, transport, maturation and storage of spermatozoa. *In utero* exposure to a single dose of the non dioxin-like PCB132 was reported to decrease epididymal weight and sperm count in adult male Sprague-Dawley rat offspring (Hsu et al. 2007) and a postnatal single dose of the same PCB decreased sperm motility in male Sprague-Dawley offspring. However, no changes were detected in sperm count, testis weight and testosterone concentration (Hsu et al. 2003). Effects of PCB exposure have been observed in female Sprague-Dawley offspring. Pre- and postnatal exposure to the anti-estrogenic dioxin-like PCB 126 showed a reduction in ovarian weight, a delay in puberty and congenital anomalies of the external genitalia (Shirota et al. 2006). Female minks (*Mustela vision*) exposed to PCB 126 through their diet failed to produce viable offspring and histological examination of their uterus indicated conception but only partial fetal development (Beckett et al. 2007). In young adult male mice, a single acute dose of PCB 99 (low affinity for AhR) and PCB 153 (low affinity for AhR), two of the major congeners detected in wildlife and humans, showed a significant increase in Leydig cell apoptosis. No changes were detected in reproductive endpoints, such as spermatogenesis and sperm chromatin (Oskam et al. 2004). Studies have also been conducted in goat (*Capra hircus*) to elucidate reproductive effects of PCBs following *in utero* and lactational exposure. Perinatal exposure to PCB 153 reduced the pre-pubertal plasma concentration of luteinising hormone (LH) and delayed onset of puberty in female goat offspring. No effects were seen following PCB 126 exposure (Lyche et al. 2004). The male goat offspring reported PCB 153-induced reduction in pre-pubertal gonadotropin concentrations, decreased testosterone, testicular diameter and an increase in the percentage of sperm cells with DNA damage. PCB 126 induced only a lower plasma concentration of testosterone (Oskam et al. 2005). Reproductive alterations have been detected in free-ranging animals exposed in their natural environment. Female polar bears (*Ursus maritimus*) showed decreased ovary length and male polar bears showed decreased phallus size with increasing level of ΣPCB (Sonne et al. 2006).

Some of the effects on the reproductive system might be explained by the fact that PCBs exhibit estrogenic or anti-estrogenic properties. Recent findings suggest that PCB 126 might have estrogenic or anti-estrogenic properties depending on the estrogen status of the individual. In estrogen-deprived tissue like the uteri of ovariectomised rats, PCB 126 showed weak estrogen agonistic activity (Lind et al. 2004; Lind et al. 1999).

Dioxin

Dioxins mainly include polychlorinated dibenzo-*p*-dioxins (PCDDs or dioxins), dibenzofurans (PCDFs or furans) and the 'dioxin-like' PCBs (Fig. 5). They are widespread pollutants and are found virtually everywhere in our environment, including animal and plant tissue. In most studies, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is used as the prototype compound when studying the effects of dioxins. Dioxins are produced as unwanted by-products during combustion and incineration, where the largest release of these chemicals originate from open burning of household waste, medical waste, municipal waste, forest fires and agricultural fires. Dioxins are lipophilic and are bioaccumulated and biomagnified in the food chain and the average half-life of TCDD in adult humans is approximately 2840 days, while in Sprague-Dawley rats the average half-life of TCDD is 19 days (Geyer et al. 2002). Humans are mainly exposed through high-fat food contaminated with dioxins, like dairy products, eggs, animal fats and some fish (Kulkarni et al. 2007). The level of PCDDs in Swedish breast milk from the Stockholm area in the late 1990s was reported to be 250 pg/g lipid. Adverse health effects of dioxins include lymphomas, chloracne (a skin lesion), and immune system and neurological effects. The sensitivity of different species differ greatly, but reproductive, carcinogenic and developmental effects have been seen following dioxin exposure (Bell et al. 2006; Cole et al. 2003; Kociba et al. 1976; Mitrou et al. 2001; WHO-ECEH/IPCS 2000). The IARC classification of TCDD is therefore into group 1 (carcinogenic to humans).

Effects on endocrine-regulated tissue

Most experimental studies performed regarding endocrine disrupting compounds in general, and dioxins in particular, involve the use of different rat and mouse strains. Studies elucidating the effects of dioxins on the male reproductive system report effects on the prostate and epididymis. The prostate serves to secrete a fluid that contributes to sperm motility and viability, and *in utero* and lactational exposure to TCDD showed decreased weight of prostate gland in rats (Lin et al. 2002) and inhibited prostate development in mice (Lin et al. 2002). The effect on testis weight show contradicting results (Lin et al. 2001; Theobald and Peterson 1997). One of the most sensitive endpoints after *in utero* and lactational exposure to TCDD is reduced epididymal sperm count (Gray et al. 1995; Theobald and Peterson 1997). In females, exposure to TCDD early in life could lead to premature ageing of the ovaries, as shown in female rats exposed to TCDD during gestation. As adults, they experienced a reduction in ovarian weight in association with an early decline in fertility (Gray and Ostby 1995). In addition, the susceptibility of chemically induced mammary gland tumours as adults was increased in female rats if exposed *in utero* to TCDD (Brown et al. 1998).

General structure of PCDDs

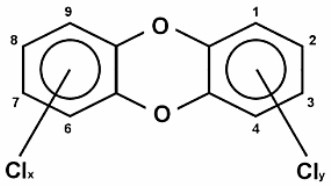


Figure 5. General structure of polychlorinated dibenzo-*p*-dioxins (PCDDs)

Species differences in sensitivity to TCDD-induced toxicity

There is a great interspecies difference in sensitivity to TCDD-induced toxicity. The guinea pig is very sensitive to TCDD, while the hamster shows a 10 000-fold greater resistence to TCDD (McConnell et al. 1978). In addition to interspecies differences, also intraspecies differences are found, ***e.g.*** Long-Evans rats are sensitive to TCDD exposure, while Han/Wistar rats are resistant. There is a 1000-fold sensitivity difference in acute lethality between the two strains (Pohjanvirta et al. 1993).

The aryl hydrocarbon receptor

Most of the toxic effects of TCDD and other dioxin-like chemicals are elicited after binding to and activation of the aryl hydrocarbon receptor (AhR), also called the dioxin receptor. This receptor belongs to the family of basic helix-loop-helix-PAS (bHLH-PAS) regulatory proteins/transcription factors. Homologues to this receptor are found in vertebrates (*e.g*. mouse and rat) as well as in invertebrates (*e.g.* the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*) (Hahn 2002; McMillan and Bradfield 2007). The mechanisms of activation of the AhR by dioxins involve binding of the compound to the AhR, which resides in the cytosol in a complex with several chaperone proteins, translocation of the ligand/receptor complex to the nucleus and dissociation of the AhR from the chaperone proteins. In the nucleus, the AhR dimerise with the aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the (bHLH-PAS) protein family. Thereafter, the AhR-ARNT dimer binds to dioxin-responsive elements (DRE) on DNA and activates gene expression of cytochrome P450s, for example (Fig 6).

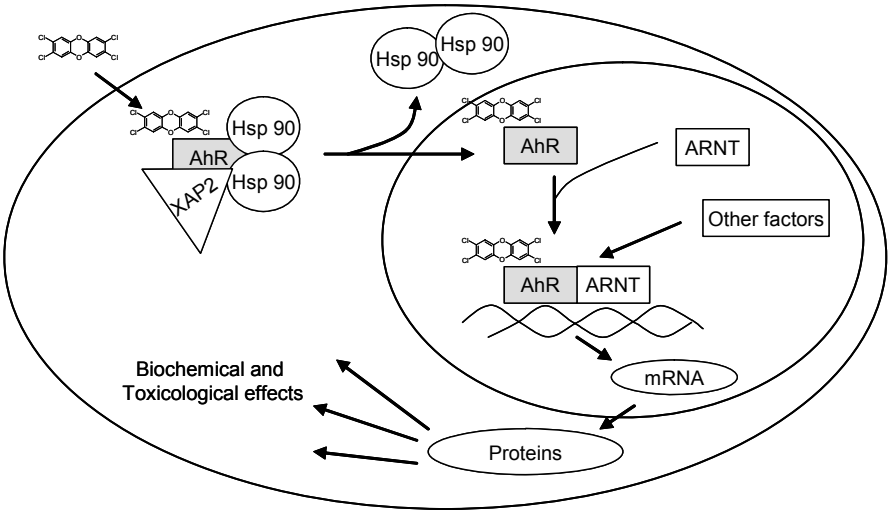


Figure 6. Mechansim of AhR transactivation of genes by TCDD, inducing biochemical and toxicological effects. *(Schematic drawing by Mattias Öberg)*

TEQ

The common mechanism of action of the dioxin-like chemicals is activation of the Ah-receptor. However, there are large differences in the biological effects elicited by the different congeners and their concentrations in the environment differ considerably. TCDD is the most studied congener, therefore risk assessment of all other dioxin-like compounds is related to TCDD. This is called the concept of toxic equivalence factors (TEF), which is very useful for estimating potential health risks. The criteria for including a compound in the TEF concept include 1) structural relationship to the PCDD/Fs, 2) bind AhR and elicit AhR-mediated biochemical and toxic responses and 3) be persistent and bioaccumulate. TCDD is the congener with the highest affinity to the AhR, and is therefore assigned a TEF of 1. All others dioxin-like congeners are given a TEF-value that reflects their toxic potency relative to that of TCDD. The TEFs are then further used to calculate the toxic equivalent (TEQ), which is the sum of the concentrations of the individual congeners present in the mixture, multiplied by their individual TEF value.

TOXICITY IN HUMANS

There have been situations where the levels of exposure to POPs have greatly exceeded the levels to which the general population is exposed (Fact box 2).

Fact box 2. Exposure in humans

Ukraine 2004

The presidential candidate of Ukraine Viktor Yushchenko was poisoned with dioxin during the 2004 election and the development of chloracne was clearly seen.

Sweden 1990s

Due to their high dietary exposure through contaminated fish, Swedish fishermen and their wives are part of an extensive epidemiological study concerning the relationship between POPs and human health effects (Svensson et al. 1994). From this cohort, links between POP exposure and increased risk for having a child with low birth weight, type 2 diabetes, lower semen function, and an increased risk for osteoporotic fractures (in the wives) have been reported (Rignell-Hydbom et al. 2004; Rignell-Hydbom et al. 2007; Rylander et al. 2005; Rylander et al. 1998; Wallin et al. 2004).

Japan 1968 and Taiwan 1979

In these two accidents, called Yusho (Japan) and Yuosheng (Taiwan), rice oil used for cooking was contaminated with PCBs and PCDFs. The exposures caused chloracne, hyperpigmentation and developmental effects (Hsu et al. 1985; Schecter et al. 2006).

Seveso, Italy 1976

A chemical factory producing a trichlorophenol herbicide (TCP) exploded in Seveso, Italy in 1976 and a chemical cloud containing TCDD contaminated the surrounding area (Bertazzi et al. 1998). Many children were exposed and some of them developed chloracne. Since the accident, the population in Seveso has been closely monitored and effects such as altered sex ratio (excess of girls) (Mocarelli et al. 1996), diabetes (Bertazzi et al. 1998) and developmental enamel defects have been observed (Alaluusua et al. 2004).

Vietnam 1960s

During the Vietnam War, US Air Force planes sprayed dioxin mixtures (Agent Orange) onto approximately 3000 villages inhabited by at least 2.1 million people. The effects that have been reported in the exposed Vietnamese population include birth defects and increased risk of cancer. Elevated levels of dioxins have also been detected in American Vietnam veterans (Stone 2007).

Turkey 1955

An accidental human exposure to the fungicide hexachlorobenzene (HCB) in Turkey between 1955-1959 was caused by ingestion of HCB treated grain. The exposure lead to hyperpigmentation, porphyrinuria (excretion of large quantities of porphyrin in the urine), smaller hands and enlarged thyroid and liver. The milk of exposed mothers contained 8 times more HCB compared to unexposed controls (Gocmen 1989).

EFFECTS OF POPs ON CALCIFIED TISSUES

IN VITRO

There are a limited number of studies performed on bone cells. In 1994, Gierthy reported decreased formation of ossification centers in a rat osteoblastic cell line exposed to TCDD, indicating a block in the osteoblastic differentiation. As a result of these observations, it is possible that chronic exposure to low environmental doses of TCDD could result in altered fetal bone development or increased risk of osteoporosis in the adult (Gierthy et al. 1994). Similarly, 3-methylcholanthrene (3MC) another Ah-receptor ligand, inhibited proliferation and differentiation of osteoblasts, both *in vitro* and *in vivo*, suggesting an anti-estrogenic effect (Naruse et al. 2002). An *in vitro* study on rat osteosarcoma cells showed opposite results compared to the two studies mentioned above; here TCDD had estrogenic effects (Partridge et al. 2000). A recent study on rat osteoblasts showed that the expression of the bone adhesion protein osteopontin is a rapid and sensitive marker for TCDD-exposure (Wejheden et al. 2006). In support of the suggestion that the AhR is involved in TCDD-mediated bone toxicity, the AhR antagonist resveratrol was reported to inhibit the TCDD-mediated effects on rat and chicken osteoblasts (Singh et al. 2000). Studies of TCDD (Ilvesaro et al. 2005) and 3MC (Naruse et al. 2004) on rat osteoclasts showed no effect on the osteoclastic activity, but inhibition of osteoclastic differentiation (Naruse et al. 2004).

EXPERIMENTAL ANIMALS

PCB

The number of studies elucidating the effects of individual PCBs and PCB-mixtures *in vivo* are also limited. Femurs of male Fisher rats exposed to a commercial PCB-mixture (Aroclor 1254) showed inhibition of periosteal growth and increased endosteal bone resorption. Also, increased density and decreases in cross-sectional area (CSA) and cortical area were seen. The changes in bone tissue caused by PCB resulted in a significantly weaker bone (Andrews 1989). A recent study reports disrupted femoral bone metabolism in adult male Wistar rats, where the bone formation marker ALP and collagen were decreased due to Aroclor 1254 exposure (Ramajayam et al. 2007). Aroclor 1254 is suggested to have anti-estrogenic properties, which is in line with the observed effects in the rats. In addition, studies have been performed using the individual dioxin-like PCB-congener PCB 126. In PCB 126-exposed female Sprague-Dawley rats, bone length, marrow cavity, torsional stiffness and collagen concentration were significantly reduced (Lind et al. 1999; Lind et al. 2000). In addition, PCB 126 might display estrogenic activity depending on the estrogen-status of the individual (Lind et al. 2004). In the jaw, dietary exposure to PCB 126 or TCDD for up to 101 days did not produce any effects in male Long-Evans rats (Aulerich et al. 2001), but alterations were seen in PCB 126- or TCDD-exposed mink (*Mustela vision*), where maxillary and mandibular osteolysis were seen (Render et al. 2001). Moreover, experimental studies on nestling

American kestrels (*Falco sparverius*) showed developmental effects of PCB 126, such as shortening of the humerus, radius-ulna and femur (Hoffman et al. 1996).

TCDD

Bone

Studies have shown that dioxins cause adverse effects on bone tissue composition and dimensions. TCDD induced dose-dependent changes in bone mineral density (BMD) and biomechanical properties (energy and stiffness) in two rat strains; the TCDD-resistant Han/Wistar and the TCDD-sensitive Long-Evans. Not surprisingly, the effects of TCDD were more pronounced in the sensitive strain (Jamsa et al. 2001). Miettinen *et al* reported that rats with dioxin-resistant alleles showed increased resistance to bone effects after *in utero* and lactational exposure to TCDD, compared to rats without resistant alleles (Miettinen et al. 2005). In mice, TCDD affected the size and shape of mandibles (Allen and Leamy 2001), while in the zebrafish impaired lower jaw development was reported (Teraoka et al. 2002).

Teeth

During development, teeth are among the most sensitive targets of TCDD. Experimental exposure of animals and accidental exposure of humans have shed some light on the effects of TCDD on teeth and tooth development. In young adult male Han/Wistar rats, a single dose of TCDD gave rise to thinner upper and lower incisor teeth, indicative of altered dentine formation. Also, the pulps of the lower incisors were exposed to the oral cavity (Alaluusua et al. 1993). In addition, TCDD can accelerate or retard the eruption of teeth in rats (Kattainen et al. 2001; Miettinen et al. 2002). Murtomaa *et al* concluded that the molar teeth could be a sensitive biomarker for PCDD/F exposure in wild bank vole populations (*Clethrionomys glareolus*) (Murtomaa et al. 2007). Moreover, effects on tooth development was also reported in rhesus monkeys, suggesting a lowest observed adverse effect level (LOAEL) between 30-300 ng/kg for TCDD in monkeys (Yasuda et al. 2005).

WILD ANIMALS

Aquatic

The seal population in the Baltic Sea has been heavily exposed to persistent environmental pollutants, such as DDT and PCB. The discovery of high levels of PCB and DDT in the 1960's lead to the idea that the decreased birth rate in seals could be connected to these contaminants (Ahnland 1994). Severe pathological changes were found in bone (Bergman 1992; Lind et al. 2003) and there have been reports concerning vertebral deformities in fish, for example in (*Myoxocephalus quadricornis*) exposed to pulp mill effluents in the Baltic Sea (Gulf of Botnia) (Bengtsson 1988; Mayer 1988).

Another free-ranging specie affected is the Beluga whale (*Delphinapterus leucas*) in the Golf of St Lawrence, Quebec, Canada, where bioaccumulation of PCBs and DDT-related compounds has been noticed, together with periodontitis (loss of teeth) and skeletal defects, such as spinal column deformation (Beland et al. 1993). The polar bears of the Arctic regions are affected by POPs even though they inhabit areas far from the pollution sources. Studies of East Greenland polar bear (*Ursus maritimus*) skulls suggests a negative correlation between bone mineral density and exposure to organochlorine contaminants, such as sumPCBs, chlordanes, DDT and dieldrin (Sonne et al. 2004).

Terrestrial

Bone alterations have been reported in terrestrial animals. Minks (*Mustela vison*) residing in a contaminated area of southwestern Michigan, USA have shown lesions of mandibles and maxillae, suggested to be due to environmental exposure to PCBs (Aroclor 1242 and 1254) (Beckett et al. 2005). The diet of the minks includes fish, frogs, clams, and snakes, which could result in dietary exposure to PCB. Moreover, bone effects have been found in birds. Free-ranging clapper rails (*Rallus longirostris*) inhabiting a PCB- and Hg-contaminated estuarine marsh system in coastal Georgia, USA displayed accelerated bone maturation (Rodriguez-Navarro et al. 2006) and grey heron (*Ardea cinerea*) nestlings in the United Kingdom had skeletal deformities, possibly associated with PCBs and PCDD/Fs (Thompson et al. 2006). In addition, bill deformities (crossed bills) have been reported in bald eagles (*Haliaeetus leucocephalus*) and in double-crested cormorants (*Phalacrocorax auritus*) residing in the Great Lakes area (Bowerman et al. 1994; Fox et al. 1991). Observations include abnormal mandibles, such as deformation (crooked) or differences in length between upper and lower mandible.

EPIDEMIOLOGICAL STUDIES IN HUMANS

Bone

Two epidemiological studies investigating human exposure to POPs in relation to bone effects suggests a weak association between serum DDE levels and reduced BMD (Beard et al. 2000; Glynn et al. 2000), while a third study fail to show such a correlation (Bohannon et al. 2000). In studies performed on a cohort of Swedish fishermen and their wives, Wallin *et al* concluded that the results only minimally supports a possible link between POP exposure and an increase in osteoporotic fractures in the wives. No such relationship was found for the fishermen (Wallin et al. 2004). Moreover, there were significant negative associations between PCB 153 concentrations in serum and BMD in both the fishermen and their wives, but after adjustment for age and body mass index, the associations did not remain (Wallin et al. 2005). Similarly, a study in Inuit women from Greenland showed negative associations between PCB 153 concentrations and BMD, but after adjustments for similar confounders, no associations were seen (Cote et al. 2006).

Teeth

Effects on teeth and tooth development have been detected in humans. Dioxins and furans may cause mineralisation defects in breast-fed children, if the concentration in the mother's milk is high enough, and if the breast-feeding period is long (Alaluusua et al. 1999). Individuals from the Seveso area in Italy displayed permanent dental alterations following childhood exposure to TCDD (Alaluusua et al. 2004).

AIMS OF THE THESIS

The overall objective of my studies has been to elucidate potential bone effects following exposure to persistent organic pollutants, in controlled experimental settings (goat and rat) and in wild animals (alligator and herring gull).

♦ Reproductive disorders have been observed in male and female alligators (*Alligator mississippiensis*) residing in the pesticide-polluted Lake Apopka in Florida, USA. These disorders are thought to be due to high concentrations of dicofol, DDT and its metabolites, released into the lake due to an industrial spill. The aim of the work on alligators that constitute paper I was to investigate if the bone tissue was affected in female alligators living in this contaminated environment. Could these pesticides possess endocrine-disrupting potential on bone tissue, and how would that affect the composition and dimensions of the bone?

♦ Some of the most widespread persistent organic pollutants are the PCBs, which can be found in most biota from all environmental compartments and in adipose tissue of humans. Studies of accidentally exposed humans and studies of the effects of dioxins and dioxin-like compounds in animals have shown detrimental effects on bone and teeth. The aim of the study reported in paper II was to investigate if the dioxin-like PCB 126 (anti-estrogen) and the non dioxin-like PCB 153 (putative estrogen) gave rise to developmental effects on bone in female goat offspring following *in utero* and lactational exposure.

♦ Chronic and sub-chronic studies in rats have shown that dioxin and dioxin-like compounds impair bone tissue homeostasis. Therefore, in the study reported in paper III, the aim was to investigate if short-term exposure to a single dose of TCDD resulted in detectable effects on bone in young male rats. In addition to pQCT and biomechanics, in this study we had the opportunity to include other analytical techniques, such as bone chemical composition and bone markers (ALP and CTX) to investigate the potential bone alterations at different levels.

♦ Studies of Great Lakes wildlife have reported negative effects of organochlorines on various hormonal systems. In addition, the eggs of herring gulls (*Larus argentatus*) have been used for over 30 years to monitor trends in concentration of persistent and bioaccumulative contaminants in Great Lakes food webs. Previous studies on the gulls reported biochemical and histopathological alterations in different tissues, and therefore the studies were extended to include bone tissue. The aim of the work presented in paper IV was to investigate if bone tissue homeostasis was disrupted in herring gulls residing in the Great Lakes, a lake system polluted with POPs and metals and situated near many large cities and industries.

MATERIAL AND METHODS

ANIMALS

Alligator

Juvenile American alligators (*Alligator mississippiensis*) were collected at night by hand capture from two lakes in central Florida. Animals were obtained from Lake Apopka (N=9) and Lake Woodruff (N=7) between September 6 and 29, 1995. The mean ages of the female animals from the reference and the contaminated lakes, respectively, were similar (5.6 vs. 5.2 years). Lake Apopka has a documented history of contamination from agricultural pesticides, municipal runoff and sewage, and a pesticide (dicofol, DDT and its metabolites) spill. Lake Woodruff, the reference lake, has had little or no modern agricultural activity and no direct municipal runoff. It has been sprayed with modern herbicides used for aquatic weed control. Following blood collection, individuals were sexed, body measurements (snout–vent length, total length) obtained and environmental data (air and water temperatures) recorded.

Goat

Fortyfive adult female goats (*Capra hircus*) with synchronised oestrus cycle were mated until pregnant. After confirmed pregnancy, 30 goats were allocated into three groups (10 goats/group) by use of block randomisation. One group was exposed to PCB 153, a second group to PCB 126, and the third group served as a control. The appropriate PCB dissolved in corn oil was administered orally with a syringe three times a week from day 60 of gestation and terminated at delivery. The control group was given corn oil only. The doses of PCB 153 and PCB 126 were 98 μg/kg body wt/day (230 μg/kg body wt/treatment), and 49 ng/kg body wt/day (115 ng/kg body wt/treatment), respectively. The pregnant does in the PCB 153 and PCB 126 treatment groups and the control animals gave birth to 7, 11 and 7 healthy female kids, respectively. The goats delivered indoors and every kidding was supervised. Health status of the kids was controlled daily and body weights were recorded once a week. The suckling period lasted for 6 weeks and the kids were euthanised at 9 months of age.

Rat

Male Sprague-Dawley rats were housed in the animal facility at Karolinska Institutet, Stockholm and kept singly under standard conditions (50% humidity, 22°C, 12/12h light/dark cycle) in ventilated, filter-top cages containing sterile sawdust bedding and environmental enrichment (plastic piping and cardboard boxes). The animals were fed standard food pellets (R70, Lactamin, Sweden). Weight matched, two months old male Sprague-Dawley rats (ca 200g) were treated with TCDD by a single intraperitoneal injection (*i.p*) of 50 μg TCDD/kg bw in corn oil (N=10) or vehicle only (N=9). Five days following the exposure, left and right tibiae were excised, blood was collected and serum was prepared.

Herring gull

Collections of herring gulls (*Larus argentatus*) were made from 10 colonies (N=102), representing all five Great Lakes and the Detroit River, and included colonies where there was or was not historical evidence of point source contamination or reproductive effects. The Great Lakes are polluted with several contaminants, including PCBs, pesticides (mirex, DDT and its metabolites), dioxins and metals *e.g.* lead (Pb), cadmium (Cd), and mercury (Hg), as determined from tissues of herring gulls (Fox et al. 2007). For comparison, a colony from Lake Winnipeg was chosen as a freshwater reference lake with comparable surface area (N=19), but without obvious point source contamination. Kent Island, in the Bay of Fundy, was chosen as a marine reference colony with very low levels of contamination (N=19).

The sampling of the collections were timed to coincide with mid-incubation (day 7 to 21 of the 28-day incubation period) based on actual (marked nests) or historical chronology. Stage of incubation for unmarked nests was determined by egg floatation prior to trapping. Collections in the Great Lakes were made between 6 and 31 May, 1991 (and 1 colony in 1993), on Lake Winnipeg on 8–9 June, 1991, and in the Bay of Fundy on 19–20 June, 1991. The incubating gulls were trapped on their nests using self-triggered drop traps. An attempt was made to collect both members of the pairs, and to obtain a sample size of 10 individuals from most Great Lakes colonies and 20 from large colonies and non-Great Lakes sites.

ETHICAL APPROVAL

The experiments were approved by University of Florida's institution of animal care and use committee project Z037 (WX01310) (paper I, alligator), Forsøksdyrutvalget, Godkjenningsdokument nr 127, avd referensnummer: 6/28 (paper II, goat), Stockholms norra djurförsöksetiska nämnd, dnr N392104 (paper III, rat) and Ontario Region Animal Care Committee, Project 91F2 (paper IV, herring gull).

PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

The method used in all papers (I-V) is peripheral quantitative computed tomography (pQCT), an established technique for the determination of bone mineral density. There are different methods to use when measuring bone density, *e.g.* pQCT and dual energy X-ray absorptiometry (DEXA), but the pQCT system has some advantages compared to DEXA; the ability to separate estimation of trabecular and cortical bone and to give a true volumetric mineral density value (3D) (g/cm^3). DEXA gives an areal density value (2D) (g/cm^2). The principle of pQCT involves the attenuation of X-rays passing through the bone while they revolve around it. Minerals have a higher atomic number compared to soft tissue or air, which attenuates the intensity of an X-ray beam passing through the bone. Thus, high density is associated with a high attenuation of the beam. The radiation dose depends on bone thickness and scanning velocity, and the dose is less than 1 mSv, which is less than the dose used in chest X-rays (Radetti et al. 2006).

Before each study, the pQCT settings have to be tested and decided upon, because the bones are from different species and are of different sizes. The attenuation thresholds were larger in paper I (alligator) and paper II (goat), than in paper III (rat) and paper IV (herring gull). The voxel size determines the resolution of the picture and a lower voxel size means higher spatial resolution. In paper III and paper IV, a lower voxel size was used for the smaller bones.

In paper I-III, the bones were measured at the diaphysis (cortical measure point) and at the metaphysis (trabecular measure point). Measures of bone composition include bone mineral density (BMD; mg/cm^3) and bone mineral content (BMC; mg/mm). The pQCT system also measures the dimensions of the bone with great accuracy, *e.g.* total cross-sectional area (CSA) (mm^2), cortical area (mm^2), trabecular area (mm^2), periosteal (outer) circumference (mm), and endosteal (inner) circumference (mm) (Table 1, Fig 7).

To evaluate the reproducibility of the pQCT measurements, the coefficients of variation (CV) for the different measures were calculated from 10 repeated measurements with a single sample being repositioned before each measurement.

Table 1. Measures determined by peripheral quantitative computed tomography (pQCT).

Measures	Unit
cortical bone mineral density (cort BMD)	mg/cm^3
trabecular bone mineral density (trab BMD)	mg/cm^3
total bone mineral density (total BMD)	mg/cm^3
cotical bone mineral content (cort BMC)	mg/mm
trabecular bone mineral content (trab BMC)	mg/mm
total cross-sectional area (CSA)	mm^2
cortical area (cort A)	mm^2
trabecular area (trab A)	mm^2
periosteal (outer) circumference	mm
endosteal (inner) circumference	mm

BIOMECHANICS

The bones were subjected to biomechanical testing to failure after the pQCT measurements. The strength and the mechanical characteristics of a bone is determined by several factors, including mineral content (inorganic content), micro architecture, geometry (*e.g.* cross-sectional area, diameter and length) and organic matrix. The pQCT can theoretically predict the mechanical strength of a bone using different models, although it is only through destructive testing by the use of a material testing machine that the true mechanical characteristics can be determined. There are four basic testing configurations that are commonly used to determine the biomechanical properties of a bone; bending, torsion, compression and tension. For long bones, bending until failure using three or four-point technique is by far the most commonly used testing configuration (Fig. 7). Whenever a long bone is subjected to bending it means that the cortical bone on one side is subjected to compression while the cortex on the opposite

side simultaneously will be subjected to distraction, or tension. Pure tension or compression testing might be of interest for assessment of the intrinsic material properties of bone matrix, such as the effect of density on the mechanical characteristics. Tension is more used for measurements of cortical bone and compression for trabecular bone. When assessing the trabecular bone in vertebrae, compression is commonly used because of the similarities to the loadings seen *in vivo*.

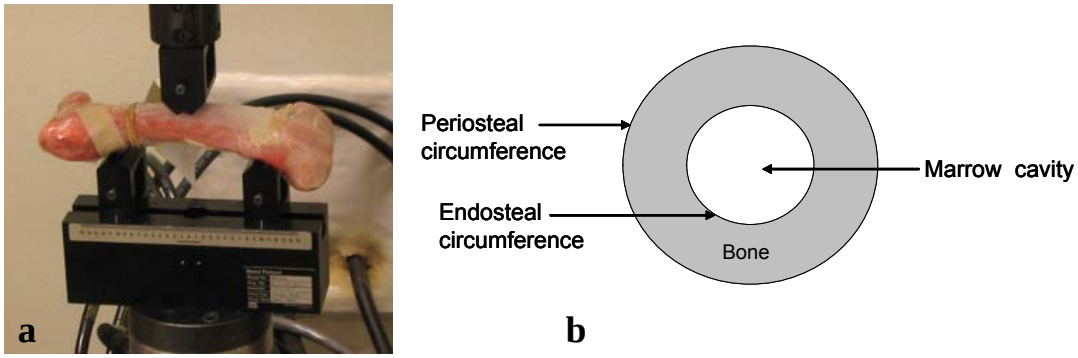


Figure 7. a) Photograph showing the three-point bending test (goat bone) and b) a schematic cross-section of a bone as seen in the pQCT; showing marrow cavity, endosteal and periosteal circumference (diaphyseal part).

In paper II-IV, the three-point bending test was used to determine the bone strength. The bone was placed on two supports after which an increasing load was applied towards the mid-diaphyseal area of the bone until failure. The three-point bending fixture was connected to a computer that registered load (N) and displacement (mm) at 50 Hz. Based on the load and displacement data it was then possible to determine load at failure (N), displacement at failure (mm), energy to failure (surface under the curve, N*mm, Joule (J)) and stiffness (slope of the load-deflection curve, N/mm) (Fig. 8). The distance between the two supports was altered for different bone types based on the size. The supports were located further apart for the longer bones in paper II (goat) compared to paper III (rat) and paper IV (herring gull). Unintentional thawing of the alligator bones during transportation might have had a negative, or at least unknown effect on the mechanical properties. We therefore decided not to do any mechanical testing on the alligator bones.

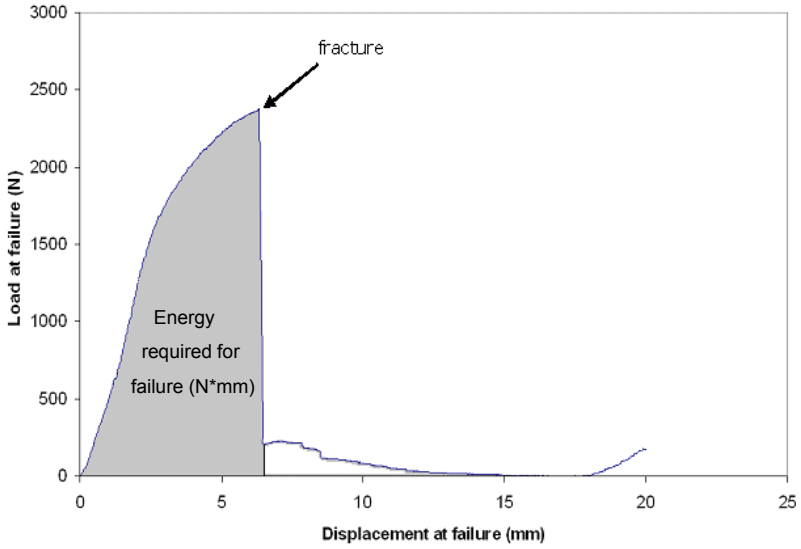


Fig. 8. A representative load–displacement curve resulting from a biomechanical test. The height of the curve represents breaking force (bone strength or load at failure), the width of the curve is the displacement at failure while the slope for the curve describes the stiffness, and the area under the curve the energy required to break the bone.

CARBOXYTERMINAL TELOPEPTIDE OF TYPE I COLLAGEN

In paper III, the widely accepted bone resorption marker carboxyterminal telopeptide of type I collagen (CTX) was measured in serum, using the RatLaps™ Enzyme-Linked Immuno-Sorbent Assay (ELISA). Serum is incubated with the primary antibody at +4 °C over night. After washing, the samples are incubated with the peroxidase-conjugated secondary antibody for one hour. Following incubation with the substrate solution, the absorbance is measured at 450 nm. In the same test set, a standard curve is included together with a negative control. All samples are analysed in triplicates. A standard curve is prepared with the absorbances from the six standards against the corresponding RatLaps concentration. The serum concentration (ng/mL) in each sample is determined by the equation of the curve, and the mean values representing each group is calculated.

ALKALINE PHOSPHATASE

In paper III, the serum level of alkaline phosphatase (ALP) was analysed with an enzyme assay based on the conversion of para-nitrophenylphosphate (*p*-NPP) to para-nitrophenol and the colorimetric determination of the resulting coloured product. Serum and H_2O are mixed with a substrate solution containing *p*-NPP. Following incubation, the absorbance is read at 405 nm. Each serum sample was analysed three times, each time in triplicates.

MINERALISATION

Bone chemical composition (mineralisation) was analysed by optical emission spectroscopy (ICP-OES) and Fourier transform infrared spectrometry (FTIR). The bones were cleaned with a scalpel, rinsed with Milli-Q water and powdered using a cryogenic mill (CertiPrep 6750 Freezer/Mill, SPEX). For the ICP-OES analyses, 50 mg of bone powder was digested in a 10 mL solution of 10% HNO_3 and 3% H_2O_2. Ca and P concentrations were measured using an ICP-OES (Mod. ARL 3410, Fisons). For quality control purposes, standard trace grade solutions containing Ca and P in sample range concentrations were prepared and analysed after every five samples. All standard solutions were within 5% of the reported values. For the FTIR analyses, 5 mg of bone powder was mixed with 90 mg of FTIR-grade KBr and pressed under vacuum. Infrared spectral data were collected on a Fourier transform infrared spectrometer (Magna IR200, Nicolet). The amounts of phosphate, carbonate, and organic matrix in bone were determined from the peak area of absorption bands associated with phosphate, carbonate, and amide groups in the infrared spectra. The following parameters were used to describe bone degree of mineralisation and carbonate content from FTIR analyses:

The degree of mineralisation of bone (mineral) is defined as the mineral to organic matrix ratio and was estimated as follows:
(1) mineral = A1200_900/A1660, where A1200_900 is the peak in the FTIR analysis representing the amount of phosphate in bone and A1660 is the peak representing the amount of amide I groups (main band from bone organic matrix).

The relative amount of carbonate in bone mineral (minCO3) was calculated as the ratio of the peak area for 1405 cm-1 (A1405; mainly carbonate type B substitution) to phosphate band area (A1200_900) and was calculated as follows:
(2) $minCO_3 = A1405 / A900\_1200$

STATISTICS

In paper I and II, the results were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc Fisher's protected least significant difference test. Adjustments were done for body weight in paper II. In paper III, pQCT, biomechanical and bone chemical composition (mineralisation) results were evaluated by ANOVA and adjusted for body weight. CTX and ALP were evaluated using Student's t-test. In paper IV, colonies were grouped as Great Lakes, freshwater reference, and marine reference. All colonies were also pooled and then divided into high TEQ (TCDD-TEQs >500 pg/g; N=55) or low TEQ (TCDD-TEQs <500 pg/g; N=85), based on their TCDD-TEQ concentrations derived from congener-specific PCB, PCDD, PCDF concentrations in their livers using World Health Organization toxic equivalency factors for birds. Similarly, they were also grouped as high PCBs (ΣPCBs >9500 ng/g; N=60) or low PCBs (ΣPCBs <9500 ng/g; N=80) based on Σ42 congeners. The results were evaluated by ANOVA followed by a Bonferroni-Dunn test. Analysis of covariance (ANCOVA) was used when adjusting for sex and the continuous variables body mass and bone length. Differences were considered significant at $p \leq 0.05$ for all studies.

RESULTS AND DISCUSSION

The results of paper I-IV will be presented together with a discussion of the findings. To see an overview of the studies and results, see Table 2. For paper III, there are results from ALP, CTX and mineralisation analyses, which are presented on p. 33. Bone composition includes BMD, BMC, total, trabecular and cortical area, bone dimensions include bone length, marrow cavity, periosteal and endosteal circumference, and total cross-sectional area, and finally biomechanics include stiffness, load, energy and displacement.

Table 2. Overview of set up and results of paper I (alligator), paper II (goat), paper III (rat), and paper IV (herring gull).

	EDC	EDC level	Type of exposure	Effects on bone		
				Composition	Dimension	Biomechanics
Alligator (F)†	DDT/ dicofol	environmental	continous	yes	no	n/a
Goat (F)	PCB153/ PCB126	PCB 153: 98 µg/kg bw/day PCB 126: 49 ng/kg bw/day (environmentally relevant)	*In utero* /lacation	yes	yes	no
Rat (M)	TCDD	50 µg/kg bw	single *i.p* injection ^	no	yes	no
H. gull (M/F)†	mixture	environmental	continous	yes**	yes*,**	yes*

† =free-ranging, * =compared to freshwater reference, **=compared to marine reference, M=male, F=female, n/a =not applicable, EDC=endocrine disrupting compound, ^ =short-term exposure

BONE COMPOSITION AND DIMENSIONS

Bone composition

In paper I (female alligator) and paper II (female goat offspring), exposure to DDT/dicofol and PCB 153, respectively, resulted in increased total and trabecular BMD, suggesting estrogenic effects. In addition, the BMC was increased in the alligators (Fig 9). In contrary, in paper IV, the herring gulls (male and female) showed decreases in both BMD and BMC, indicative of anti-estrogenic effects (Table 3). However, the decreases in BMD and BMC were significant only when the Great Lakes gulls were compared to gulls from the marine reference. Another lake, Lake Apopka (inhabited by the alligators) is heavily contaminated with dicofol, DDT and its metabolites. In several studies, many of these compounds have shown the ability induce estrogen-like effects, *e.g* dicofol, *o,p*'-DDT, and also *p,p´*-DDE, which is mainly thought to display anti-androgenic properties, but has previously shown estrogenic effects in reptiles (Bitman and Cecil 1970; Hoekstra et al. 2006; Leanos-Castaneda et al. 2007; Willingham and Crews 1999). Therefore, one suggestion as to why the BMD and BMC are increased in the alligators could be the estrogenic environment of the lake. The estrogenicity of the compounds could be achieved by their binding to the ERs on both osteoblasts and osteoclasts, increasing the production of TGF-ß, a growth factor that enhances osteoclastic apoptosis (Hughes et al. 1996). This results in remodelling favouring bone formation. Mainly, estrogen reduces the rate of bone turnover by acting on the osteoclasts, but effects on osteoblasts and bone formation have been reported (Chow et al. 1992). In the case of the putative estrogen PCB 153, the same mechanisms as for the alligators are proposed for the observed effects in female goat

offspring. In this case, PCB 153 acts as a ligand to the ERs and elicits estrogenic actions in the bone.

Table 3. Bone composition obtained from pQCT measurements of bone from alligator, goat, rat and herring gull, following exposure to environmental contaminants, either in laboratory settings or in their natural habitat (free-ranging).

	BMD (mg/cm^3)	Total BMD (mg/cm^3)	BMC (mg/mm)	Trabecular A (mm^2)
Alligator (paper I)	↑#	↑	↑#	-
Goat (paper II)	↑#	-	-	-
Rat (paper III)	-	-	-	↓
Herring gull (paper IV)	↓**, ^	-	↓**, ^	-

BMD = bone mineral density, BMC = bone mineral content , A = area, ** = compared to marine reference, # = trabecular, ^ = cortical

Bone mineralisation is linked to BMD thorough the osteoblasts. Mature osteoblasts secrete proteins that create the bone matrix, which is converted into hard mineralised bone tissue. Reduced bone turnover increases the number of active osteoblasts, and hence, increases the amount of bone matrix and mineral content. This pattern, where BMD and BMC follow each other, is seen in the alligators and in the herring gulls; in the alligators, an increased BMD is followed by an increased BMC and in the herring gulls, a decrease in BMD is followed by a decrease in BMC. The observed decreases in the herring gulls might possibly be mediated by the anti-estrogenic environment of the Great Lakes. Alternatively, metals could have affected the bones negatively, thus Cd is suggested to increase bone resorption in a population-based study of women (Akesson et al. 2006). However, the contaminant mixture in the Great Lakes area is not as defined as in Lake Apopka, therefore one can only, with even more uncertainty, speculate about causes giving bone effects in Great Lakes gulls.

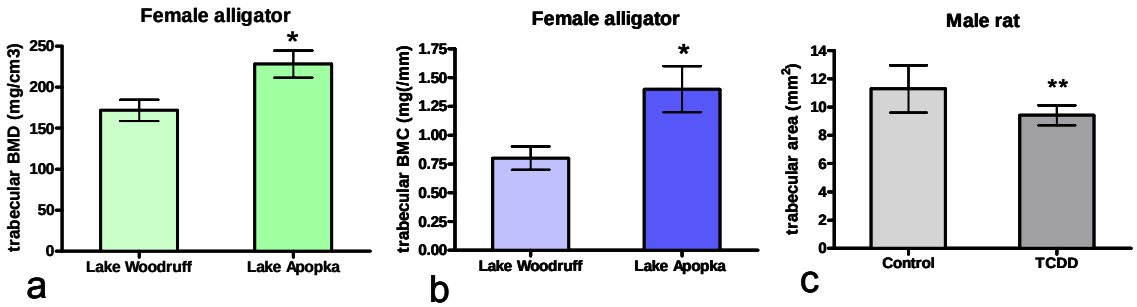


Figure 9 a) Trabecular BMD (mg/cm^3) of free-ranging alligators from the pesticide-polluted (dicofol, DDT and its metabolites) Lake Apopka, compared to females from a control lake (Lake Woodruff). b) Trabecular BMC (mg/mm) of free-ranging alligators from the pesticide-polluted (dicofol, DDT and its metabolites) Lake Apopka, compared to females from a control lake (Lake Woodruff). c) Trabecular area (mm^2) of young male Sprague/Dawley rats exposed to one single *i.p* injection of 50 µg TCDD/kg bw for five days, compared to controls receiving corn oil.

In paper III (rat), the only change in bone composition involved a decrease in trabecular area. The BMD seems to be unaffected, which could be due to the short exposure time (5 days). In the other studies (I, II, and IV), the time of exposure was much longer; the alligators and the herring gulls have probably been exposed as early as *in ovo* and then continued exposure as juveniles up to adult age. Therefore, the bone effects seen in the alligators and herring gulls could be the result of developmental toxicity and/or juvenile/adult dietary exposure to a mixture of environmental contaminants. Similarly, the goats were exposed *in utero* and during lactation, which could result in developmental effects or effects induced during the growth period coinciding with lactation. However, a study on rats exposed to TCDD *in utero* and during lactation reported effects on the developing bone, but gestational exposure was not enough to cause alterations. Instead, the lactational exposure was more important with regard to bone effects. The effects detected include interference with mechanical strength and bone growth, such as length and BMD, and the effects were reversible (Miettinen et al. 2002).

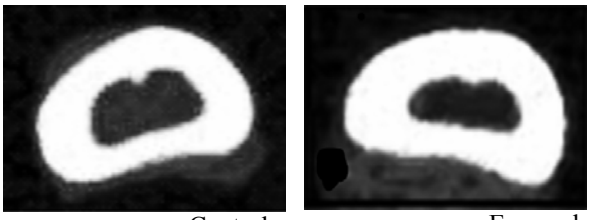
Another possible suggestion for the observed alterations in bone tissue in papers II-IV (rat, goat and herring gull) involves cross-talk between the ER- and the AhR-signalling pathway (Kietz et al. 2004). However, the molecular mechanisms for this cross-talk are unclear, but could involve; 1) AhR-mediated decrease in estrogen level resulting from TCDD-induced up-regulation of estrogen-metabolising enzymes *e.g.* CYP1A1, 1A2, and 1B1, 2) TCDD-induced suppression of the transcription of many estrogen-induced genes by blocking or disrupting binding of ER to DNA at estrogen responsive elements (ERE) sites, adjacent to or overlapping with AhR-binding sites (DRE), 3) competition between AhR and ERs for shared co-factors 4) increased degradation of ER (Brunnberg et al. 2003; Kietz et al. 2004; Matthews et al. 2005; Wormke et al. 2003).

Moreover, enzymes involved in the steroid biosynthesis have previously shown alterations following TCDD-exposure and the short heterodimer partner orphan nuclear receptor (SHP) was down-regulated (Fletcher et al. 2005), which is particularly interesting, since it has been shown to inhibit the activity of the osteoclasts (Granot-Attas et al. 2007) and suppress the transcriptional activity of retinoid and estrogen hormone receptors (Johansson et al. 1999; Lee et al. 2000) Thus, a decreased activity of SHP together with a decreased level of estrogen might lead to more osteoclasts actively resorbing bone tissue and reduced bone formation.

Trabecular bone is metabolically more active than cortical bone due to its larger surface area. Therefore, not surprisingly, most of the effects seen are located at the metaphyseal part of the bone (paper I-III). Any insults to the remodelling process firstly affect the trabecular bone, as seen in the alligator, goat, and rat. In the alligator and the goat, the trabecular density is increased, while the trabecualar area is decreased in the rat.

Bone dimensions

Changes in bone dimensions were seen in the goats and herring gulls only (Table 4). In goat offspring decreases include marrow cavity (Fig. 9), CSA (Fig. 10), and moment of resistance (a biomechanical measure determined by the pQCT) and in the herring gulls decreases include bone length, CSA, and periosteal circumference. Thus, the bones in both studies have become thinner. As mentioned previously, the estrogenic action on different parts of the bone may change the shape of it; estrogen inhibits periosteal bone formation and promotes endosteal bone formation (Åkesson 2006). In the goat bone, estrogen may have caused decreased CSA due to periosteal inhibition, and a decreased marrow cavity due to endosteal promotion. The bones of the herring gulls may have experienced estrogenic inhibition of periosteal bone formation, as seen by decreases in CSA and periosteal circumference.



Control Exposed

Figure 9. Representative images from pQCT scans at the diaphyseal 18% measure point from female goat offspring. The white outer part is cortical bone and the dark inner part is bone marrow cavity. *Note* the small area of the marrow cavity in bone from the exposed animal.

The alterations in bone dimensions in the goats and herring gulls are partly in accordance with previous experimental studies in rats, where male Fisher rats exposed to a commercial PCB-mixture (Aroclor 1254) showed inhibition of periosteal growth, but increased endosteal bone resorption (Andrews 1989). However, in ovariectomised female Sprague-Dawley rats, the marrow cavity was reduced following exposure to the anti-estrogenic PCB126 (Lind et al. 2000), indicative of weak estrogenic activity, due to promotion of endosteal bone formation. In a previous paper concerning the same PCB 126-exposed rats, the authors conclude that PCB 126 might display estrogenic activity depending on the estrogen-status of the individual (Lind et al. 2004; Lind et al. 1999).

Table 4. Bone dimensions obtained from pQCT measurements of bone from alligator, goat, rat and herring gull, following exposure to environmental contaminants, either in laboratory settings or in their natural habitat.

	Marrow cavity (mm^2)	CSA (mm^2)	Moment of resistance (mm^3)	Periosteal circumference (mm)
Alligator (paper I)	-	-	-	-
Goat (paper II)*	↓	↓	↓	-
Rat (paper III)	-	-	-	-
Herring gull (paper IV)	-	↓	-	↓

* = Results from the 18% diaphyseal measure point, CSA = cross-sectional area

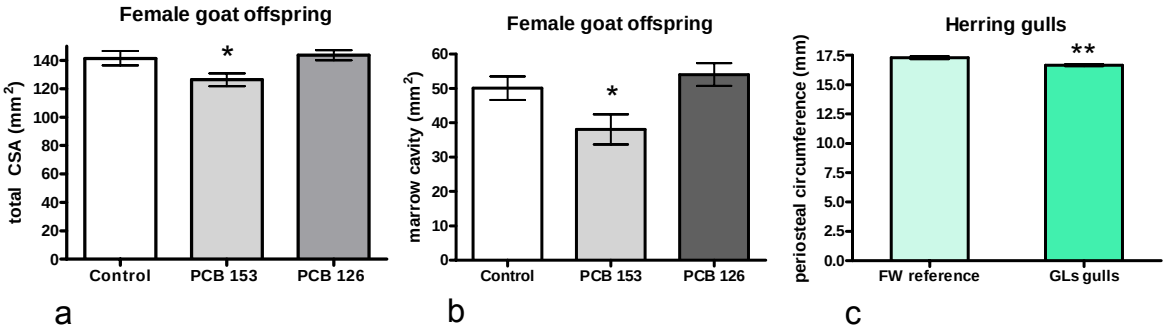


Figure 10. a) Total CSA (mm^2) at the diaphyseal 18% measure point, located at a distance of 18 % of the total bone length from the proximal tip, of female goat long bone after exposure to PCB 126, PCB 153 or vehicle (corn oil). b) Marrow cavity (mm^2) at the diaphyseal 18% measure point, located at a distance of 18 % of the total bone length from the proximal tip, of female goat long bone after exposure to PCB 126, PCB 153 or vehicle (corn oil). c) Periosteal circumference (mm) of femur of herring gulls residing in the Great Lakes or in the freshwater reference (Lake Winnipeg), in the early 1990s.

Regarding the length of the bones, only the bones of the herring gulls (paper IV) were changed, and shorter bones indicate an early closure of the growth plate. During normal physiological conditions, the growth plate closes at the end of adolescence following increased estrogen-signalling. The herring gulls have been exposed to an unknown mixture of contaminants, triggering the closure of the plate, suggesting estrogenic activity of the contaminants. Contradictory, when the gulls are pooled (GLs and references) and divided into high TEQ and low TEQ groups, differences in biomechanical measures were detected in the HTEQ, compared to the LTEQ group (these biomechanical measures will be discussed shortly). This suggests exposure to dioxin-like chemicals, and not *ortho*-substituted PCB congeners (estrogen-like). The reason for the contradicting results in the herring gull bones is unknown, most likely the Great Lakes area contains both estrogenic and anti-estrogenic compounds, all with different affinity for their respective receptor and different capability to induce alterations. In addition, metals present in the Great Lakes could have the potential to alter bone tissue homeostasis.

BIOMECHANICS

Changes in the biomechanical properties, such as a decrease in stiffness, and increases in displacement at failure and energy required for failure, were detected only in the herring gulls (paper IV; compared to freshwater reference, Table 5). This might be due to the large number of contaminants in the Great Lakes area, creating a plethora of effects on bone tissue due to its dependence on hormonal and nutritional factors for normal remodelling and homeostasis. Also, the nutritional status of the GLs gulls could have affected the individual, irrespective of contaminant level. The pQCT measurement of the bones showed decreased CSA and periosteal circumference, hence, thinner bones (with unaffected bone composition) are easier to break. Decreases in energy, stiffness, CSA, periosteal circumference and endosteal circumference have been reported in Han/Wistar rats and Long-Evans rats, subcutaneously dosed with 20 weekly doses of TCDD (total dose of 0.17-170 µg TCDD/kg bw) (Jamsa et al. 2001). It is possible that the effects seen in herring gull bone could be connected to exposure to dioxin-like chemicals, since

changes were not only seen in Great Lakes gulls vs freshwater reference, but also in high TEQ vs low TEQ, where load, energy required for failure and displacement at failure were increased.

Table 5. Biomechanical properties of bone from alligator, goat, rat and herring gull, following exposure to environmental contaminants, either in laboratory settings or in their natural habitat.

	Load at failure (N)	Energy (N*mm)	Stiffness (N/mm)	Displacement (mm)
Alligator (paper I)	-	-	-	-
Goat (paper II)	-	-	-	-
Rat (paper III)	-	-	-	-
Herring gull (paper IV)	-	↑	↓	↑
Herring gull (paper IV)*	↑	↑	-	↑

* = high TEQ vs low TEQ

The altered biomechanical properties of the herring gull bones could be due to inadequate mineralisation or a mineralisation defect. Contaminants could have altered the bone mineralisation process in Great Lakes gulls by interfering biochemically (affecting Ca and P concentration) or cellularly (disturbing osteoblastic formation of bone matrix). The measured plasma concentrations of Ca and P in the gulls show that individuals from the Great Lakes area had significantly lower concentrations compared to gulls from the freshwater reference (Lake Winnipeg). The median plasma P concentrations were inversely associated with liver TCDD-TEQs while median plasma Ca was inversely associated with kidney cadmium (Fox et al. 2007). Another study in birds reported decreased plasma P concentrations in breeding colonies of white stork (*Ciconia ciconia*) in southwestern Spain, accidentally exposed to large amounts of acidic and metal-rich water (Smits et al. 2007). A low level of Ca and P could potentially result in pathological changes in bone tissue, such as osteomalacia (Shoback 2007), a condition characterised by soft and demineralised bones. However, osteomalacia may also be due to vitamin D-deficiency. Long-Evans rat dams and their offspring showed reduced serum levels of 1,25-dihydroxyvitamin D_3 following exposure to a mixture of PCBs (Lilienthal et al. 2000). The mineralisation process could also be affected on a cellular level; the osteoblastic formation of bone matrix could have been altered by contaminants; *e.g.* anti-estrogens. Decreased estrogen-signalling could reduce the secretion of type I collagen by osteoblasts, potentially leading to decreased formation of bone matrix. Another important constituent of bone mineral is Mg; a deficiency in Mg inhibits the osteoblasts (Palacios 2006), which could have an impact on mineralisation. Gulls from the Great Lakes had altered plasma magnesium concentrations, which were inversely associated with liver concentrations of non-*ortho*-PCBs and TCDD-TEQs (Fox et al. 2007). Surprisingly, the load at failure was significantly higher in femurs of gulls from the high TEQ group than in those from the low TEQ group. The reason for this is presently not known.

BIOCHEMICAL MARKERS OF BONE TURNOVER

The levels of the biochemical markers ALP (bone formation) and CTX (bone resorption) were measured in serum of the TCDD-treated rats (paper III). ALP was 30% lower and CTX was 4.6% higher compared to controls, five days following exposure. This indicates increased bone resorption and decreased bone formation, which is in accordance with the decrease in trabecular area detected by the pQCT. However, the level of ALP measured in the rats was total ALP, not bone-specific ALP, therefore no certain conclusions can be drawn regarding the rate of remodelling in the bone tissue. Similar to our finding of ALP, a previous *in vitro* study showed a TCDD-induced decrease of ALP in rat osteoblasts, by acting through an AhR-dependent mechanism (Ryan et al. 2007). Similarly, dioxin-like compounds seem to alter the blood concentration of ALP, as seen in free-ranging loggerhead sea turtles (*Caretta caretta*), where the blood concentration of dioxin-like PCBs was negatively correlated with ALP activity (Keller et al. 2004). Moreover, in experimental American kestrels (*Falco sparverius*), the dioxin-like PCB 126 caused a decrease in ALP activity (Hoffman et al. 1996). Thus, TCDD and dioxin-like PCBs may reduce bone formation by inhibiting the differentiation of new osteoblasts.

In contrary to the ALP measurement, the measurement of CTX is bone-specific, and the serum level of CTX was increased in TCDD-exposed rats. Estrogen is suggested to attenuate bone resorption by inhibiting the osteoclastogenesis and the activity of the osteoclasts (Blair and Zaidi 2006). In this study, the level of CTX was increased, thus in our experiment TCDD might have an anti-estrogenic effect on the CTX release, indicating an increased bone resorption.

MINERALISATION

In paper III (rat), treatment with TCDD did not affect the degree of mineralisation, but it significantly altered the chemical composition of bone at the molecular level. Specifically, the bone mineral in treated rats showed a lower relative amount of acid phosphate (HPO_4) and a higher relative amount of crystalline phosphate components compared to controls. During bone maturation there is a progressive loss of acid phosphate content, which is converted into crystalline phosphate. Thus, the observed changes in the relative amount of phosphate groups in different molecular environments are consistent and indicate that TCDD-treatment results in the formation of bone mineral with a composition characteristic of more mature bone. These compositional changes could result from a reduced metabolic activity of bone induced by exposure to toxicants (Rodriguez-Navarro et al. 2006). This is in agreement with the observed reduction in area of the metabolically most active bone (trabecular bone) determined from pQCT data.

CONCLUDING REMARKS

♦ As inhabitants of Lake Apopka, Florida, USA, the female alligators showed increases in density and mineral content compared to female alligators from a control lake, while no changes in bone length or bone strength were seen. An increase in density suggests estrogenic effects on bone tissue homeostasis, favouring bone formation. The DDT-metabolite *p,p'*-DDE has in studies mainly shown anti-androgenic properties, but in the female alligators it was shown to produce effects indicating estrogenic exposure. Exposure to dicofol, which is considered to be mainly estrogenic, could also have increased the density. This study shows that the endocrine-regulated bone tissue might have suffered from exposure to persistent organic pollutants. However, the mechanisms are not known.

♦ Environmental doses of the non dioxin-like and estrogenic PCB 153 elicited developmental effects on bone tissue in female goat offspring following *in utero* and lactational exposure. No effects were seen following exposure to the dioxin-like anti-estogenic PCB 126. The exposure to PCB 153 resulted in thinner bones (decreased cross-sectional area) with increased density, while no changes were detected in bone length or bone strength. The PCB 153-induced bone alterations suggest estrogenic actions on bone tissue, hence in this study the putative estrogen PCB 153 can in fact be considered to be estrogen-like.

♦ Young adult male rats exposed to a single dose of TCDD exhibited smaller trabecular bone area, an increase of the bone resorption marker CTX, a decrease of the bone formation marker ALP, and altered chemical composition of the bone. The results of the analyses of bone markers in serum were in accordance with the effects observed using pQCT. In addition, the results of the chemical analyses revealed a chemical composition which is normally seen in more mature bones. No effects were seen in bone length or bone strength.

♦ We observed that male and female herring gulls residing in the Great Lakes area in USA/Canada, have thinner (decreased cross-sectional area) and shorter bones, which are more elastic, and possibly also less mineralised, compared to gulls from a freshwater reference. The effects seen could have been induced by the mix of environmental contaminants in the Great Lakes area. However, it is difficult to draw any certain conclusion as to which specific toxicant(s) induced the observed alterations in bone tissue. Persistent organic pollutants as well as metals are present in the Great Lakes.

♦ Persistent organic pollutants, including dicofol, DDT, PCBs and dioxins might be responsible for the observed alterations in bone tissue in species as different as alligator, goat, rat and herring gull. The suggested main mechanisms of action involves endocrine disruption at different levels; 1) agonistic binding of ligand to estrogen receptors (ER) (PCB 153, dicofol, and *p,p'*-DDE), 2) AhR-mediated up-regulation of estrogen-metabolising enzymes, *e.g.* CYP P450s, decreasing the level of estrogen (dioxins/TCDD/dioxin-like PCBs), 3) cross-talk between the AhR/ER pathways (dioxins/TCDD/PCBs), 4) disturbed mineralisation due to interference of the osteoblastic activity.

FINAL COMMENTS

The bone effects seen in the wild and experimental animals, what does it tell us, and how is this related to humans? The effects of pollutants on bone are of great interest since we are constantly exposed to chemicals in our environment; in air, food, water, and soil. Some of us are also exposed accidentally or occupationally. However, the area of bone toxicology is still quite unexplored, and there still remains much to investigate. Due to their lipophilicity and persistence, some pollutants have the potential to bioaccumulate in fat tissue in our bodies. This puts the sensitive newborns at risk, since studies have shown that breast-feeding mothers may transfer their accumulated contaminants to the child through the fatty breast milk.

So what does the bone effects seen in bone tissue of alligator, goat offspring, rat and herring gull tell us? Well, it suggests that environmental contaminants, such as POPs and metals, may have the potential to disrupt bone tissue homeostasis. The mechanisms are still unknown, but could involve direct binding of the pollutant to the estrogen receptor, induction of estrogen-metabolising enzymes, crosstalk between the AhR/ER signalling pathways or a disruption of the mineralisation process.

How is this related to humans? Since alterations can be seen in the bone tissue of these four animal species, it is likely that also bone tissue of humans is at risk of being affected by persistent contaminants. However, the alligators and the herring gulls reside in polluted waters and are constantly exposed to the contaminants, from *in ovo* until adults, and the rats received a quite high dose of TCDD. The endocrine-disrupting properties of pollutants are of concern regarding both the effects on reproductive tissue as well as the effects on bone. Alterations in the bone remodelling process might result in conditions such as osteoporosis and osteomalacia, significantly increasing the risk of fracture and hospitalisation, which leads to pain for the individual and increased medical costs for the society. It is in our interest to reduce the production and use of environmental pollutants, in order to protect us, the wild animals and our environment.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vad har avhandlingen visat?
I denna avhandling har flera miljögifter, alla med olika egenskaper att påverka hormonbalansen i kroppen, undersökts i fyra olika djurarter och alla djur uppvisar förändringar i benvävnaden. Dessa förändringar skulle kunna bero på miljögifterna, men exakt hur de har påverkat benvävnaden är ännu oklart. Det har gjorts väldigt få studier på miljögifters och kemikaliers påverkan på benvävnaden. Några teorier involverar direkt påverkan på bencellerna, eller att gifterna sänker koncentrationen av hormoner, till exempel östrogen. En lägre östrogennivå ger upphov till lägre bentäthet (och eventuell benskörhet), vilket man bland annat har sett i kvinnor som passerat klimakteriet, där en naturlig sänkning av östrogennivån sker.

Benvävnad
Benskörhet (osteoporos) är en sjukdom som har blivit allt vanligare sedan andra världskriget, framför allt i industrialiserade länder, där Skandinavien ligger i topp vad gäller antalet frakturer. Vad denna ökning beror på vet man inte. En frisk benvävnad är beroende av hormoner såsom östrogen och testosteron för att upprätthålla en normal balans och funktion. En rubbning av denna balans orsakar en ökning eller minskning av bentätheten.

Miljögifter
En del miljögifter har visat sig vara hormonstörande och kan därigenom påverka organ i kroppen som styrs av hormoner, såsom reproduktionsorganen och benvävnaden. Miljögifter som dioxiner, DDT och PCB har kemiska egenskaper som gör att de lätt kan lagras i fettvävnad i människor och djur. Vissa gifter är svåra att bryta ned i naturen och stannar därmed kvar länge i mark och i vattendrag. Dessa egenskaper gör att långlivade miljögifter ansamlas i djur ju högre upp i näringskedjan de befinner sig; plankton äts av fisk, fisk äts av säl och säl äts av isbjörn. På så sätt kan halter av miljögifter i isbjörn vara mycket höga, vilket bland annat kan få negativa effekter på fortplantning och benvävnad.

Vad var syftet med avhandlingen?
Syftet med den här avhandlingen har varit att undersöka ett eventuellt samband mellan dioxiner, DDT, och PCB och påverkan på benvävnad i fyra olika djurarter; alligator, get, råtta och gråtrut. De ben som har undersökts är bland annat lårben och skenben.

Djuren som ingår i avhandlingen
Alligatorerna har levt i en sjö i Florida som är starkt förorenad med bekämpningsmedlen dicofol, DDT och deras metaboliter. På 1980-talet skedde en olycka i en fabrik som tillverkade bekämpningsmedel, vilket förorenade sjön på grund av oavsiktligt utsläpp. Tidigare studier på alligatorerna har visat en rad förändringar, till exempel minskad testosteronhalt, abnormala reproduktionsorgan, och minskad kläckbarhet hos äggen. Gråtrutarna som undersökts har levt i sjöområdet Great Lakes i Nordamerika, vilket är omgivet av stora städer och industrier. På grund av detta så är sjöarna starkt förorenade med olika miljögifter, såsom dioxiner, PCB, DDT och metaller. De effekter man sett på gråtrutarna i tidigare studier är bland annat skador på lever och njure. De djur som har

studerats experimentellt, det vill säga de är inte viltlevande utan har exponerats för miljögifter endast i laboratoriet, är get och råtta. I getstudien undersöktes hur benvävnaden påverkas av två lika typer av PCB:er, en som är östrogenlik (PCB 153) och en som inte är östrogenlik (PCB 126). Av intresse i denna studie var att se hur benen har påverkats i avkomman efter att de har exponerats under fosterstadiet och genom modersmjölken. Råttornas benvävnad undersöktes redan fem dagar efter att de hade fått en hög dos av dioxin, vilket är att betrakta som en mycket kort tidsperiod.

Vilka metoder har används?
De metoder som har använts för att undersöka benen är:
1) röntgen, för att mäta densitet och area
2) biomekanik, där benen knäcks för att se hur starka de är
3) mineralanalys, där mängden av olika mineraler mäts
4) blodanalys, för att undersöka koncentrationen av två specifika proteiner, vilka kan ge en fingervisning om ifall ben bildas eller bryts ned

Vad blev resultatet av undersökningarna?
Resultaten visade att benvävnaden var påverkade i alla djuren. Alligatorerna hade högre bentäthet och ett högre mineralinnehåll än normalt, vilket tyder på östrogena effekter av DDT och dicofol. Östrogena effekter av PCB 153 påvisades i getterna, där högre bentäthet och mindre märghåla (hålrummet inne i skelettet som löper längs hela benet, där benmärgen återfinns) observerades. Efter exponering för dioxin (TCDD) kunde en lägre bentäthet observeras i råttorna och även en förändring i benets mineralinnehåll, en förändring som liknar den som ofta ses i mer mogna ben. Dessa effekter är anti-östrogena, det vill säga TCDD förhindrar östrogenets funktion på benvävnaden, vilket normalt är att stimulera bildning av benvävnad. Den biomekaniska analysen på gråtrutsbenen visar att mineraliseringen kan ha påverkats negativt av exponering för miljögifter. Lårbenen var även kortare och hade en mindre tvärsnittsarea jämfört med kontrollerna. Då Great Lakes är förorenat med ett stort antal kemikalier är det svårt att veta vilken som har mest påverkan på benvävnaden.

Vad betyder detta för människor?
De förändringar som har setts i benvävnaden i dessa studier gäller djur, men miljögifter kan potentiellt även orsaka förändringar i benvävnaden hos människor, speciellt då vi befinner oss högt i den så kallade näringskedjan. Vi får i oss miljögifter främst genom födan och speciellt höga nivåer återfinns i feta produkter, som fisk, kött, ägg och mjölkprodukter. Det återstår dock mycket forskning för att säkerställa om och hur hormonstörande miljögifter orsakar benpåverkan.

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