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Bone tissue alterations in ewes and their foetuses due to sewage sludge exposure

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CONTENTS

PREFACE

This report is part of a project which leads to a Master of Science in Ecotoxicology at Uppsala University, Sweden. The project took place at the Department of Environmental Toxicology, Uppsala University and the Department of Orthopaedics at Uppsala University Hospital and is collaboration with the Institute of Environmental Medicine at Karolinska Institutet, Solna, Sweden.

Thanks to my supervisor, Associate Professor Monica Lind at the Institute of Environmental Medicine at Karolinska Institutet for all your wisdom and inspiration through the entire project. Thanks also to Associate Professor Jan Örberg at the Department of Environmental Toxicology who contributed many interesting reflections and advice. I'm grateful, also, for all the material and help provided by Dr Stewart Rhind and his colleagues at the Macaulay Institute in Scotland. Despite a troublesome start with the transportation of the material, all worked out well. Thank you, Professor Sune Larsson at Uppsala University Hospital, for all the help with the bones. For all the cooperation and interesting discussions, I finally would like to thank Dan Arvidsson, my working partner during this project.

SUMMARY

Sewage sludge is used as a fertilizer on pasture due to its high content of organic matter, nitrogen, phosphorus and other nutrients. However, sludge also contains environmental contaminants which can be endocrine disruptive. To determine whether or not bone tissue was affected by these substances, pregnant ewes were allowed to graze on pasture fertilized twice annually with thermally dried pellets of sewage sludge (2.25 tonnes dry matter/ha). Control ewes were grazing on similar pasture treated twice annually with inorganic fertilizer containing the same amount of applied nitrogen (225 kg/ha/year) as was applied to the treated pasture. The ewes grazed on pasture fertilized with sewage sludge from the age of 18 months until slaughter, at 110 d gestation, when they were 4-6 years old. The femur bones from the ewes and foetuses were analyzed with pQCT (peripheral Quantitative Computed Tomography and three-point bending test. The areas of interest in the bones from ewes were the metaphyseal part, analyzed with pQCT at 3.6 % of the total length of the bone from the reference point located at the trochlea ossis femori in the distal part of the femur, and the diaphyseal part, located at 40 % and 50 % of the total length of the bone in the distal part of the femur. The foetal bones's metaphyseal measure point was at 20 % of the total length of the bone, and the diaphyseal measure point at 50 %. The three-point bending test was conducted on all femurs at the mid-diaphyseal measure point (50 %).

Results from the pQCT analysis showed alterations in the trabecular bone in the metaphyseal part of the femur from ewes exposed to sewage sludge. Compared to the control group, the trabecular bone mineral content and density, as well as total and trabecular cross sectional area, was significantly lower in the treated group which indicates an anti-estrogenic effect. Biomechanical bending showed that increased force was required to break the femurs of treated ewes, compared with controls. The load at failure and energy to failure were significantly increased ($p<0.05$) in the treated ewes but this may reflect the presence of heavier individuals within the group which causes a natural development of stronger bones.

The female foetuses were affected at the mid-diaphyseal part of the bone where endocortical circumference and marrow cavity were significantly lower in the treated individuals than in the controls. In exposed foetal males the total bone mineral content was higher in the mid-diaphyseal part than in controls. No adverse effect was seen in the biomechanical bending of the treated foetuses' bones compared to controls.

In conclusion, ewes grazing on sewage sludge fertilized pasture show alterations in their bone tissue compared to controls in the metaphyseal part of the femur.

1 INTRODUCTION

1.1 BONE

Bone is a living tissue and is constantly renewed, in a process called remodelling, which occurs throughout life. The organic matrix of the bone comprises of 95 % collagen I and 5 % proteoglycans and several types of non-collagenous proteins with embedded hydroxyapatite crystals which give strength (Marks and Odgren 2002). During the first 20 years in a normal human life, the skeleton undergoes modelling. This period is characterized by greater rates of renewal of bone than of loss. The bone grows in length, width and cross section and by the age of 20-30 years the human peak bone density is reached and now the formation rate is equal to the resorption rate. In later years, bone density decreases as the resorption activity exceeds the renewal (Ljunggren 1998). Because bone is a mineralized tissue, it can withstand external forces such as bending, pressure or torsion which makes it suited for protection of inner organs. It also acts as a basin reservoir for minerals, such as calcium and phosphate (Eroschenko 1996).

Bone consists of two different types of bone; cortical or trabecular. Compact cortical bone surrounds the inner porous trabecular bone which can be seen in figure 1. The function of cortical bone is to give structure and protection, like trabecular bone, but it also has metabolic functions (Marks and Odgren 2002). Bone receives its blood and nourishment from tiny blood vessels (Ljunggren 1998). The end part of a long bone, such as a femur, is called epiphysis. The shaft flanked by the epiphysis is called diaphysis and between the diaphysis and the epiphysis is the metaphysis.

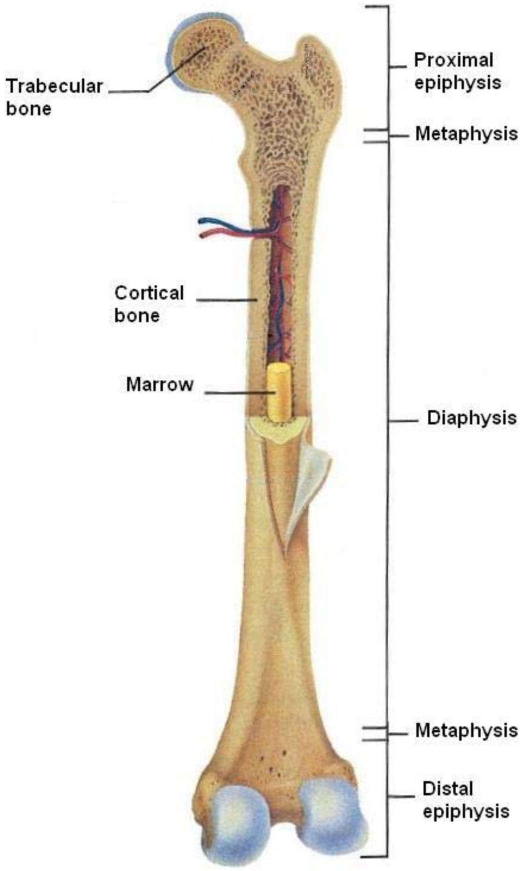

Figure 1. Schematic picture over a femur showing basic structure. Modified from Hole and Koos 1994.

1.1.1 Bone cells

Four different cell types: osteoclasts, osteoblasts, osteocytes and lining cells are important for bone tissue homeostasis.

Osteoclasts are created when stem cells from the bone marrow, called preosteoclasts, form multi-nucleated cells (Ljunggren 1998). When the osteoclast binds to the bone surface, it acts in a macrophage-like manner and breaks down collagen, minerals and bone cells by creating a "pH-dip" in the micro environment which arises between the bone surface and the cell's ruffled membrane (Teitelbaum 2000). After the osteoclast has broken down the bone tissue, it leaves a cavity which is later refilled by a cell type that produces bone instead, the osteoblast.

The osteoblasts synthesize bone tissue and fill in the cavities with osteiod (uncalcified bone). The osteiod then goes through mineralization and becomes solid bone (Eroschenko 1996). Osteoblasts are differentiated in two different ways, either through intramembranous- or endochondral ossification. In intramembranous differentiation the mesenchymal cells transform directly to osteoblasts. This occurs in the formation of mandibles, cranium and clavicle, for example (Karaplis 2002). When cartilage is replaced with bone tissue, the process is called endochondral ossification. Here, the osteoblasts are proliferated from precursor cells and bone construction occurs from within with the mineralized matrix as base (Karaplis 2002). Blood vessels invade the cartilage whilst the chondrocytes die and mineralization of the matrix takes place (Ducy *et al.* 2000).

Osteocytes are mature osteoblasts which are embedded in small cavities in the bone tissue. The cells have connections between each other and with cells on the bone surface through channels called canaliculi. Osteocytes are presumed to be the bone cells which respond to mechanical stimuli (Klein-Nulend *et al*. 1995).

Lining cells (Nakamura 2007) are inactive osteoblasts which are thought to have significance for the mineral homeostasis (Miller and Jee 1987). The cells pull away from the bone surface and enable the osteoclast to attach and start the resorption of bone tissue (Ljunggren 1998).

1.1.2 Bone and estrogen

When bones grow and mature, the cells are dependent on many different hormones, including estrogen and testosterone. Both osteoblasts and osteoclast have estrogen receptors (Eriksen *et al.* 1988, Oursler *et al.* 1991), and osteoblasts also have androgen receptors (Colvard *et al.* 1989). Estrogen works by inhibiting interleukin-6 which further stimulates osteoclast production (Jilka *et al.* 1992). Osteoblast differentiation increases under estrogenic influence (Qu *et al.* 1998). Estrogen is responsible for the fusion of the epiphyseal growth plate that completes the skeleton’s growth, and the expression of both estrogen- and androgen receptors have been detected in the human growth plate throughout pubertal development (Nilsson *et al.* 2003).

Estrogen deficit in post menopausal women can result in lower bone density (Kröger *et al.* 1993). The reduction in serum estrogen creates an imbalance in the interaction between osteoclasts and osteoblasts. To prevent the exaggerated degradation of bone mass in women, sex hormone therapy and the drug bisphosphonates (see osteoporosis below) can be used.

1.1.3 Osteoporosis

Osteoporosis is a disease in which bones are weakened, leading to an increasing risk of fractures. Every year in Sweden, about 70 000 fractures occur which are linked to osteoporosis (The Swedish Council on Technology Assessment in Health Care 2003). The disease occurs in one woman in three within the age of 70-79 years. In the US, 68 % of all osteoporosis patients are women (National Institutes of Health- Osteoporosis and Related Bone Diseases 2006).

Women who have reached menopause have an increased risk of osteoporosis. Their bodies cease to produce estrogen, which is a vital hormone in the bone remodelling process (Kröger *et al.* 1994). Even men with low serum estradiol levels can develop osteoporosis (Carlsen *et al.* 2001). Studies show that women who undergoes treatment with sex hormone replacement therapy exhibit reduced bone loss, and a lower risk of fractures (Rozenberg *et al.* 1995). However, the value of this method is questioned because of the increased risk of developing breast cancer and heart decease (The Swedish Council on Technology Assessment in Health Care 2003). A common way to prevent osteoporosis is by taking the drug bisphosphonate which inhibits osteoclasts from breaking down the bone (Watts 2003).

Physical inactivity, high age, heredity, corticosteroid intake, alcohol consumption, smoking and a low body weight are all risk factors for developing osteoporosis (Hemenway *et al.* 1988, Omland *et al.* 2000, Haugeberg *et al.* 2002, National Institutes of Health- Osteoporosis and Related Bone Diseases 2005, Lorentzon and Ohlsson 2006).

1.2 TOXIC ENVIRONMENTAL COMPOUNDS

1.2.1 Endocrine disrupting compounds (EDCs)

EDCs can affect the various organs and functions in the body regulated by hormones. Their actions can perturb behaviour, reproduction, growth etc (Bernes 1998). Some environmental contaminants are estrogenic or anti-estrogenic, while others are androgenic or anti-androgenic (Walker *et al.* 2006). They can act in different ways :

- Mimic natural hormones and bind to receptors which causes over stimulation
- Block the endogenous hormones from binding to receptors
- Interfere with the production of hormones or their receptors

EDCs can be of natural origin, such as isoflavones in alfalfa, or man-made chemicals, like some PCBs (Thigpen *et al.* 2004). Humans are exposed to EDCs through their diet and through use of plastics, pesticides and flame retardant compounds in clothes. Potential additional routes through which free ranging animals may be affected include rainwater, air and the soil (Rhind 2005).

1.2.2 Sewage sludge

Sewage sludge contains a large amount of organic material as well as nutrients and is presently used as fertilizer within agriculture (Tenenbaum 1997). Thus, important nutrients are recycled and the soil is enriched for the crops. Unfortunately, the sludge not only contains desirable substances, but also organic pollutants such as PCB, PAH, dioxins, phthalates which may have endocrine disrupting capabilities. In addition, heavy metals, pathogens and other toxic compounds can be added to the soil and may be transported to the ground water or be assimilated in plants or crops which are consumed by farm animals or by humans (Harms 1996).

Erhard and Rhind (2004) found that exposure to sewage sludge affected exploratory behaviour in sheep. Animals which had been grazing on pasture fertilised with sewage sludge spent less time exploring new surroundings than controls that were exposed to inorganic conventional fertilizer. The ewes in the control group spent a greater amount of time exploring than the rams. This difference was absent in the treated group, suggesting hormonal disturbance which resulted in a demasculinisation of the rams in the treatment group and made them as active as the ewes.

Other studies have shown that male sheep foetus, whose mothers have been grazing on pasture fertilized with sewage sludge, have a lower number of functional somatic cells in the testis compared to control animals (Paul *et al.* 2005).

1.2.2.1 *PCB and dioxins*

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a highly toxic compound which binds to the Ah-receptor and causes an upregulation of several proteins such as the enzyme cytochrome P4501A1. Dioxin can give rise to cancer, chloracne and affect the immune system. Polychlorinated biphenyls (PCBs) were manufactured during the 1920 for use within industries as additives in colour and glue and as softeners in plastics (Bernes 1998). The substance was banned from the Swedish market in 1978 when several findings of its toxicity became known. There are 209 congeners of PCB were the chlorine atoms are localized in different positions. The more chlorines that are localized in ortho-position, the more stable the molecule becomes. Those congeners which lack chlorines in ortho-position can be very toxic because of their co-planar dioxin-like structure. The chemical binds to the Ah-receptor which can give rise to dioxin-like effects in the organism (Safe *et al.* 1985).

Both TCDD and dioxin-like PCBs are anti-estrogenic and can affect the reproductive functions (Bernes 1998, Buchanan *et al.* 2000). Guiding value for the contents of PCB which can be spread on agricultural soil in Sweden is 0.4 mg/kg dry matter (Swedish Environmental Protection Agency 1995). The value was the results of a voluntary agreement between the Swedish Environmental Protection Agency, the Federation of Swedish Farmers and the Swedish Water & Wastewater Association.

1.2.2.2 *Alkylphenols*

Nonylphenols are used as an active substance in laundry detergents and in cleaning- and degreasing products in industries. Alkylphenols are endocrine disrupting in an estrogenic way which means they can impair reproductive functions (White *et al.* 1994). In Sweden the contents of nonylphenols in sewage sludge are declining, as shown through sampling from several sewage-treatment plants in Stockholm between 1981 and 2003. The guiding value (50 mg/kg dry matter) has not been breached since the year of 2000 (Thuresson and Haapaniemi 2005). Sewage sludge used in experimental studies in Scotland contained nonylphenols exceeding this guiding value (209±21 mg/kg dry matter) (Rhind *et al.* 2007). The risk of bioaccumulation of alkylphenol is although considered small because of a probable rapid biodegradation in the environment (Rhind *et al.* 2002).

1.2.2.3 *Phthalates*

Phthalates are used mainly in plastic products as softeners. Rhind *et al.* (2002 and 2005) stated that the risk of exposure to this endocrine disrupting chemical via fertilization with sewage sludge is slightly elevated for farm animals. Di-2-ethylhexyl phthalate (DEHP) seems to cause estrogenic effects in vitro and anti-androgenic effects in vivo (Blom *et al.* 1998). Disturbances in the reproduction of rats have been observed as a result of anti-androgenic action (Grey *et al.* 2000, Stroheker *et al.* 2004). Comparative experiments made on long-tailed macaque *(Macaca fascicularis)* show that the monkeys are less sensitive to DEHP than rats

(Pugh *et al.* 2000). The question whether the rat is a representative species for the evaluation of human health has been asked. The phthalate content of sewage sludge has declined annually in Europe, but investigations show that samples from Norwegian, Swedish and Danish sewage-treatment plants still exceeds the Danish guiding value of 60 mg/kg dry matter (Langenkamp *et al.* 2001, de Jonge *et al.* 2002).

1.2.2.4 *PAH*

Polycyclic aromatic hydrocarbons (PAH) form when organic material is not burned completely, and is spread effectively in the air. It also comes off as particles when rubber tires containing high aromatic-oils are worn down. The source of PAH in digested sludge mainly comes from automobile traffic and then transportation with particles entering the surface water (KemI 2003). Metabolites from benzo[a]pyrene (BaP) are both carcinogenic and can cause sterility in mice (Bernes 1998). Some hydroxylated PAHs interact with ERα and ERβ in vitro but it remains uncertain whether they can act as an estrogenic compound in vivo since they don't affect the uterine weight in rats (Fertuck *et al.* 2001).

1.2.2.5 *DDT*

The use of the insecticide DDT (1,1,1-trichloro-2,2-bis (4-chlorophenyl)ethane) in Swedish agriculture was banned in the early seventies due to severe effects on non-target organisms. Traces of the biocide have been found in sludge from several sewage-treatment plants in Sweden in 2005 which could be due to, as the author says, "old sins" (Kylin 2005). Long range transport could be one possible explanation to this finding. The metabolite DDE is a slowly degradable product that bioaccumulates in lipophilic tissue (Bernes 1998). Birds of pray which have had high contents of *p,p'*-DDE in their body, have produced eggs with such a thin eggshell, that they were crushed during brooding (Ratcliffe 1967). This most probably caused a rapid decline in the population. A hormonal disturbance, caused by the *p,p'*-DDE, affects the transport of calcium in the eggshell gland in female birds (Lundholm 1997). *p, p'*-DDT is an estrogenic compound and *p, p'*-DDE has been shown to be anti-androgenic in rats (Bustos *et al.* 1988, Kelce *et al.* 1997).

1.2.2.6 *PBDE*

Polybrominated diphenyl ether (PBDE) is part of the brominated flame retardant group and mainly used as flame retardants mostly in electrical devices such as computers and in furniture. Concentrations of decaBDE 209 (banned from Swedish market) ranging between 1.0 and 275 µg/kg dry matter has been found in Swedish waste water plants (Hellström 2000, de Wit *et al.* 2002). The concentration of PBDE in breast milk has increased exponentially between 1972 and 1992 (Norén and Meironyté 2000). PBDE 153 results in neurotoxic effects and impaird learning and memory in neonatal mice which were given the substance orally (Viberg *et al.* 2003). Developing male rats that were given low doses of PBDE 47 had a disturbance in their thyroid homeostasis (Andrade *et al.* 2004). Further studies is needed to see if this can cause an alteration in the differentiation and growth of the testis and later on cause a decline in the sperm count.

1.3 EXPERIMENTAL STUDIES REGARDING BONE TOXICITY

1.3.1 Rat

Rats which have been exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) via subcutaneous injection showed changes in the bone growth, modelling, as well as a reduction in the resistance when put under mechanical stress (Jämsä *et al.* 2001). This study also demonstrated that the effects were dose related. Studies regarding the underlying mechanism of TCDD on bone toxicity are very few. One in vitro study has demonstrated TCDD as a down regulator of the transcription of adhesion protein osteopontin in osteoblasts from rat, which is considered to have a vital role in the bone formation (Wejheden *et al.* 2006).

Exposure to the anti-estrogenic and dioxin-like compound 3,3’,4,4’,5-Pentachlorobiphenyl (PCB 126) resulted in an increased osteoid surface, cortical thickness and organic material in the tibia in sham-operated female rats compared to controls (Lind *et al.* 1999). When ovariectomized (no endogenous production of estrogen) rats were given 17 β-estradiol (E2)-supplement together with PCB 126, the trabecular bone volume increased. Intact (intact ovaries producing estrogen) females which were given PCB 126 showed a decrease in trabecular bone volume instead. PCB 126 therefore seems to induce negative effects in trabecular bone in an estrogenic environment (Lind *et al.* 2004a).

Ovariectomized (ovx) rats given a mixture of E_2-supplimentation + BaP/7,12-dimethylbenz(a)anthracene (DMBA), showed a significant decrease in bone mineral density in a lumbar vertebra compared to rats which were not exposed to the chemicals or individuals which had been ovariectomized (Lee *et al*. 2002). A three-point bending test was performed on the femur and showed a decrease in failure force in ovariectomized rats exposed to the chemicals compared to intact, exposed or non exposed individuals as well as ovariectomized non exposed .

1.3.2 Goat

PCB 153 results in changes in the bone tissue in female goats which have been exposed to the substance prenatally and via lactation (Lundberg *et al.* 2006). An increased trabecular bone density in diaphyseal bone was expected because PCB 153 has a presumed estrogen effect. A reduction of the total cross-sectional area, bone marrow cavity and moment of resistance in the metaphyseal bone was also noted. PCB 126 was also investigated because of its suspected anti-estrogenic properties. However, no anti-estrogenic effect was found on the bone tissue of goat.

1.3.3 Frog

In experiments of short-term exposure on male Common frog (*Rana temporaria*) to the suspected anti- androgen chemical *p,p'*-DDE, a significantly lower cortical bone mineral density in the diaphyseal bone was detected compared with bone from control animals (Lundberg *et al.* 2007).

1.3.4 Mink

Mink (*Mustela vison*) were given food containing PCB 126 during 1 to 2 months and lesions on the upper- and lower jaw and more porous bone were found (Render *et al.* 2000). Gingivitis and misplaced and loose teeth were also effects from the exposure. In another study, offspring of female mink fed with dioxin like congeners of PCB showed a lower bone mineral density than control individuals (Lind *et al.* 1996).

1.3.5 Bird

Nestlings of the American kestrel (*Falco sparverius*) were exposed to BDE 100, 153 and 183. They had developed longer bones and were heavier than the controls. This may be a response to a change in the thyroid system (Fernie *et al.* 2006). The authors believe that the excessive weight may alter the bone structure and cause an increase in energetic cost for the birds.

1.4 FREE RANGING ANIMALS SHOWING BONE TISSUE ALTERATIONS AFTER EXPOSURE TO EDCs

1.4.1 American alligator

Lake Apopka in Florida, USA, has received a lot of agricultural runoff in form of nutrients and pesticides. There was also a pesticide spill of dicofol (similar structure to DDT) in the 1980 which further contaminated the lake. Juvenile and neonatal American alligators (*Alligator mississippiensis*) in Lake Apopka showed differences in hormone concentration in the plasma, as well as morphological deviation in their reproductive organs compared to alligators from control lakes (Guilette *et al.* 2000). Female alligators from lake Apopka have a significant increase in bone density in trabecular bone compared to individuals from a control lake. This is probably due to an additional estrogen effect in the animals which have led to a reduction in bone resorption (Lind *et al.* 2004b).

1.4.2 Polar bear

Persistent organic pollutants (POPs) bioaccumulate in the tissues of organisms (Bernes 1998). Compounds from industrialized countries are transported through the atmosphere to the arctic. The polar bear has a high percentage of fatty tissue and is therefore a particularly exposed specie. Bone mineral densities in skulls from young East Greenland polar bears (*Ursus maritimus*) were found to be higher during the period before large discharges of industrial environmental contaminants (1892-1932) compared to skulls after high discharges (1966-2002) (Sonne *et al.* 2004). The study showed that there was a correlation between the changes in bone mineral density and increased content of multiple contaminants in the environment. Polar bears from East Greenland and Svalbard collected between 1892 and 2002 had several pathological abnormalities (Sonne *et al.* 2007). Caries, misplaced teeth, gingivitis and exostoses were some of the findings, but only the prevalence of tooth wear and periodontitis could be statistically analyzed. There are, however, no apparent connections between the pathological abnormalities and an elevated content of POPs in the environment.

1.4.3 Seal

Depending on from which period bones from Baltic grey seals (*Halichoerus grypus*) were collected, a difference in the bone density can be seen. During the period when discharges of organochlorines (OC) were at the highest (1965-1985), the bones showed the lowest trabecular bone density compared to other periods with low contamination (1850-1955 and 1986-1997) (Lind *et al.* 2003). Another study on Baltic gray seal displayed an elevated frequency of skull lesions during distinct periods of exposure to pollutants. This is thought to be linked with contents of PCB and DDT which increased at the same time in the environment (Bergman *et al.* 1992).

The frequencies of jaw lesions among Harbour seals (*Phoca vitulina*) has increased since the 1900th century. In an analysis on skulls from Swedish and Danish museums, exostosis of alveolar bone was detected (Mortensen *et al.* 1992).

1.4.4 Beluga whale

A long term study of a small population of Beluga whales (*Delphinapterus leucas*) living in the St Lawrence estuary showed a high concentration of PCBs and DDT in the tissues (Béland *et al.* 1993). Females had a lower amount of PCB and DDT than males which can be the result of transfer to the calves through the placenta or lactation. Beluga whales may have the ability to metabolize dioxin-like compounds since dioxins were only found in low concentrations. Tooth loss and spinal deformations were noted in the population of Beluga whales. Due to the small size of the population it is not known if the skeletal disorder is a result of chemicals or genetics traits.

1.5 HUMAN BONE TISSUE ALTERATIONS DUE TO EDC EXPOSURE

There are few studies of the effects of EDCs on bone tissue in humans. The numbers of individuals in these studies, however, are very limited. A few catastrophic events in the last 50 years, involving these substances, have shown some effects. In Turkey between the years of 1955 and 1961, 3000 people were food poisoned after consumption of hexachlorobenzen contaminated wheat. 20-30 years later, some exposed individuals had orthopaedic abnormalities and 44 % of the follow up-patients that were exposed before puberty had a small stature (Cripps *et al.* 1984). Another misfortune regarding hazardous food occurred in Japan 1968 when children whose mothers have been exposed to rice oil contaminated with

PCB, developed spotty calcification of the skull-bones and unusually large fontanels (Miller 1985).

Swedish women living on the east coast who ate more than one meal per month containing fatty fish from the Baltic Sea had an increased risk of fracture than women from the same area which ate one meal or less per month of fatty fish (Wallin *et al.* 2004). Glynn *et al.* (2000) found a weak correlation between the contents of *p, p'*-DDE in serum and a lower bone density in men. Several other studies have tried to prove a connection between serum levels of POP and a reduction in bone mineral density, but only weak correlations were found. Although, these findings could be the result of other risk factors like smoking (Alveblom *et al.* 2002).

1.6 AIM

Sewage sludge contains several chemicals that are potentially endocrine disruptive and harmful to organisms. The aim of this study is to determine whether pregnant ewes which have been grazing on pasture fertilized with sewage sludge show any difference in their own and in their foetuses bone tissue, compared to control ewes and foetuses.

2 MATERIAL AND METHODS

2.1 ANIMAL EXPOSURE

The exposures were conducted at the Macaulay Institute research station at Hartwood, Scotland. From the age of 18 months, Texel ewes (*Ovis aries*) grazed either on sewage treated pasture (treated) or on pasture treated with conventional, inorganic fertilizer (control). Both treated and control ewes were pregnant during the last period of exposure.

Thermally dried sludge pellets (2.25 tonnes dry matter/ha) were applied twice annually to a 9 ha pasture. About 225 kg nitrogen/ha/year was applied to the pasture on these occasions (Erhard and Rhind 2004, Rhind *et al.* 2007). Directly after the application, the ewes were contained on previously-treated plots for 3 weeks, according to British law (Paul *et al.* 2005), before they were allowed to start grazing within the newly treated plot. The sludge was similar to that used in previous studies and contained measurable amounts of nonyl phenol (mean±SE; 209±21 mg/kg dry matter) and DEHP (mean ± SE; 108±63 mg/kg dry matter (Rhind *et al.* 2007). The contents of PCB (28, 52, 101, 118, 138, 153, 180) and PBDE (28, 47, 99, 100, 153, 154, 183) in sludge were recently analyzed according to an accredited procedure at Macaulay Analytical Laboratories (Rhind, personal communication). The highest concentrations of PCBs were 24.3 µg/kg dry matter and of PBDEs 417 µg/kg dry matter from three subsamples. However, the contents may differ with every batch of sludge and vary ten fold in soil samples just a few meters apart (Rhind *et al.* 2002). The control animals grazed on a 9 ha pasture which had been treated with inorganic fertilizer with approximately the same amount of nitrogen.

The ewes were slaughtered at 5 or 6 years of age and when at 110 days gestation, about 35 days before birth. 23 control and 22 treated ewes and 47 control and 51 treated foetuses were used.

2.2 PREPARATION OF BONES AND MEASUREMENTS

The femur bones used in this study came from ewes and their foetuses. The bones were dissected, cleaned and maintained in a freezer (-18 C˚). They were then thawed in a refrigerator (+8 C˚) for 24 hours before pQCT (peripheral Quantitative Computed Tomography)-analyses.

2.2.1 Bones from ewes

The length of the bone were measured with a slide calliper (accuracy 0.1 mm) from the patellar groove located at the distal part of the bone, to the groove between head and greater trochanter at the proximal end (for visual guidance see figure 2).

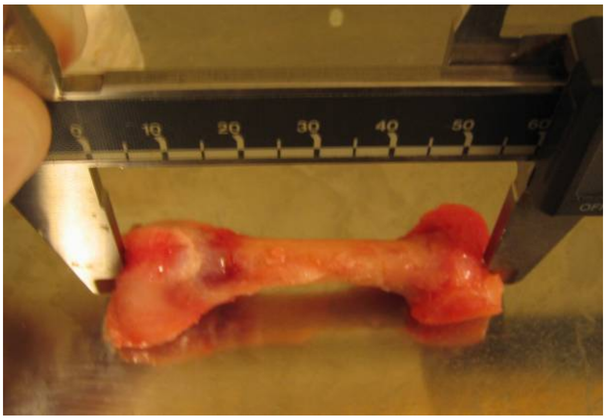

Figure 2. The sheep femur bones were measured with a slide calliper from the patellar groove at the distal part of the bone, to the area between head and greater trochanter at the proximal end.

The reference point was decided before the x-ray with a wood screw (size 2.5*12 mm) which was placed dorsal of the trochlea ossis femori in the metaphysis at the distal end of the femur, see figure 3.

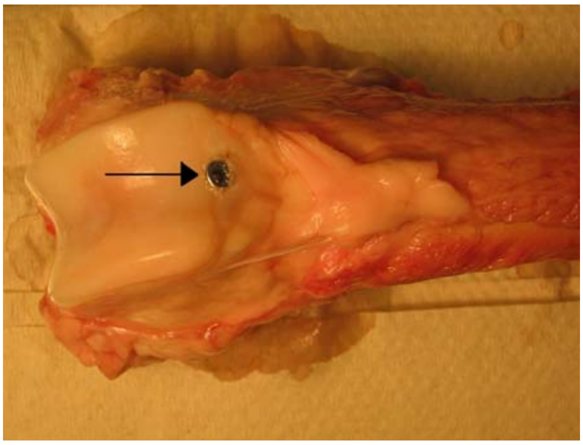

Figure 3. The reference point in the sheep femur was set before pQCT analysis by inserting a wood screw (size 2.5*12 mm) dorsal of the trochlea ossis femori in the metaphysis at the distal part of the bone.

The bones were covered with gauze bandage moistened with Ringer solution (pH 7.4, 0.3 g Tris, 9 g NaCl, 0.24 g $CaCl_2$ * H_2O, 0.4 g KCl and 2.05 ml 1 M HCl/ 1 l H_2O) and then packed in polyethene film to prevent dehydration. The diaphyseal area was sellotaped, partially to keep the polythene in place, and partially as an aid to be able to mark 50 % and 40 % of the total length of the bone.

2.2.2 Bones from foetus

Bones from the foetuses were measured with a digital calliper (accuracy 0.01 mm) in the same way as the ewes. When entering the values in the pQCT, the accuracy was however lowered to 0.1 mm because of the software settings. The bones were placed in a 50 ml test tube each and the tubes were filled with Ringer solution to prevent dehydration. The bone's midpoint was marked with a pencil directly on the bone surface after measurement with the digital calliper. The reference point and scan line was placed in the scan view of the pQCT system at the beginning of the clearly detectable growth plate in the distal part of the bone.

2.2.3 pQCT

pQCT (Stratec XCT 960 A with software version 5.21, Norland Stratec Medizintechnik, Pforzheim, Germany) was used to measure the bone mineral density and dimensions of the bones. The machine was calibrated once a week with a hydroxyapatite phantom with a known density (Gasser 2003). The measurements took place at Uppsala University Hospital, Uppsala, Sweden.

2.2.3.1 *Reproducibility*

To create a valid method, 10 repeated pQCT measurements were performed on a single bone which was repositioned between each measurement. The coefficient of variation in percent (CV = standard deviation/mean × 100) was calculated.

2.2.3.2 *Metaphyseal measurements of bones from ewes*

The numbers of bones used were 23 from the control group and 22 from the treated group. The criteria which were set when the trabecular measure points were to be determined were:

- High trabecular area
- Cortical bone was to surround trabecular bone and be clearly separated in density
- Low variation of the values of the measures within and between bones

To measure trabecular bone, the bones were placed horizontally in the pQCT machine. Using the scout view option of the instrument the scan line was placed at a distance equal to 3.6 % of the total bone length from the reference point towards the diaphysis. For the analysis the following settings were used: voxelsize 689 µm, peel mode 2, threshold 270 mg/cm^3, inner threshold 500 mg/cm^3 and contour mode 1. The inner threshold is the density limit value which separates cortical and trabecular bone. The outer contour of the bones is detected when using contour mode. The measures obtained from the analysis were total bone mineral content (Total BMC), volumetric total bone mineral density (Total BMD), trabecular bone mineral content (Trabecular BMC), volumetric trabecular bone mineral density (Trabecular BMD), total cross sectional area (Total CSA), trabecular cross sectional area (Trabecular CSA) and periosteal circumference.

2.2.3.3 *Metaphyseal measurements of foetus bone*

The numbers of bones used were 28 from the control group of females and 25 from the treated. Number of bones from the male controls were 18 and from the treated group 25. The measure point for foetal bone was set to 20 % of the total bone length from the reference point at the beginning of the growth plate in the distal part. For the analysis the following settings were used: voxelsize 295 µm, peelmode 2, threshold 270 mg/cm^3, inner threshold 500 mg/cm^3 and contour mode 1. Due to technical problems during the analysis two bones from males were excluded from this part of the study. The measures obtained from the analysis were total BMC), total BMD, trabecular BMC, trabecular BMD, total CSA, trabecular CSA and periosteal circumference.

2.2.3.4 *Diaphyseal measurements of bones from ewes and foetal bone*

The numbers of bones from ewes used were 23 from the control group and 22 from the treated group. The numbers of foetus bones used were 28 from the control group of females and 25 from the treated. Number of foetus bones from the male control and treated group differ from the metaphyseal measurements due to damaged bones and there were 19 controls and 26 treated. To examine the cortical bone, the measure point was set to 50 % of the total length of the bone and analysed with the light point in the pQCT system. Bones from ewes were also measured at 40 % of the total length of the bone from the reference point in the distal part of the bone. The measures obtained from the analysis were total BMC, total BMD, total CSA, cortical BMC, cortical BMD, cortical CSA, mean cortical thickness circular ring model, periosteal circumference, endocortical circumference, moment of resistance and marrow cavity.

2.2.4 Three-point bending test

The three-point bending test is a biomechanical method to measure the mechanical properties of the bone (Aspden 2003). A vertical load is forced upon the bone until it breaks. Both bones from ewes and foetuses were thawed for 24 hours before the experiment. The load test was performed at the mid-diaphyseal pQCT measure point (50 % of total bone length). The machine used for the ewe bones was a servohydraulic MTS 858 Mini Bionix (Avalon Technologies, MN, USA) with an axial capacity of 10 000 N. The displacement rate was

1mm/sec and the span length was set to 100 mm to support the ends of the bones. The foetal bones were tested in an electromechanical testing machine (Avalon technologies, MN, USA) with an axial capacity of approximately 1112 N (250 lbs). The displacement rate was 1mm/sec and the span length was 30 mm. Load (N) was plotted as a function of displacement (mm) in a curve (Figure 4). Energy to failure (area under curve, N×mm) and maximal stiffness (steepest slope, N/mm) were calculated. Due to technical problems during the analysis three foetal bones were excluded from this part of the study.

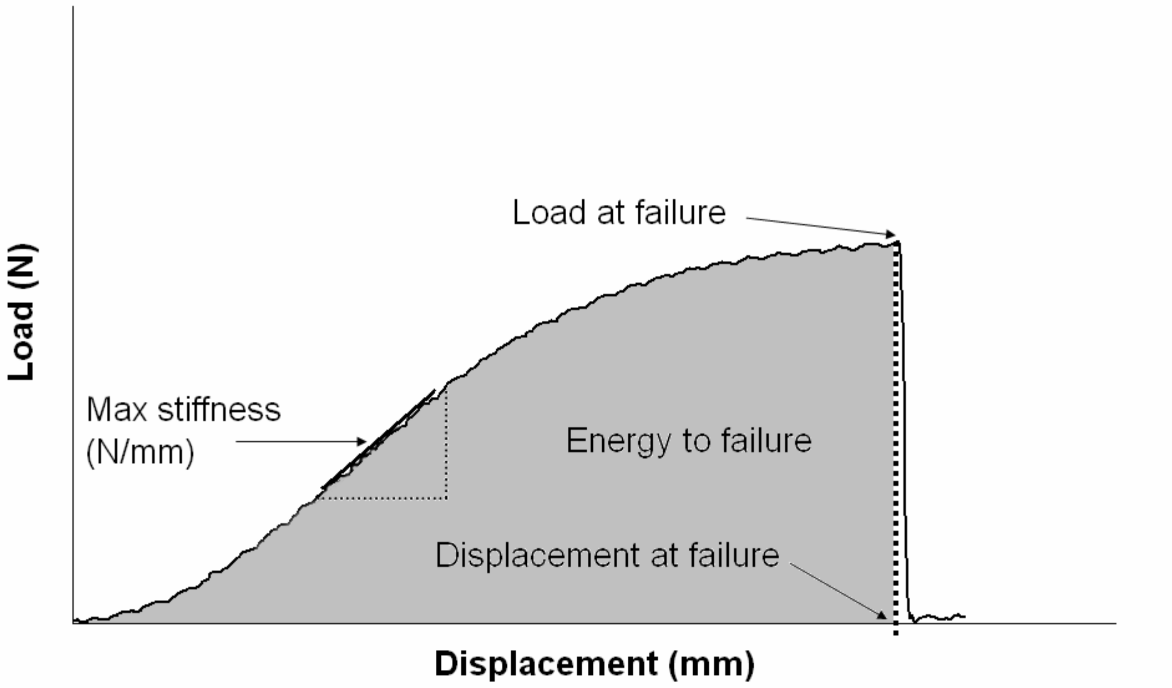

Figure 4. A three-point bending test results in a curve were load (N) is a function of displacement (mm). Maximal stiffness (N/mm) can be calculated from the slope in the steepest part of the curve. Energy to failure (N×mm) is the area under the curve.

2.3 STATISTICS

The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body weight. Differences were considered significant at $p<0.05$ (StatView, version 5.0; SAS Institute Inc., Cary, NC, USA).

3 RESULTS

3.1 pQCT MEASUREMENTS

The results from the pQCT measurements on ewe and foetal femurs at the metaphyseal and diaphyseal measure points are presented in the tables 6 to 12.

3.1.1 Reproducibility

3.1.1.1 *Bones from ewes*

The results from the pQCT reproducibility measurements are presented in table 1-3. 10 repeated pQCT measurements of the bone from one of the ewes gave a coefficient of variation (CV) that varied from 0.26 % to 2.21 % in the metaphyseal measure point (3.6 %, table 1). The CV for the mid-diaphyseal measure point (50 %) ranged between 0.20 % and 1.54 % (table 2). The CV for the diaphyseal measure point (40 % of the bone's total length) ranged between 0.16 % and 1.46 % (table 3). The bone was repositioned between the measurements.

Table 1. The reproducibility (mean±SD, CV) calculated from 10 repeated measurements of a single femur from a **ewe** analysed with peripheral quantitative computed tomography (pQCT). The bone was repositioned between the measurements. The metaphyseal measure point was located 3.6 % of the total length of the bone from the reference point towards the diaphysis. BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area.

Metaphyseal bone, 3,6 %	Mean	±	SD	CV (%)
Total BMC (mg/mm)	355.2	±	1.8	0.52
Total BMD (mg/cm³)	529.9	±	2.3	0.44
Trabecular BMC (mg/mm)	76.8	±	1.7	2.21
Trabecular BMD (mg/cm³)	216.5	±	2.9	1.35
Total CSA (mm²)	670.3	±	3.5	0.53
Trabecular CSA (mm²)	354.5	±	3.7	1.05
Periosteal circumference (mm)	91.8	±	0.2	0.26

Table 2. The reproducibility (mean±SD, CV) calculated from 10 repeated measurements of a single femur from a **ewe** analysed with peripheral quantitative computed tomography (pQCT). The bone was repositioned between the measurements. The mid-diaphyseal measure point was located at 50 % of the total length of the bone. BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area.

Diaphyseal bone, 50 %	Mean	±	SD	CV (%)
Total BMC (mg/mm)	301.3	±	1.3	0.42
Total BMD (mg/cm³)	602.8	±	2.8	0.47
Total CSA (mm²)	499.8	±	2.0	0.40
Cortical BMC (mg/mm)	268.2	±	0.9	0.35
Cortical BMD (mg/cm³)	1336.2	±	4.2	0.31
Cortical CSA (mm²)	200.7	±	1.0	0.51
Cortical thickness (circular ring model)(mm)	2.9	±	0.0	0.79
Periosteal circumference (mm)	79.3	±	0.2	0.20
Endocortical circumference (mm)	61.3	±	0.3	0.47
Moment of resistance (mm³)	1856.0	±	23.1	1.24
Marrow cavity (mm²)	299.1	±	2.8	0.95

Table 3. The reproducibility (mean±SD, CV) calculated from 10 repeated measurements of a single femur from a **ewe** analysed with peripheral quantitative computed tomography (pQCT). The bone was repositioned between the measurements. The diaphyseal measure point was located at 40 % of the total length of the bone from the reference point towards the diaphysis. BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area.

Diaphyseal bone, 40 %	Mean	±	SD	CV (%)
Total BMC (mg/mm)	314.0	±	0.9	0.28
Total BMD (mg/cm³)	618.1	±	2.2	0.36
Total CSA (mm²)	507.9	±	1.6	0.31
Cortical BMC (mg/mm)	278.6	±	1.9	0.69
Cortical BMD (mg/cm³)	1314.7	±	6.5	0.49
Cortical CSA (mm²)	212.0	±	2.4	1.14
Cortical thickness (circular ring model) (mm)	3.0	±	0.0	1.46
Periosteal circumference (mm)	79.9	±	0.1	0.16
Endocortical circumference (mm)	61.0	±	0.4	0.62
Moment of resistance (mm³)	1867.3	±	23.7	1.27
Marrow cavity (mm²)	296.0	±	3.7	1.23

3.1.1.2 *Bones from foetuses*

The results from the pQCT reproducibility measurements are presented in table 4-5. 10 repeated pQCT measurements of bone from a foetus gave a coefficient of variation that varied between 0.50 % and 6.76 % in the metaphyseal measure point (20% of the total length of the bone from growth plate) (table 4). The CV for the mid-diaphyseal measure point (50 %) ranged between 0.39 % and 2.94 % (table 5). The bone was repositioned between the measurements.

Table 4. . The reproducibility (mean±SD, CV) calculated from 10 repeated measurements of a single femur from a **foetus** analysed with peripheral quantitative computed tomography (pQCT). The bone was repositioned between the measurements. The metaphyseal measure point was located 20 % of the total length of the bone from the reference point towards the diaphysis. BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area.

Metaphyseal bone, 20 %	Mean	±	SD	CV (%)
Total BMC (mg/mm)	28.1	±	0.2	0.55
Total BMD (mg/cm³)	566.2	±	4.6	0.82
Trabecular BMC (mg/mm)	6.6	±	0.4	6.76
Trabecular BMD (mg/cm³)	419.3	±	4.7	1.12
Total CSA (mm²)	49.6	±	0.5	0.99
Trabecular CSA (mm²)	15.7	±	0.9	5.84
Periosteal circumference (mm)	25.0	±	0.1	0.50

Table 5. The reproducibility (mean±SD, CV) calculated from 10 repeated measurements of a single femur from a **foetus** analysed with peripheral quantitative computed tomography (pQCT). The bone was repositioned between the measurements. The mid-diaphyseal measure point was located at 50 % of the total length of the bone. BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area.

Diaphyseal bone, 50 %	Mean	±	SD	CV (%)
Total BMC (mg/mm)	26.2	±	0.1	0.40
Total BMD (mg/cm³)	673.0	±	4.4	0.66
Total CSA (mm²)	39.0	±	0.3	0.78
Cortical BMC (mg/mm)	17.7	±	0.4	2.09
Cortical BMD (mg/cm³)	900.9	±	6.7	0.74
Cortical CSA (mm²)	19.7	±	0.4	1.91
Cortical thickness (circular ring model) (mm)	1.0	±	0.0	2.57
Periosteal circumference (mm)	22.1	±	0.1	0.39
Endocortical circumference (mm)	15.6	±	0.2	1.47
Moment of resistance (mm³)	25.2	±	0.4	1.79
Marrow cavity (mm²)	19.3	±	0.6	2.94

3.1.2 Bone analysis

3.1.2.1 *Bones from ewes*

3.1.2.1.1 *Metaphyseal measurements*

The results from the metaphyseal pQCT measurement on bones from ewes are presented in table 6. The total bone mineral density was significantly higher in the treated group compared to the control group (8.9 %, $p<0.05$). Trabecular bone mineral content (-18.4 %, $p<0.05$) and trabecular cross sectional area (-11.1 %, $p<0.05$) were however significantly lower in the treated group compared to the control group. When adjusted for weight with the ANCOVA, the total bone mineral density was no longer different between the groups. Trabecular bone mineral content and density (-8.5 %, $p<0.05$) as well as total (-4.0 %, $p<0.05$) and trabecular cross sectional area were all significantly lower in the treated group compared to the control group.

Table 6. Results from pQCT analysis (mean±SD) performed on femur bones from two groups of **ewes**. The metaphyseal measure point was located 3.6 % of the total length of the bone from the reference point towards the diaphysis. The treated sheep had been grazing on pasture, fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Control sheep had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=23	Treated		n=22	p-value	
Metaphyseal bone, 3,6 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (kg)	81.7	±	8.8	88.6	±	5.0	*	na
Total BMC (mg/mm)	357.9	±	39.0	372.8	±	34.7	ns	ns
Total BMD (mg/cm³)	501.4	±	45.3	546.2	±	63.0	*	ns
Trabecular BMC (mg/mm)	78.6	±	11.4	64.1	±	19.3	*	*
Trabecular BMD (mg/cm³)	195.5	±	25.1	179.0	±	44.7	ns	*
Total CSA (mm²)	715.0	±	61.4	686.7	±	59.8	ns	*
Trabecular CSA (mm²)	403.9	±	48.9	359.0	±	63.6	*	*
Periosteal circumference (mm)	94.7	±	4.1	92.8	±	4.1	ns	ns

3.1.2.1.2 *Diaphyseal measurements*

The results from the diaphyseal pQCT measurements on bones from ewes are presented in table 7-8. When using ANOVA, the total (5.8 %, $p<0.05$) and cortical bone mineral content (6.3 %, $p<0.05$), cortical cross sectional area (5.8 %, $p<0.05$) and cortical thickness (6.0 %, $p<0.05$) were significantly higher in the mid-diaphyseal bone tissue of the treated group compared to the control group. The diaphyseal measurement point located at 40 % of the total length of the bone showed significant increase in several bone tissue measurements; the total bone mineral density (5.1 %, $p<0.05$), cortical bone mineral content (7.0 %, $p<0.05$), cortical cross sectional area (6.3 %, $p<0.05$) as well as cortical thickness (7.0 %, $p<0.05$). The treated ewes weighed more than the ones in the control group. The mid-diaphyseal measurements and at 40% of the total length of the bone gave no significant difference between measures in the control and treated groups when analysed with ANCOVA which adjusted for the individuals' weight.

Table 7. Results from pQCT analysis (mean±SD) performed on femur bones from two groups of **ewes**. The mid-diaphyseal measure point was located at 50 % of the total bone length. The treated sheep had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Control sheep had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=23	Treated		n=22	p-value	
Diaphyseal bone, 50 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (kg)	81.7	±	8.8	88.6	±	5.0	*	na
Total BMC (mg/mm)	320.5	±	28.3	339.1	±	32.6	*	ns
Total BMD (mg/cm³)	673.6	±	46.8	702.7	±	64.2	ns	ns
Total CSA (mm²)	477.9	±	53.6	484.4	±	46.3	ns	ns
Cortical BMC (mg/mm)	287.8	±	27.0	306.1	±	30.9	*	ns
Cortical BMD (mg/cm³)	1366.9	±	15.6	1373.2	±	17.8	ns	ns
Cortical CSA (mm²)	210.5	±	18.8	222.8	±	21.2	*	ns
Cortical thickness (circular ring model) (mm)	3.1	±	0.2	3.3	±	0.3	*	ns
Periosteal circumference (mm)	77.4	±	4.3	77.9	±	3.7	ns	ns
Endocortical circumference (mm)	57.8	±	4.4	57.2	±	4.2	ns	ns
Moment of resistance (mm³)	1928.4	±	304.8	2009.3	±	261.6	ns	ns
Marrow cavity (mm²)	267.4	±	40.7	261.6	±	38.5	ns	ns

Table 8. Results from pQCT analysis (mean±SD performed on femur bones from two groups of **ewes**. The diaphyseal measure point was set to 40 % of the total bone length of the bone from the reference point towards the diaphysis. The treated sheep had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Control sheep had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=23	Treated		n=22	p-value	
Diaphyseal bone, 40 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (kg)	81.7	±	8.8	88.6	±	5.0	*	na
Total BMC (mg/mm)	313.2	±	31.2	331.0	±	31.7	ns	ns
Total BMD (mg/cm³)	610.2	±	38.8	641.3	±	55.3	*	ns
Total CSA (mm²)	514.5	±	55.4	517.8	±	47.7	ns	ns
Cortical BMC (mg/mm)	277.8	±	28.7	297.1	±	29.7	*	ns
Cortical BMD (mg/cm³)	1326.0	±	22.4	1334.4	±	18.8	ns	ns
Cortical CSA (mm²)	209.4	±	20.5	222.5	±	20.7	*	ns
Cortical thickness (circular ring model) (mm)	2.9	±	0.2	3.2	±	0.3	*	ns
Periosteal circumference (mm)	80.3	±	4.2	80.6	±	3.7	ns	ns
Endocortical circumference (mm)	61.8	±	4.0	60.8	±	4.1	ns	ns
Moment of resistance (mm³)	1918.4	±	295.7	2014.4	±	274.8	ns	ns
Marrow cavity (mm²)	305.1	±	40.2	295.3	±	39.5	ns	ns

3.1.2.2 *Bones from foetuses*

3.1.2.2.1 ***Metaphyseal measurements***

The results from the metaphyseal pQCT measurements on foteal bones are presented in table 9-10. There were no significant differences in the variables at the metaphyseal measure point between the two groups of foetuses.

Table 9. Results from pQCT analysis (mean±SD) performed on femur bones from two groups of **foetal** female sheep (110 days old). The metaphyseal measure point was set to 20 % of the total bone length of the bone from the reference point towards the diaphysis. The treated mothers had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Pregnant control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time before mating. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=28	Treated		n=25	p-value	
Metaphyseal bone, 20 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (g)	1895.7		229.2	1827.7		270.8	ns	na
Total BMC (mg/mm)	31.2	±	5.2	30.0	±	5.1	ns	ns
Total BMD (mg/cm³)	526.9	±	80.2	528.0	±	74.0	ns	ns
Trabecular BMC (mg/mm)	7.3	±	3.1	7.7	±	3.9	ns	ns
Trabecular BMD (mg/cm³)	371.2	±	69.4	374.0	±	64.4	ns	ns
Total CSA (mm²)	59.3	±	6.3	57.3	±	9.2	ns	ns
Trabecular CSA (mm²)	21.2	±	10.4	22.1	±	12.1	ns	ns
Periosteal circumference (mm)	27.3	±	1.4	26.7	±	2.1	ns	ns

Table 10. Results from pQCT analysis (mean±SD) performed on femur bones from two groups of **foetal** male sheep (110 days old). The metaphyseal measure point was set to 20 % of the total bone length of the bone from the reference point towards the diaphysis. The treated mothers had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Pregnant control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time before mating. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at p<0.05. *= p<0.05, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=18	Treated		n=25	p-value	
Metaphysis, 20 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (g)	1890.2	±	342.7	1954.0	±	203.0	ns	na
Total BMC (mg/mm)	33.2	±	6.6	33.4	±	5.1	ns	ns
Total BMD (mg/cm³)	569.5	±	81.8	545.8	±	74.9	ns	ns
Trabecular BMC (mg/mm)	5.8	±	4.3	7.8	±	4.2	ns	ns
Trabecular BMD (mg/cm³)	395.6	±	65.7	389.8	±	56.9	ns	ns
Total CSA (mm²)	58.6	±	11.3	61.6	±	7.7	ns	ns
Trabecular CSA (mm²)	16.0	±	13.4	21.3	±	12.6	ns	ns
Periosteal circumference (mm)	27.0	±	2.6	27.8	±	1.8	ns	ns

3.1.2.2.2 *Diaphyseal measurements*

The results from the diaphyseal pQCT measurements on foteal bones are presented in table 11-12.The endocortical circumference (-7.0 %, p<0.05) and marrow cavity area (-13.8 %, p<0.05) in foetal mid-diaphyseal bone tissue of females were significantly lower in the treated group than in the control group. The treated male foetuses had a significantly higher total bone mineral content (2.8 %, p<0.05) than the control males when analyzed with ANCOVA.

Table 11. Results from pQCT analysis (mean±SD) performed on femur bones from two groups of **foetal** female sheep (110 days old). The mid-diaphyseal measure point was located at 50 % of the total bone length. The treated mothers had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Pregnant control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time before mating. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at p<0.05. *= p<0.05, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=28	Treated		n=25	p-value	
Diaphyseal bone, 50 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (g)	1895.7	±	229.2	1827.7	±	270.8	ns	na
Total BMC (mg/mm)	28.1	±	5.4	27.2	±	4.6	ns	ns
Total BMD (mg/cm³)	649.5	±	87.0	674.2	±	65.8	ns	ns
Total CSA (mm²)	43.2	±	5.2	40.4	±	5.7	ns	ns
Cortical BMC (mg/mm)	18.9	±	5.7	19.4	±	4.1	ns	ns
Cortical BMD (mg/cm³)	875.9	±	38.0	893.9	±	27.7	ns	ns
Cortical CSA (mm²)	21.4	±	5.9	21.7	±	4.3	ns	ns
Cortical thickness (circular ring model) (mm)	1.1	±	0.3	1.1	±	0.2	ns	ns
Periosteal circumference (mm)	23.2	±	1.4	22.5	±	1.6	ns	ns
Endocortical circumference (mm)	16.4	±	1.8	15.3	±	1.5	*	*
Moment of resistance (mm³)	27.7	±	7.8	27.5	±	6.1	ns	ns
Marrow cavity (mm²)	21.8	±	4.6	18.8	±	3.7	*	*

Table 12. Results from pQCT analysis (mean±SD) performed on femur bones from two groups of **foetal** male sheep (110 days old). The mid-diaphyseal measure point was set to 50 % of the total bone length. The treated mothers had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Pregnant control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time before mating. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at p<0.05. *= p<0.05, n= number of individuals, BMC= bone mineral content, BMD= bone mineral density, CSA= cross sectional area, ns= non significant, na= non applicable.

	Control		n=19	Treated		n=26	p-value	
Diaphyseal bone, 50 %	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (g)	1889.6	±	333.1	1940.9	±	209.8	ns	na
Total BMC (mg/mm)	29.7	±	5.8	30.6	±	4.5	ns	*
Total BMD (mg/cm³)	702.6	±	68.9	691.7	±	71.1	ns	ns
Total CSA (mm²)	42.4	±	8.0	44.4	±	6.2	ns	ns
Cortical BMC (mg/mm)	21.5	±	4.8	21.9	±	4.2	ns	ns
Cortical BMD (mg/cm³)	906.6	±	28.0	896.4	±	33.2	ns	ns
Cortical CSA (mm²)	23.7	±	5.1	24.4	±	4.2	ns	ns
Cortical thickness (circular ring model) (mm)	1.2	±	0.2	1.3	±	0.2	ns	ns
Periosteal circumference (mm)	23.0	±	2.2	23.6	±	1.7	ns	ns
Endocortical circumference (mm)	15.2	±	2.2	15.7	±	2.1	ns	ns
Moment of resistance (mm³)	149.6	±	54.3	164.6	±	39.0	ns	ns
Marrow cavity (mm²)	18.7	±	5.6	20.0	±	5.3	ns	ns

3.2 THREE-POINT BENDING TEST

The results from the analysis from the three-point bending test conducted on ewe and foetal femurs gave the results presented in the tables 13-15.

3.2.1 Bones from ewes

The results from the mid-diaphyseal three-point bending test on the bones are presented in table 13. Load at failure (16.1 %, p<0.05), energy at failure (27.1 %, p<0.05) and max stiffness (14.2 %, p<0.05) were all significantly higher in the treated group than in the control group with ANOVA. When adjusted for weight with ANCOVA, the load to failure and energy to failure were still significantly higher amongst the treated individuals.

Table 13. Results (mean±SD) from three point bending test performed on femur bones from two groups of **ewes** (control and treated). The bending took place at the mid-diaphyseal measure point (50 % of the total length of the bone) with a servohydraulic MTS 858 Mini Bionix (MTS inc Minneapolis, MN, USA) with an axial capacity of 10 000 N. The displacement rate was 1mm/sec and the span length was set to 100 mm. The treated sheep had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Control sheep had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= numbers of individuals, ns= non significant, na= non applicable.

	Control		n=23	Treated		n=22	p-value	
	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (kg)	81.7	±	8.8	88.6	±	5.0	*	na
Displacement at failure (mm)	4.6	±	1.2	4.5	±	0.9	ns	ns
Load at failure (N)	4958.0	±	556.0	5758.1	±	795.7	*	*
Energy at failure (N*mm)	9502.4	±	3505.3	12073.0	±	2526.1	*	*
Max stiffness (N/mm)	2238.6	±	469.7	2555.9	±	496.9	*	ns

3.2.2 Bones from foetuses

The results from the mid-diaphyseal three-point bending test on female and male foteal bones are presented in table 14-15. There were no significant differences in the variables at the mid-metaphyseal measure point between the two groups of foetuses.

Table 14. Results (mean±SD) from three point bending test performed on femur bones from two groups of female **foetal** sheep (110 days old) (control and treated). The bending took place at the mid-diaphyseal measure point (50 % of the total length of the bone) in an electromechanical testing machine (Avalon technologies, MN, USA) with an axial capacity of approximately 1112 N (250 lbs). The displacement rate was 1mm/sec and the span length was 30 mm. The treated mothers had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Control sheep had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time before mating. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= numbers of individuals, ns= non significant, na= non applicable.

	Control		n=28	Treated		n=23	p-value	
	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (g)	1898.2	±	246.9	1853.7	±	266.3	ns	na
Displacement at failure (mm)	6.6	±	1.2	7.0	±	1.0	ns	ns
Load at failure (N)	267.8	±	80.2	271.8	±	61.7	ns	ns
Energy at failure (N*mm)	999.7	±	284.6	976.9	±	305.8	ns	ns
Max stiffness (N/mm)	73.3	±	19.3	64.4	±	15.4	ns	ns

Table 15. Results (mean±SD) from three point bending test performed on femur bones from two groups of male **foetal** sheep (110 days old) (control and treated). The bending took place at the mid-diaphyseal measure point (50 % of the total length of the bone) in an electromechanical testing machine (Avalon technologies, MN, USA) with an axial capacity of approximately 1112 N (250 lbs). The displacement rate was 1mm/sec and the span length was 30 mm. The treated mothers had been grazing on pasture fertilized twice annually with sewage sludge (2.25 tonnes of dry matter/ha), since the age of 18 months, until slaughter at 5-6 years of age. The chemical analysis of the sludge is presented on page 18. Control sheep had been grazing on pasture fertilized with conventional, inorganic fertilizer for the same amount of time before mating. The results obtained were evaluated by analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used when adjusting for the continuous variable body mass. Differences were considered significant at $p<0.05$. *= $p<0.05$, n= numbers of individuals, ns= non significant, na= non applicable.

	Control		n=19	Treated		n=25	p-value	
	Mean		SD	Mean		SD	ANOVA	ANCOVA
Weight (g)	1889.6	±	333.1	1975.1	±	196.4	ns	na
Displacement at failure (mm)	7.2	±	0.9	6.8	±	1.2	ns	ns
Load at failure (N)	293.2	±	78.9	299.7	±	78.9	ns	ns
Energy at failure (N*mm)	1092.8	±	372.0	1084.2	±	273.5	ns	ns
Max stiffness (N/mm)	70.1	±	21.6	76.6	±	20.5	ns	ns

4 DISCUSSION

This project is part of an ongoing study of effects on bone tissue in sheep grazing on pasture fertilized with sewage sludge. In the previous study changes in bone variables resembling those caused by estrogens were found in the cortical bone in the femurs of ewes (Broman 2006). The previous study was based on relatively few adult individuals (ewes) compared to the present study that has a greater number of adult individuals and also includes foetuses. Ewes that were exposed to sewage sludge show an alteration in bone tissue composition compared to controls. The trabecular BMC, BMD and CSA were all significantly lower and the body weight significantly higher in the treated ewes compared to individuals in the control group. This suggests an anti-estrogenic effect caused by the exposure to sewage sludge when grazing on fertilized pasture.

Depending on the sources of inputs to a specific waste water plant the composition of sewage sludge can be highly variable (Thuresson and Haapaniemi 2005). Batches of sludge can exhibit a ten fold difference in the amount of any one anthropogenic substance. Taking this into consideration, it's not very surprising that putatively different estrogenic and anti-estrogenic influences have resulted in differences in the results of the preceding study and the present study. Some EDCs may be well overrepresented in previous batches of sludge compared to the ones used in this study and in the absence of comprehensive analyses of the chemical content of the sludges it is impossible to draw specific conclusions concerning mechanisms of action.

The calculated reproducibility from ten repeated pQCT measurements performed on bones from ewes at the diaphyseal- and metaphyseal measure points gave a low coefficient of variation (CV) that never exceeded 2.3 %, making it a valid method. The CV from measurements on bone from foetus was higher, reaching 6.76 %. This can be explained by a lower precision in the placement of the light point in the pQCT system due to the small size of the bones.

Trabecular area was significantly lower in the femurs of exposed ewes in comparison to the control animals. A small trabecular area in the humerus was also seen in a study on rats exposed to the anti-estrogenic substance PCB-126 (Lind *et al.* 2000). However, in a study of trabecular density in goats, no observed effects of PCB 126 were detected in the bones of female goat offspring following exposure perinatally (Lundberg *et al.* 2006). These apparently anomalous results may reflect differences in the dosage given, time of exposure and species used.

After correction for body weight there were no statistically significant differences regarding pQCT variables of the cortical bone between treated and control ewes. The three-point bending test, however, showed a significantly higher mechanical strength of femur diaphysis in exposed ewes than in controls. This finding might partly be explained by the slight but non-significant difference in geometry – periosteal circumference, cortical thickness and cortical cross sectional area were greater in femur from exposed than in femur from control ewes.

The metaphyseal CSA were lower in the treated heavier ewes compared to controls. This points to an anatomical diversion also seen in American kestrel exposed to BDE 100, 153 and 183 (Fernie *et al.* 2006) who developed longer bones and were heavier than the unexposed.

Foetuses usually show a greater sensitivity to environmental contaminants due to their delicate stage of development. The foetuses used in this study did not show clear effects in their bone tissue composition in either the pQCT measurements or biomechnical bending. What has been noted is a reduction in the endocortical circumference and marrow cavity in the mid-diaphyseal measure point in treated female foetus. Treated male foetus show a higher total bone mineral content in the mid-diaphyseal measurepoint. Because the contents of the sewage sludge is still uncertain whether or how these chemicals are transported from the ewe to her foetus, it is difficult to known the pathway by which the toxic substances operate.

The uptake and assimilation of toxic substances in plants grown on sewage sludge treated land is considered to be low (Petersen *et al.* 2003). However, if the sludge contains heavy metals, a build-up of metal concentration in the soil is possible and it may, with time, become unfit for food production. The risk of metal leaching to groundwater is dependent of the soil pH, so liming could be a good prevention method (Speir *et al.* 2003). Depending on what kind of plants that are growing on the pasture, they can contain different amounts of natural EDCs such as isoflavones. Red clover, for instance, is estrogenic and may have an affect on grazing animals (Zava *et al.* 1998). In theory, this could explain the estrogenic results from the previous study, but this is highly unlikely as no clover was growing on the pastures because of the unsuitable geographic location (Rhind, personal communication).

Rhind (2005) has brought several aspects to light when considering whether farm animals and their health can be affected by endocrine disruptive compounds. The type of compound and its properties determine how it affects organisms. Persistence, chemical structure, bioavailability, effects when mixed with different compounds and effects on subsequent generations are all aspects which should be taken into account. Investigations are required to determine the potential risk from these compounds for human- and animal health.

There have been many toxicity studies on laboratory animals involving single EDCs and large doses. Sewage sludge, by contrast, contains a cocktail of substances in different concentrations and can't be properly evaluated on the basis of its individual components. The chemicals, when spread in the environment, are often present at a low dose but, nevertheless, can affect wildlife through a prolonged exposure. An animal may also be more susceptible to one substance because of the presence of other harmful contaminants, i.e. synergism is possible (Bernes 1998). Different species of mammals may differ in sensitivity when exposed to EDCs (Pugh *et al.* 2000) and even differences in the genetic line within the same species can exhibit different responses (Bernes 1998). The results obtained from animal studies are therefore not always representative when considering human exposure and health effects.

In conclusion, ewes grazing on pasture fertilized with sewage sludge show an anti-estrogenic effect in their trabecular bone. There are still only a few studies that have been conducted on effects of sewage sludge on exposed organisms. Because of the great variation of contents in the sewage sludge, it's hard to know exactly what chemical is causing what adverse changes in the body. However, adverse effects of exposure to EDCs on testis development in sheep male foetuses whose mothers have been grazing on sewage sludge fertilized pasture have been reported (Paul *et al*. 2005) and a probable demasculinisation of male sheep exposed to sewage sludge causes a modification in exploratory behaviour, compared to control animals (Erhard and Rhind 2004). The chemical or chemicals that are causing these effects are not known but the fact that this kind of fertilizer is used on agricultural land makes means that humans, may be at risk as well.

REFERENCES

Alveblom A-K., Rylander L., Johnell O. and Hagmar L., 2003. Incidence of hospitalized osteoporotic fractures in cohorts with high dietary intake of persistent organochlorine compounds. International Archives of Occupational and Environmental Health 76: 246–248

Andrade A. J. M., Kuriyama S. N., Akkoc Z., Talsness C. E. and Chahoud I., 2004. Effects of developmental low dose PBDE 47 exposure on thyroid hormone status and serum concentrations of FSH and inhibin B in male rats. Organohalogen compounds 66: 3907-3912

Aspden R. M., 2003. Mechanical testing of bone ex vivo. In Helfrich M. H. and Ralston S. H. (eds), Methods in molecular medicine, volume 80, Bone research Protocols, pp. 369-379. Humana Press Inc., New Jersey

Béland P., DeGuise S., Girard C., Lagacé A., Martineau D., Michaud R., Muir D. C. C., Norstrom R. J., Pelletier E., Ray S. and Shugart L. R., 1993. Toxic Compounds and Health and Reproductive Effects in St. Lawrence Beluga Whales. Journal of Great Lakes Research 19: 766-775

Bergman A., Olsson M. and Relland S., 1992. Skull-bone lesions in the Baltic gray seal (halichoerus grypus). Ambio 21: 517-520

Bernes C., 1998. Persistant organic pollutants. Monitor 16. Swedish Environmental Protection Agency, Stockholm

Blom A., Ekman E., Johannisson A., Norrgren L. and Pesonen M., 1998. Effects of Xenoestrogenic Environmental Pollutants on the Proliferation of a Human Breast Cancer Cell Line (MCF-7). Archives of Environmental Contamination and Toxicology 34: 306-310

Broman F., 2006. Effects on bone tissue in sheep reared on pasture treated with sewage sludge. Project report from the Department of Environmental Toxicology, Uppsala University, no 111

Buchanan D. L., Sato T., Peterson R. E. and Cooke P. S., 2000. Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in mouse uterus: critical role of the aryl hydrocarbon receptor in stromal tissue. Toxicological Sciences 57: 302-311

Bustos S., Denegri J. C., Diaz F., and Tchernitchin A. N., 1988. *p,p'*-DDT is an Estrogenic Compound. Environmental Contamination and Toxicology 41: 496-501

Carlsen C. G., Soerensen T. H. and Eriksen E. F., 2000. Prevalence of Low Serum Estradiol Levels in Male Osteoporosis. Osteoporosis International 11: 697-701

Colvard D. S., Eriksen E. F., Keeting P. E., Wilson E. M., Lubahn D. B.,, French F. S., Riggs B. L. and Spelsberg T. C., 1989. Identification of androgen receptors in normal human osteoblast-like cells. Proceedings of the National Academy of Sciences 86: 854-857

Cripps D. J., Peters H. A., Gocmen A. and Dogramici I., 1984. Porphyria turcica due to hexachlorobenzene: a 20 to 30 year follow-up study on 204 patients. British Journal of Dermatology 111: 413-422

de Jonge H., de Jonge L. W, Blicher B. W. and Moldrup P., 2002. Transport of Di(2-ethylhexyl)phthalate (DEHP) Applied with Sewage Sludge to Undisturbed and Repacked Soil Columns. Journal of Environmental Quality 31: 1963–1971

de Wit C., Eriksson U., Nylund K., Haglund M., Berggren D., Kierkegaard A., Allan A. and Asplund L., 2002. Bromerade flamskyddsmedel i avloppsslam. Swedish Environmental Protection Agency report no 5188, Stockholm

Ducy P., Schinke T. and Karsenty G., 2000. The Osteoblast: A Sophisticated Fibroblast under Central Surveillance. Science 289: 1501-1504

Erhard H. W. and Rhind S. M., 2004. Prenatal and postnatal exposure to environmental pollutants in sewage sludge alters emotional reactivity and exploratory behaviour in sheep. Science of the Total Environment 332: 101-108

Eriksen E. F., Colvard D. S., Berg N. J., Graham M. L., Mann K. G., Spelsberg T. C., and Riggs B. L., 1988. Evidence of Estrogen Receptors in Normal Human Osteoblast-Like Cells. Science 241: 84-86

Eroschenko, V. P, 1996. Di Fiore's atlas of histology with functional correlations, 8th edition. Williams & Wilkins

Fertuck K. C., Kumar S., Sikka H.C., Matthews J. B. and Zacharewski T. R., 2001. Interaction of PAH-related compounds with the α and β isoforms of the estrogen receptor. Toxicology Letters 121: 167–177

Fernie K. J., Shutt J. L. , Ritchie I. J., Letcher R. J., Drouillard K. and Bird D. M., 2006. American Kestrels (Falco sparverius) Exposed to Environmentally Relevant Polybrominated Diphenyl Ethers. Journal of Toxicology and Environmental Health, Part A, 69: 1541–1554

Gasser J. A., 2003. Bone measurements by peripheral Quantitative computed tomography in rodents. In Helfrich M. H. and Ralston S. H. (eds), Methods in molecular medicine, volume 80, Bone research Protocols, pp. 323-341. Humana Press Inc., New Jersey

Glynn A. W., Michaëlsson K., Lind P. M., Wolk A., Aune M., Atuma S., Darnerud P. O. och Mallmin H., 2000. Organochlorines and Bone Mineral Density in Swedish Men from the General Population. Osteoporosis International 11: 1036–1042

Gray L. E. Jr., Ostby J., Furr J., Price M., Veeramachaneni D. N. R. and Parks L., 2000. Perinatal Exposure to the Phthalates DEHP, BBP, and DINP, but Not DEP, DMP, or DOTP, Alters Sexual Differentiation of the Male Rat. Toxicological Sciences 58: 350-365

Guillette L. J. Jr., Crain D. A. , Gunderson M. P., Kools S. A. E., Milnes M. R., Orlando E. F., Rooney A.. A. and Woodward A.. R., 2000. Alligators and Endocrine Disrupting Contaminants: A Current Perspective. American Zoology 40: 438–452

Harms H. H., 1996. Bioaccumulation and metabolic fate of sewage sludge derived organic xenobiotics in plants. Science of total environment 185: 83-92

Haugeberg G., Ørstavik R. E., Uhlig T., Falch J. A., Halse J. I. and Kvien T. K., 2002. Bone Loss in Patients With Rheumatoid Arthritis. Arthritis & Rheumatism 46: 1720–1728

Hemenway D., Colditz G. A., Willett W. C., Stampfer M. J., and Speizer F. E., 1988. Fractures and Lifestyle: Effect of Cigarette Smoking, Alcohol Intake, and Relative Weight on the Risk of Hip and Forearm Fractures in Middle-Aged Women. American Journal of Public Health 78: 1554-1558

Hellström T., 2000. Brominated Flame Retardants (PBDE and PBB) in Sludge – a Problem? VAV- The Swedish Water and Wastewater Association, Report 113

Hole J. W. and Koos K. A., 1994. Human anatomy. 2nd edition. Wm. C. Brown Communications, Dubuque

Jilka R. L., Hangoc G., Girasole G., Passeri G., Williams D. C., Abrams J. S., Boyce B., Broxmeyer H. and Manolagas S. C., 1992. Increased Osteoclast Development After Estrogen Loss: Mediation by Interleukin-6. Science 257: 88-91

Jämsä T., Viluksela M.,. Tuomisto J. T., Tuomisto J. and Tuukkanen J., 2001. Effects of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin on Bone in Two Rat Strains with Different Aryl Hydrocarbon Receptor Structures. Journal of Bone and Mineral research 16: 1812-1820

Karaplis A. C., 2002. Embryonic development of bone and molecular regulation of intramembranous and endochondral bone formation. In Bilezikian J. P., Raisz L. G., Rodan G. A., (eds) Principles of bone biology, volume 1, 2nd edition, pp 33-55. Academic Press, San Diego

Kelce W. R., Lambright C. R., Gray L. E. Jr. and Roberts K. P., 1997. Vinclozolin and *p,p'*-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism. Toxicology and applied pharmacology 142: 192–200

KemI 2003. HA oils in automotive tyres. Prospects of a national ban. The Swedish National Chemicals Inspectorate report 5/03

Klein – Nulend J., Van der Plas A., Semeins C. M., Ajubi N. E., Frangos J. A., Nijweide P. J. and Burger E., 1995. Sensitivity of osteocytes to biomechanical stress in vitro. The Federation of American Societies for Experimental Biology 9: 441-445

Kröger H., Tuppurainen M., Honkanen R., Alhava E. and Saarikoski S., 1994. Bone Mineral Density and Risk Factors For Osteoporosis-A Population-Based Study of 1600 Perimenopausal Women. Calcified Tissue International 55: 1-7

Kylin H., 2005. Växtskyddssubstanser i avloppsvatten och -slam från sju svenska reningsverk, rapport 2005:29. Institutionen för miljöanalys, Sveriges lantbruksuniversitet

Langenkamp H., Part P., Erhardt W. and Prüeß A., 2001. Organic contaminants in sewage sludge for agricultural use. EU-project

Lee L. L., Lee J. S. C., Waldman S. D., Casper R. F. and Grynpas M. D., 2002. Polycyclic Aromatic Hydrocarbons Present in Cigarette Smoke Cause Bone Loss in an Ovariectomized Rat Model. Bone 30: 917–923

Lind M., Brunstrom B., Ljunggren O., Ljunghall S., and Orberg J., 1996. Impaired bone mineralization in mink pups following chronic PCB exposure of the dam. Toxicology Letters 88: 111

Lind P. M., Eriksen E. F., Sahlin L., Edlund M., och Örberg J., 1999. Effects of the Antiestrogenic Environmental Pollutant 3,3’,4,4’,5-Pentachlorobiphenyl (PCB #126) in Rat Bone and Uterus: Diverging Effects in Ovariectomized and Intact Animals. Toxicology and Applied Pharmacology 154: 236–244

Lind P. M., Larsson S., Johansson S., Melhus H., Wikström M., Lindhe Ö. och Örberg J., 2000. Bone tissue composition, dimensions and strength in female rats given an increased dietary level of vitamin A or exposed to 3,3’,4,4’,5-pentachlorobiphenyl (PCB126) alone or in combination with vitamin C. Toxicology 151: 11–23

Lind P. M., Bergman A., Olsson M. and Örberg J., 2003. Bone Mineral Density in Male Baltic Grey Seal (Halichoerus grypus). Ambio 32: 385-388

Lind P. M., Eriksen E. F., Lind L., Örberg J. and Sahlin L., 2004a. Estrogen supplementation modulates effects of the endocrine disrupting pollutant PCB126 in rat bone and uterus. Diverging effects in ovariectomized and intact animals. Toxicology 199: 129–136

Lind P. M., Milnes M. R., Lundberg R., Bermudez D., Örberg J., and Guillette L. J. Jr., 2004b. Abnormal Bone Composition in Female Juvenile American Alligators from a Pesticide-Polluted Lake (Lake Apopka, Florida). Environmental Health Perspectives 112: 353-362

Lundberg R., Lyche J. L., Ropstad E., Aleksandersen M., Rönn M., Skaare J. U., Larsson S., Örberg J. and Lind P. M., 2006. Perinatal exposure to PCB 153, but not PCB 126, alters bone tissue composition in female goat offspring. Toxicology 228: 33-40

Lundberg R., Munro Jenssen B., Leiva-Presa À., Rönn M., Hernhag C.,

Wejheden C., Larsson S., Örberg J. and Lind P. M., 2007. Effects of Short-term Exposure to the DDT Metabolite *p,p'*-DDE on Bone Tissue in Male Common Frog (Rana temporaria). Journal of Toxicology and Environmental Health, Part A 70: 614–619

Lundholm C. E., 1997. DDE-Induced Eggshell Thinning in Birds: Effects of *p,p'*-DDE on the Calcium and Prostaglandin Metabolism of the Eggshell Gland. Comparative biochemistry and physiology 118: 113-128

Ljunggren Ö., 1998. Det levande benet. Sparre medical, Stockholm

Lorentzon M., Ohlsson C., 2006. Osteoporos – en ärftlig sjukdom. Läkartidningen 103: 2970-2971

Marks S. C. and Odgren P. R, 2002. Structure and development of the skeleton. In Bilezikian J. P., Raisz L. G., Rodan G. A., (eds) Principles of bone biology, volume 1, 2nd edition, pp. 3-15. Academic Press, San Diego

Miller R. W., 1985. Congenital PCB Poisoning: A Reevaluation. Environmental Health Perspectives 60: 211-214

Miller S.C. and Jee W. S. S., 1987. The Bone Lining Cell: A Distinct Phenotype? Calcified Tissue International 41: 1-5

Mortensen P., Bergman A., Bignert A., Hansen H. J., Härkönen T. and Olsson M., 1992. Prevalence of Skull lesions in harbor seals *(Phoca vitulina)* in Swedish and Danish Museum collections. Ambio 21: 520-524

Nakamura H., 2007. Morphology, Function, and Differentiation of Bone Cells. Journal of Hard Tissue Biology 16: 15-22

National Institutes of Health- Osteoporosis and Related Bone Diseases 2005. Bed Rest and Immobilization: Risk Factors for Bone Loss

National Institutes of Health- Osteoporosis and Related Bone Diseases, 2006. Osteoporosis Overview

Nilsson O., Chrysis D., Pajulo O., Boman A., Holst M., Rubinstein J., Ritzén E M. and Sävendahl L., 2003. Localization of estrogen receptors-alpha and -beta and androgen receptor in the human growth plate at different pubertal stages. Journal of Endocrinology 177: 319-26

Norén K. and Meironyté D., 2000. Certain organochlorine and organobromine contaminants in Swedish human milk in perspective of past 20-30 years. Chemosphere 40: 1111-1123

Omland L. M., Tell G. S., Øfjord S. and Skag A., 2000. Risk factors for low bone mineral density among a large group of Norwegian women with fractures. European Journal of Epidemiology 16: 223-229

Oursler M. J., Osdoby P. , Pyfferoen J. , Riggs B. L. and Spelsberg T. C., 1991. Avian osteoclasts as estrogen target cells. Proceedings of the National Academy of Sciences 88: 6613-6617

Paul C., Rhind S. M., Kyle C. E., Scott H., McKinnell C. and Sharpe R. M., 2005. Cellular and Hormonal Disruption of Fetal Testis Development in Sheep Reared on Pasture Treated with Sewage Sludge. Environmental Health Perspectives 113: 1580-1587

Petersen S. O., Henriksen K., Mortensen G. K., Krogh P. H., Brandt K. K., Sørensen J., Madsen T. and Grøn C., 2003. Recycling of sewage sludge and household compost to arable land: fate and effects of organic contaminants and impact on soil fertility. Soil & Tillage Research 72:139–152

Pugh, G. Jr., Isenberg J. S., Kamendulis L. M., Ackley D. C., Clare L. J., Brown R., Lington A. W., Smith J. H. and Klaunig J. E., 2000. Effects of Di-isononyl Phthalate, Di-2-ethylhexyl Phthalate, and Clofibrate in Cynomolgus Monkeys. Toxicological Sciences 56: 181-188

Qu Q., Perälä-Heape M., Kapanen A., Dahllund J., Salo J., Väänänen H. K., and Harkonen P., 1998. Estrogen Enhances Differentiation of Osteoblasts in Mouse Bone Marrow Culture. Bone 22: 201-209

Ratcliffe D. A., 1967. Decrease in Eggshell Weight in Certain Birds of Prey. Nature 215: 208-210

Render J. A., Aulerich R. J., Bursian S. J. and Nachreiner R. F., 2000. Proliferation of maxillary and mandibular periodontal squamous cells in mink fed 3,3',4,4',5-pentachlorobiphenyl (PCB 126). Journal of Veterinary Diagnostic Investigation 12: 477–479

Rhind S. M., 2005. Are Endocrine Disrupting Compounds a Threat to Farm Animal Health, Welfare and Productivity? Reproduction in Domestic Animals 40: 282–290

Rhind S. M., Smith A., Kyle C. E., Telfer G., Martin G., Duff E. and Mayes R. W., 2002. Phthalate and alkyl phenol concentrations in soil following applications of inorganic fertiliser or sewage sludge to pasture and potential rates of ingestion by grazing ruminants. Journal of Environmental Monitoring 4: 142–148

Rhind S. M., Kyle C. E., Telfer G., Duff E. I., and Smith A., 2005. Alkyl Phenols and Diethylhexyl Phthalate in Tissues of Sheep Grazing Pastures Fertilized with Sewage Sludge or Inorganic Fertilizer. Environmental Health Perspectives 113: 447-453

Rhind S. M., Kyle C. E., Mackie C. and Telfer G., 2007. Effects of exposure of ewes to sewage sludge-treated pasture on phtalate and alkyl phenol concentrations in their milk. Science of the Total Environment 383: 79-80

Rozenberg S., Kroll M., Pastijn A. and Vandromme J., 1995. Osteoporosis Prevention and Treatment with sex Hormone Replacement Therapy. Clinical rheumatology 14: 14-17

Safe S., Bandiera S., Sawyer T., Zmudzka B., Mason G., Romkes M., Denomme M. A., Sparling J., Okey A. B., and Fujita T., 1985. Effects of Structure on Binding to the 2,3,7,8-TCDD Receptor Protein and AHH Induction Halogenated Biphenyls. Environmental Health Perspectives 61: 21-33

Sonne C., Dietz R., Born E. W., Riget F. F., Kirkegaard M., Hyldstrup L., Letcher R. J. and Muir D. C. G., 2004. Is Bone Mineral Composition Disrupted by Organochlorines in East Greenland Polar Bears (Ursus maritimus)? Environmental Health Perspectives 112: 1711-1716

Sonne C., Rigét F. F., Dietz R., Wiig Ø., Kirkegaard M. and Born E. W., 2007. Skull pathology in East Greenland and Svalbard polar bears (Ursus maritimus) during 1892 to 2002 in relation to organochlorine pollution. Science of the Total Environment 372: 554–561

Speir T. W., van Schaik A. P., Percival H. J., Close M. E. and Pang L., 2003. Heavy metals in soil, plants and groundwater following high-rate sewage sludge application to land. Water, Air, and Soil Pollution 150: 319–358

Stroheker T., Cabaton N., Nourdin G., Régnier J-F., Lhuguenot J-C., Chagnon M-C., 2005. Evaluation of anti-androgenic activity of di-(2-ethylhexyl)phthalate. Toxicology 208: 115–121

Swedish Environmental Protection Agency, 1995. Användning av avloppsslam i jordbruket. Report 4418, Stockholm

Teitelbaum S., 2000. Bone resorption by osteoclasts. Science 289: 1504 – 1508

Tenenbaum D. J., 2004. POPs in Polar Bears, Organochlorines Affect Bone Density. Environmental Health Perspectives 112: 1011

The Swedish Council on Technology Assessment in Health Care 2003. Osteoporos – prevention, diagnostik och behandling.

Thigpen J. E, Setchell K. D. R., Saunders H. E., Haseman J. K.. Grant M. G. and

Forsythe D. B., 2004. Selecting the Appropriate Rodent Diet for Endocrine Disruptor Research and Testing Studies. Institute for Laboratory Animal Research Journal 45: 401-416

Thuresson M. and Haapaniemi U., 2005. Slam från avloppsreningsverk. Mängder, kvalitet samt användning i Stockholms län under perioden 1981 till 2003. Länsstyrelsen i Stockholms län, rapport 2005:10

Turner R. T., Riggs B. L. och Spelsberg T. C., 1994. Skeletal Effects of Estrogen. Endocrine Review 15: 275-300

Viberg H., Fredriksson A. and Eriksson P., 2003. Neonatal exposure to polybrominated diphenyl ether (PBDE 153) disrupts spontaneous behaviour, impairs learning and memory, and decreases hippocampal cholinergic receptors in adult mice. Toxicology and Applied Pharmacology 192: 95-106

Walker C. H., Hopkin S. P., Sibly R. M. and Peakall D. B, 2006. Principles of ecotoxicology, 3rd edition. Taylor and Francis

Wallin E., Rylander L., Hagmar L., 2004. Exposure to persistent organochlorine compounds through fish consumption and the incidence of osteoporotic fractures. Scandinavian Journal of Work and Environmental Health 30: 30–35

Watts N. B., 2003. Bisphosphonate treatment of osteoporosis. Clinics in geriatric medicine 19: 395-414

Wejheden C., Brunnberg S., Hanberg A. and Lind P. M., 2006. Osteopontin: A rapid and sensitive response to dioxin exposure in the osteoblastic cell line UMR-106. Biochemical and Biophysical Research Communications 341: 116–120

White R., Jobling S., Hoare S. A., Sumpter J. P. and Parker M. G., 1994. Environmentally Persistent Alkylphenolic Compounds Are Estrogenic. Endocrinology 136: 175-182

Zava D. T., Dollbaum C. M. and Blen M., 1998. Estrogen and progestin bioactivity of foods, herbs, and spices. Proceedings of the Society for Experimental Biology and Medicine 217: 369-78