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Toxicity studies on bone tissue from sheep grazing on a pasture treated with sewage sludge

Magnus Gustafsson

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PREFACE

This report is a graduate project in environmental toxicology. It leads to a Master of Science degree in Biology at Uppsala University. The work was carried out at the Department of Environmental Toxicology at Uppsala University and at the Institute of Environmental Medicine at Karolinska Institutet. This report is a part of a collaboration project, and focuses on effects on bone tissue in sheep exposed to sewage sludge.

SUMMARY

In this study, femur bones and serum from rams and ewes (*Ovis aries*) bred on pastures fertilized twice annually with sewage sludge (2.25 tonnes dry matter/ha) or on control pastures were analysed. The control pastures was treated with conventional inorganic fertilizer. Both rams and ewes were exposed during development *in utero*, from conception, and after birth, during lactation, to weaning at 4 months of age. After that the rams were moved to pastures not fertilized with sewage sludge. The ewes, however, were retained on pastures treated with sewage sludge. The animals were slaughtered at 18 months of age and the femur bone was dissected.

The peripheral Quantitative Computed Tomography (pQCT) and three-point bending test analyses of the femur bone were evaluated. In rams, the total bone mineral density (BMD) at the metaphyseal part of femur was significantly greater (10.5 %, $p<0.05$) in treated than control animals. Treated animals also exhibited a reduction in the total cross sectional area (CSA, 11.5 %, $p<0.05$), the trabecular CSA (17.1 %, $p<0.05$) and the periosteal circumference (5.7 %, $p<0.05$). Analysis of the mid-diaphyseal part revealed multiple effects *e.g.* the total BMD (13.8 %, $p<0.05$) increased significantly whereas the total CSA (12.1 %, $p<0.05$) and the marrow cavity (25.8 %, $p<0.05$) were significantly reduced in the treated rams. The three-point bending test of the ram femur at the mid-diaphyseal part revealed an increased stiffness (6.4 %, $p<0.05$) in treated animals. In ewes, the trabecular bone mineral content (BMC) at the metaphyseal part of femur was significantly increased (41.6 %, $p<0.05$) in the treated ewes. pQCT analysis of the mid-diaphyseal part showed no adverse effects in the treated ewes. Femurs from treated ewes showed a reduction in the load at failure (17.3 %, $p<0.05$) and stiffness (10.7 %, $p<0.05$). The serum levels of the bone formation marker BAP, the bone resorption marker CTX and the vitamin D marker 25-OH D did not differ between exposed and control animals, neither for rams nor for ewes.

In conclusion, this study shows that exposure to sewage sludge disrupt bone tissue homeostasis in sheep. As the rams were only exposed via placental transfer and mother's milk, the obtained effects; increased cortical thickness, decreased circumference and decreased marrow cavity must be considered as developmental effects. The increase in femoral trabecular BMC in the ewes is thought likely to be attributable to estrogenic influences in the sludge.

ABBREVIATIONS

ANCOVA	analysis of covariance
BAP	bone-specific alkaline phosphatase
BMC	bone mineral content
BMD	bone mineral density
Cort	cortical
CortTHKC	cortical thickness circular ring model
CSA	cross sectional area
CTX	carboxyterminal telopeptide of type 1 collagen
***p,p'*-DDE**	dichlorodiphenyldichloro-ethylene
***p,p'*-DDD**	1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethane
DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
DEHP	di-2-ethylhexyl phthalate
EDCs	endocrine disrupting chemicals
ENDOC	endosteal circumference
IPCMW	polar moment of inertia
NP	nonylphenol
PCB	polychlorinated biphenyls
PERIC	periosteal circumference
pQCT	peripheral quantitative computed tomography
RPCMW	moment of resistance
STPs	sewage treatment plants
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
Trab	trabecular
25-OH D	25-hydroxy vitamin D

INTRODUCTION

Rhind *et al.* and Swanson *et al.* suggest that the reuse of sewage sludge in agriculture will increase as a result of the current ban on dumping sewage sludge at sea in both Europe and the United States (Rhind *et al.* 2005; Swanson *et al.* 2004). Human health is at risk as we consume, directly or indirectly, products that may have accumulated inorganic and organic pollutants present in the sludge. Information regarding tissue concentrations of these pollutants in animals reared on pasture fertilized with sewage sludge is of interest and essential for risk assessment of animal and human health.

This report is a part of a collaboration project between Stewart Rhind at the Macaulay Institute, Hartwood, Scotland, Jan Örberg at the Department of Environmental Toxicology, Uppsala University, Sweden and Monica Lind at the Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden. It focuses on effects on bone tissue in sheep exposed to sewage sludge. Within the same project Broman has presented results from studies on adult ewes and Öberg from studies on adult ewes and on male and female foetuses (Broman 2006; Öberg 2008). Broman found that the maximal energy required for breaking the bones (energy to failure) of treated ewes increased significantly after exposure to sewage sludge. It was suggested that this might be due to a minor increase in cortical cross sectional area of the bones (Broman 2006). Öberg discovered a significant reduction in trabecular bone mineral density, trabecular bone mineral content and total and trabecular cross sectional area in the treated ewes. It was suggested that this might be attributable to exposure to compounds with antiestrogenic activities (Öberg 2008). Earlier studies in this area have shown that exposure of sheep to endocrine disrupting chemicals in sewage sludge through application on pastures can cause disruption of fetal testis development (Paul *et al.* 2005). In another study, ram lambs born to ewes that had been kept throughout their lives on pastures fertilized with sewage sludge exhibited reduced exploratory activity, suggesting a feminization of the animals with respect to this behavior (Erhard and Rhind 2004).

BONE

Bone is a supporting tissue that is important for locomotion. It also constitutes a large reservoir of ions such as calcium, phosphorus, magnesium, and sodium (Seibel and Bilezikian 1999). It is composed of a protein matrix (organic phase) which becomes hard when mineralized by calcium phosphate (inorganic phase) as hydroxyapatite crystals, and other minerals (Marks and Odgren 2002). Bone consists of approximately 70 % inorganic material, 10 % water and 20 % organic material of which 90 % is collagen type 1 (Nakamura 2007). There are two types of bone tissues: cortical and trabecular. The cortical bone is compact with a high mineral density and constitutes the outer layer of long bones. The porous trabecular bone is found in the inner part close to the ends of the long bones. This bone tissue forms a dense network of thin bone needles. The mid-part of long bones is called the diaphysis (fig 1), the ends are the epiphyses and the part between these, situated close to the epiphyses are the metaphyses (Seibel and Bilezikian 1999).

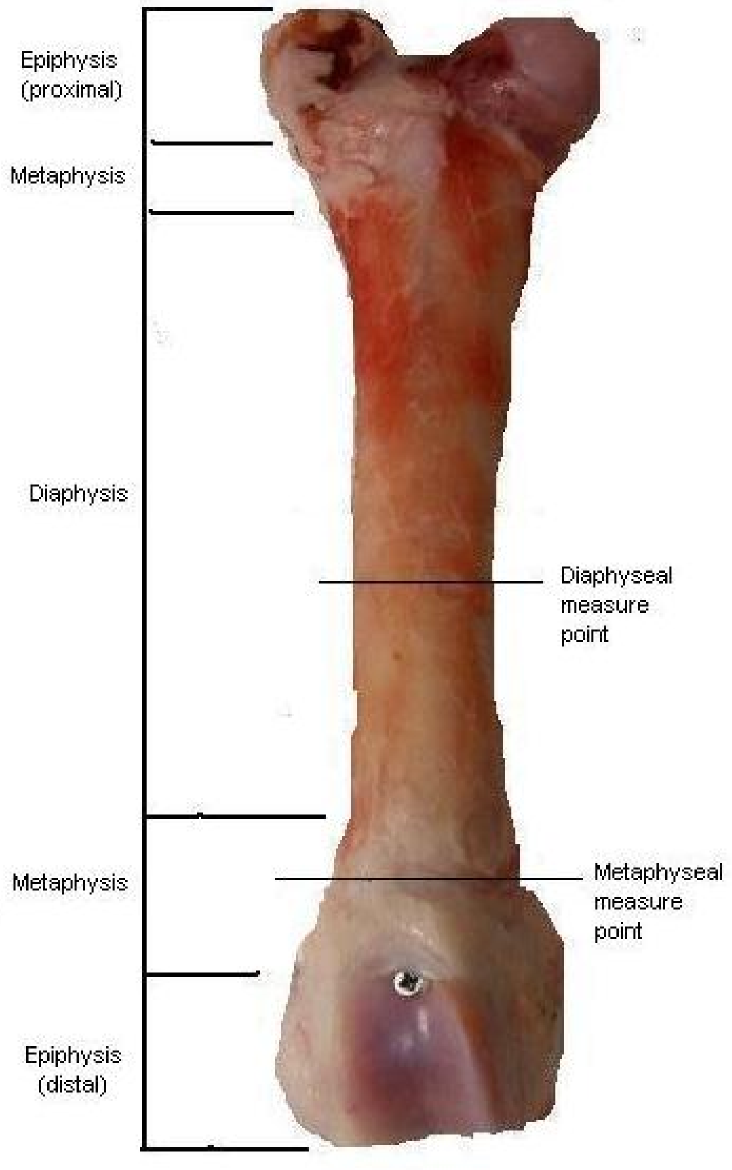

Fig.1. Femur bone showing the metaphyseal measure point at 3.6% and the diaphyseal measure point at 50% of the total length from the distal end of the bone for the pQCT analysis.

The growth plate which is composed of cartilage has a smooth, silky appearance and is situated between the epiphyses and metaphyses at both the distal and the proximal part of the long bones. It governs most of the longitudinal growth during development, until puberty when the epiphyseal cells stops proliferating and the cartilage is replaced by bone (Van Der Erden *et al.* 2003). The cortical and trabecular bone contribute to the bone strength and the trabecular bone is most important for the calcium homeostasis (Marks and Odgren 2002).

Bone tissue homeostasis

In bone tissue four types of cells are found: osteoblasts, osteoclasts, lining cells and osteocytes (Marks and Odgren 2002). Osteoblasts are derived from precursor mesenchymal stem cells of the bone marrow (Manolagas and Jilka 1995). They are differentiated bone lining cells with a round shape and are responsible for bone formation by synthesizing and secreting uncalcified matrix, called osteiod (Nakamura 2007). Osteoclasts are large multinucleated cells (Nakamura 2007) originating from hematopoetic granulocyte-macrophages in the bone marrow. They resorb bone tissue by causing a decrease in pH which dissolves the calcium salts (Teitelbaum 2000). Lining cells have a compact shape and few organelles and are present at bone surfaces. The function of these cells is unclear but they excrete small amounts of osteoid but are probably not involved in bone formation (Van Der Erden *et al.* 2003). Osteocytes are the final differentiation stage of osteoblasts which have been buried in the bone matrix. They have a large surface area and contain several actin filaments that sense mechanical stress (Nakamura 2007). They are connected to other osteocytes by channels which mediate transport of nutrients and waste between blood and bone tissue (Nomura and Takano-Yamamoto 2000).

Before puberty bone develops by lengthening and thickening in a process called modelling. Remodelling occurs in parallel with bone modelling and compensates for bone turnover. In this process there are two phases: formation (bone synthesis) and resorption (bone degradation). The remodelling enables adaptation to mechanical stress, repairs micro damage and maintains calcium ion homeostasis. Optimal remodelling requires *e.g.* sufficient intake and absorption of nutrients such as calcium from the intestine, facilitated by vitamin D. If the intake of calcium is high, calcium is incorporated into the bone by mineralization (Holick 2007). On the other hand, low calcium levels lead to release of PTH from the parathyroid gland. PTH increases the reuptake of calcium in urine from the kidneys and mobilizes calcium from bone (Guyton and Hall 1996). Furthermore, sexsteroids are required for remodelling. Both osteoclasts and osteoblasts have estrogen receptors (Eriksen *et al.* 1988; Oursler *et al.* 1991). Transforming growth factor beta (TGFβ) mediates the action of estrogen on bone tissue which exerts inhibiting effect on osteoclasts and stimulates the effect on osteoblasts, and hence is important for the modelling and remodelling of bone (Krassas and Papadopoulou 2001).

In order to estimate the activity of these cells biochemical markers are used. They are useful in assessment of bone turnover *in vivo*. The most used marker for bone formation (osteoblast activity) is BAP (bone specific alkaline phosphatase) (Nakamura 2007). The most used marker for bone resorption (osteoclast activity) is CTX (carboxyterminal telopeptide of type 1 collagen). The most used marker for vitamin D status is 25-OH D (25-Hydroxy Vitamin D).

Metabolic bone disorders

Many disorders in bone tissue are due to a disturbed bone homeostasis. One of them is osteoporosis, which is characterized by a low bone mass, microarchitectural deterioration and increased fragility and fracture risk. Osteoporosis often occurs in the elderly and is associated with low levels of estrogen. Postmenopausal women are especially predisposed to develop osteoporosis due to reduced estrogen levels. Women's bone is thinner than men's and therefore more susceptible to fracture. The incidence of fractures related to osteoporosis is highest in Scandinavia and North-America (Sambrook and Cooper 2006). Rickets and osteomalacia are both diseases caused by vitamin D deficiency. They lead to defective calcification which results in decreased amount of mineralized bone. Rickets occurs in children during bone development and osteomalacia (soft bone tissue) occurs when the bone has completed its normal development. Common alterations include deformed ribcage and soft skull bones (craniotabes). Osteopetrosis is a hereditary bone disorder originating from malfunctioning osteoclastic activity. Defective osteoclastic activity in these individuals leads to the deposition of abnormally thickened, greatly mineralized and abnormally brittle bone. Apart from increased risk of fractures, osteopetrosis implies an enhanced risk of anemia and infections in the affected individuals due to a decreased amount of red bone marrow, responsible for blood cell formation (hematopoiesis). Additional complications include compressed nerves caused by thickened bone tissue (Kumar *et al.* 2003).

SEWAGE SLUDGE

Wastewater from households and industries is transported to sewage treatment plants (STPs), were sewage sludge is formed as a by-product of processing. Sewage sludge is used as fertilizer owing to its high content of nutrients such as phosphor and nitrogen. Reuse would decrease the loading of nutrients at sea and hence the eutrophication effect. Approximately two thirds of the content in sewage sludge consists of natural organic matter, mainly carbohydrates, humic substances, lipids, and proteins (Brunner *et al.* 1988). However, it also contains metals, pathogens and medical residues which may pose a threat to human and animal health. Another component of the sewage sludge are the EDCs (endocrine disrupting chemicals), defined as exogenous substances or mixtures that alter functions of the endocrine system and cause adverse health effects in an intact organism, or its progeny, or populations (CSTEE 1999). Exposure to EDCs may disrupt endocrine controlled functions such as reproduction. Purdom *et al.* observed hermaphroditic fish in recipients of water from STPs, suggesting presence of compounds with estrogenic effects in the effluent (Purdom *et al.* 1994). A study with caged rainbow trout at the effluent of STPs showed increased vitellogenin (a protein produced in the liver in response to estradiol stimulation) concentration in the plasma of the fish. It was suggested that the effluent water contained etinylestradiol from pharmaceutical use or alkylphenol-ethoxylates from the biodegradation of surfactants and detergents during sewage treatment (Purdom *et al.* 1994).

Route of exposure

The uptake routes of sludge-borne organo-chemicals by animals can be divided into three primary pathways; direct contamination of the plants consumed, transport via soil to vegetation consumed, and ingestion of contaminated soil (Fries 1996) and in addition uptake via inhalation of volatile components (Rhind *et al.* 2005). Precipitation, plant growth and soil absorption decreases the uptake of compounds present in the sludge by animals (Fries 1996).

SEWAGE SLUDGE CONTAMINANTS AND EFFECTS

Lipophilic halogenated hydrocarbons in sludge are known to bioaccumulate. It is the bioavailability and the degradability of a substance that determines its ability to accumulate. Chemical analyses of sewage sludge of various origin have revealed fairly high concentrations of a large number of environmental contaminants such as alkyl phenols, phthalates, polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), *p,p'*- dichlorodiphenyldichloro-ethylene (*p,p'*-DDE) and metals (Brunner *et al.* 1988; Giger *et al.* 1984). Some of these might impair bone tissue homeostasis.

Alkyl phenols

Alkyl phenols are a product of industrial synthesis of detergents formed by alkylation of phenols. Sludge contains levels of alkyl phenols higher than those normally found in soil. These chemicals are toxic and readily degradable. Alkylphenols are found in PVC plastics used in the food processing and packaging industries and one, nonylphenol, is also a component in intravaginal spermicides. There is a concern that alkylphenols can mimic sexsteroids and disrupt endocrine systems. It has been demonstrated that the nonylphenol (extracted from polystyrene centrifuge tubes) cause estrogenic effects in rodents and in the human breast cancer MCF_7 cell model, *i.e.*, it induced both cell proliferation and progesterone receptor (Soto *et al.* 1991). Contaminants with estrogenic activity such as NP may cause a feminization of male fish in recipients downstream STPs and hence a reduction in reproductive success (Lee and Peart 1995).

Phthalates

Phthalates are used as softening agents in materials such as PVC (Bernes 1998). Biotransformation by microorganisms is an important degradation path of phthalates (Jianlong *et al.* 2000). One of the phthalates - di-2-ethylhexyl phthalate (DEHP) has been detected, previously, in sewage sludge (Rhind *et al.* 2007). Some phthalates are believed to disrupt reproduction and studies have confirmed that DEHP has an antiandrogen effect in male rats (Gray *et al.* 2000; Mylchreest *et al.* 1998).

PCBs

Polychlorinated biphenyls (PCBs) were used as plasticizers in plastic and in paint, etc and have been identified in sewage sludge. The theoretical number of congeners is 209 (Bernes 1998). A few PCB congeners are resistant to degradation and bioaccumulate in the environment, and many of these are very toxic to aquatic organisms and impede their reproduction. In a study on bone tissue, Lind *et al*. exposed rats to the dioxin like PCB 126 and this resulted in increased osteoid surface, cortical thickness and organic content of the tibia (Lind *et al.* 1999). In another study on female rats, Lind *et al.* showed that PCB 126 reduced the trabecular area and increased trabecular density and cortical thickness. Other effects observed were reduced maximum torque and stiffness of the humerus and serum osteocalcin levels (Lind *et al.* 2000). Ovarietomized rats exposed to PCB 126, in combination with estradiol, caused increased trabecular bone volume, compared to the sham-operated rats were the trabecular bone volume decreased, suggesting that the PCB 126 effects are dependent on the endogenous estrogen status of the exposed individual (Lind *et al.* 2004a). Another study revealed that perinatal exposure of female goats to PCB 153 but not PCB 126 disrupts bone tissue homeostasis. PCB 153 exposure resulted in significantly decreased cross sectional area, marrow cavity and moment of resistance at the diaphyseal part of the bone (Lundberg *et al.* 2006). Exposure of rat *in vivo* to the commercial PCB product Aroclor 1254 caused reduced femur length, narrowing of the marrow cavity and weaker bone (Andrews 1989).

TCDD

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is produced as a by product of organic matter combustion in the presence of chlorine. It is a contaminant in the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). 2,4,5-T that was used widely in forestry, until the 1970s when it was phased out, and 2,4,5-T was also a component in the defoliant Agent Orange used by the United States in the Vietnam War. TCDD is the most toxic member of the dioxin family and it degrades slowly and can bioaccumulate and biomagnify in the food chain. Jamsa *et al*. showed that TCDD exposure causes reduced tibial size in rats (Jamsa *et al.* 2001). Further biomechanical analyses of the tibia revealed a reduction in breaking force and stiffness. Miettinen *et al.* showed that *in utero* and lactational TCDD exposure to sensitive rat lines caused decreases in bone length, cross sectional area of cortex and in bone mineral density. Mechanic testing showed that exposure to TCDD led to a reduction in breaking force in the tibia, femur and femoral neck. The changes observed were found to be mainly reversible because one year after treatment, the dioxin effect had subsided (Miettinen *et al.* 2005).

p,p'-DDE

DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) was first used as a pesticide in World War II. DDT is metabolized in the liver to *p,p'*-DDE (*p,p'*- dichlorodiphenyldichloro-ethylene) and *p,p'*-DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethane). *p,p'*-DDD is rapidly excreted. However, both DDT and *p,p'*-DDE are stored in adipose tissues and are very slowly metabolized and excreted (Beard *et al.* 2000). In 1967 Ratcliffe showed that peregrine falcon (*Falco peregrinus*), sparrowhawk (*Accipiter nisus*) and golden eagle (*Aquila chrysaëtos*) in Britain had high concentrations of *p,p'*-DDE in their eggs which were reduced in weight and had such thin eggshell that they were broken during brooding (Ratcliffe 1967). An experiment carried out on frogs (*Rana temporiana*) showed that exposure to *p,p'*-DDE causes a decrease in cortical bone mineral density (Lundberg *et al.* 2007).

Metals

When metals reach soils or sediments they are stored for a long time before being transported further to other environmental compartments (Walker *et al.* 2006). Sewage sludge contains a lot of metals which generally originate from industrial waste. A high content of organic compounds in sewage sludge effectively binds metals that leach slowly through the soil (Walker *et al.* 2006). This implies a risk for *e.g.* cattle to be exposed via ingestion of soil while grazing (Rhind *et al.* 2002).

Risk

The application of sewage sludge to pasture has been shown to induce adverse changes in exposed animals, including behavioural disturbance and perturbed fetal testis and fetal ovary development (Erhard and Rhind 2004; Fowler *et al.* 2008; Paul *et al.* 2005). Other physiological systems may also be perturbed. Human health is at risk since we are consuming products from agriculture that may have accumulated pollutants present in sewage sludge.

AIM

The aim of this study was to study effects in bone tissue from rams and ewes bred on pastures fertilized with sewage sludge.

MATERIALS AND METHODS

The femur bones and serum used in this study were derived from Texel sheep (*Ovis aries*) bred on a pasture fertilized with sewage sludge, at the Macaulay Institute Research Station in Hartwood, Scotland. The exposure scenario of this study has been described previously (Erhard and Rhind 2004).

In brief, the pastures were fertilized with either thermally dried pellets of sewage sludge (2.25 tons per hectare, twice annually; T plots, or with conventional inorganic fertilizer containing the same amount of nitrogen; C plots. In total, 24 rams and 24 ewes were exposed *in utero*, from conception, and after birth, during lactation, until weaning at approximately 4 months of age. A total of 48 individuals were allocated to treated (T) or control (C) groups, (12 rams and 12 ewes per group). All lambs were maintained on the T or C experimental plots from conception until weaning at approximately 4 months of age. Thereafter the ram lambs were separated from the ewes and moved from the T or C plots at Hartwood Research Station to Glensaugh Research Station, Scotland, and kept on control pastures until slaughter. During the same period the ewe lambs were maintained at Hartwood on either T or C pastures. The sheep were slaughtered at 18 months of age. At this occasion blood samples were taken and serum prepared and stored at -18ºC. After the slaughter femur bones were dissected and stored at -18ºC. The exposure procedure had an ethical permit and followed U.K. legislation (Paul *et al.* 2005).

The preparation of the bone tissue material and the measurements has been described previously by Broman (2006) and Öberg (2008). In brief, femurs were moved to a refrigerator (+8ºC) and thawed approximately 24 hours prior to the analyses. The femurs length was measured using a slide calliper with an accuracy of 0.1 mm. A screw (2.5×12 mm; DynaPlus, zinc plated) inserted dorsal to the trochlea ossis femori was used as a reference point for the pQCT analyses (fig 2). The bones were covered with gauze bandage (Mollelast, elastic, Lohmann & Rauscher AG, Switzerland), moistened with Ringer solution (pH 7.4; Tris (0.3 g/l), NaCl (9 g/l), $CaCl_2{\cdot}H_2O$ (0.24 g/l), KCl (0.4 g/l)) and then wrapped in polyethylene plastic foil to prevent the femurs from drying.

Fig.2. A screw was inserted dorsal to the trochlea ossis femori at the distal end of the femur and used as a reference point in the pQCT measurements.

pQCT

pQCT (peripheral Quantitative Computed Tomography, Stratec XCT 960 A with software version 5.21, Norland Stratec Medizintechnik, Pforzheim) was used to estimate dimensions and densiometric variables of the femurs. The pQCT instrument was calibrated once a week with a hydroxyapatite phantom with a known density (Gasser 2003). The femurs were placed horizontally in the pQCT instrument with the distal end at the front and the anterior side up. The position of the bone was examined with the scout view of the pQCT. The femurs were analysed using voxelsize 590 µm. The settings used were peel mode 2, contour mode 1, threshold 270 mg/cm^3, inner threshold 500 mg/cm^3 and outer threshold 690 mg/cm^3.

Metaphysis

The metaphysis of the bone was analysed by examining a 3 mm thick slice at 3.6 % of the total bone length from the reference point located at the trochlea ossis femori in the distal part of the femur. At this site there is a continuous layer of cortical bone surrounding the inner trabecular bone. The variables evaluated were total bone mineral content (BMC, mg/mm), total bone mineral density (BMD, mg/cm^3), trabecular (trab) BMC (mg/mm), trab BMD (mg/cm^3), total cross sectional area (CSA, mm^2), trab CSA (mm^2), and periosteal circumference (PERIC, mm).

Diaphysis

The diaphysis of the bone was analysed by examining a 1 mm thick slice at 50 % of the total bone length. This site mainly consists of cortical (cort) bone. The variables evaluated were total BMC (mg/mm), total BMD (mg/cm^3), total CSA (mm^2), cort BMC (mg/mm), cort BMD (mg/cm^3), cort CSA (mm^2), cort thickness circular ring model (cortTHKC, mm), PERIC (mm), endosteal circumference (ENDOC, mm), moment of resistance (RPCMW, mm^3), polar moment of inertia (IPCMW, mm^4) and marrow cavity (mm^2).

Reproducibility

To evaluate the reproducibility of the pQCT measurements, the coefficients of variation (CV %= standard deviation ×100/mean) for the different variables were calculated from 10 repeated measurements with a single sample being repositioned before each measurement. The CV for the ten different pQCT measurements at the metaphyseal measure point was as follows: 0.4 % (total BMC), 0.3 % (total BMD), 1.5 % (trab BMC), 1.5 % (trab BMD), 0.6 % (total CSA), 1.1 % (trab CSA), 0.3 % (PERIC). The CV for the ten different pQCT measurements at the diaphyseal measure point was as follows: 0.2 % (total BMC), 0.2 % (total BMD), 0.2 % (total CSA), 0.2 % (cort BMC), 0.2 % (cort BMD), 0.3 % (cort CSA), 0.5 % (cortTHKC), 0.1 % (PERIC), 0.3 % (ENDOC), 0.3 % (RPCMW), 0.3 % (IPCMW), and 0.2 % (marrow cavity).

THREE-POINT BENDING TEST

The three-point bending test (mid-part bent until fracture) measurements were conducted at the mid-diaphyseal part of the femur. A vertical load with an axial capacity of 10 000 N was applied using a MTS 858 Mini Bionix with hydraulic grip control (Avalon Technologies, MN, USA). The span length was 100 mm and the loading speed 1 mm/s. In brief, approximately 24 hours prior to analyses the bones were thawed at +8ºC. The load was applied on the same point used in the diaphyseal pQCT measurement (50 % of the total bone length). The load (N) applied and the displacement (mm) of the bone was recorded and the stiffness (N/mm) and energy absorption (N×mm) calculated.

BONE MARKERS

BAP

The analysis of the levels of the biochemical marker BAP (bone specific alkaline phosphatase, Ostase BAP Immunoenzymetric Assay) for bone formation (osteoblast activity) was performed according to the manufacturer's instructions (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). This technique allows the identification of alkaline phosphatase produced by the liver and bone, respectively. Briefly, 50 µL of standards, controls and serum specimen (duplicates) were incubated with 100 µL of conjugate (primary antibody) for one hour at +18-25˚C. After washing, the specimens were incubated for 15 minutes at +18-25˚C. After stopping the reaction the absorbance was measured at 450 and 650 nm (Molecular Devices Kinetic Reader). The results were analysed using point-to-point curve fit (SoftMax Pro). A standard curve was retrieved and used for the determination of the concentration (µg BAP/L) in the serum samples.

CTX

The levels of the CTX (carboxyterminal telopeptide of type 1 collagen) were analysed according to the manufacturer's instructions (Serum Crosslaps ELISA, Nordic Bioscience Diagnostics A/S, Herlev, Denmark). This is an *in vitro* enzyme immunological test for the quantification of degradation products of CTX. Briefly, 50 µL (duplicates) of standards, controls and specimens and 150 µL of antibody solution were incubated at +18-22 ºC on a 96-well plate for two hours. After washing and adding 100 µL of substrate solution the plate was incubated for 15 minutes at +18-22˚C in the dark. After quenching the reaction the absorbance was measured at 450 and 595 nm (Tecan Magellan Plate Reader). The results were analysed using quadratic curve fit (Assay Zap software 3.1, Biosoft, Cambridge, UK). A standard curve was retrieved with absorbance values from the standards and the CrossLaps concentrations. Serum concentrations (ng/mL) were determined by the equation of the curve.

25-OH D

Concentrations of vitamin D were analysed according to the manufacturer's instructions (25-Hydroxy Vitamin D (25-OH D) EIA, Immunodiagnostic Systems Ltd). This is an enzyme immunoassay for determination of 25-OH D and other hydroxylated metabolites. Briefly, 200 μL of calibrators and specimen were incubated at +18-25˚C on a 96-well plate for two hours. After washing, 200 μL enzyme conjugate was added and the plate was incubated at +18-25˚C for 30 minutes. After a second washing 200 μL TMB (tetramethylbenzedine) was added and the plate was incubated at +18-25˚C for 30 minutes. After quenching the reaction, the absorbance was measured at 450 and 650 nm (Tecan Magellan Plate Reader). The results were analysed using four parameter logistic curve fit (4PL, Assay Zap software 3.1, Biosoft, Cambridge, UK). A standard curve with percent binding (B/B %=(mean absorbance/(mean absorbance for 0 calibrator)×100) of each calibrator was retrieved. The standard curve was used for the determination of the concentration of 25-OH D (nmol/L) in the samples.

STATISTICS

The results were evaluated with t-test (Graph Pad Prism, version 4.03) and analysis of covariance (ANCOVA, StatView, version 5.0; SAS Institute Inc., Cary, NC, USA). ANCOVA were used to adjust for differences in weight between animals of the same gender. $P<0.05$ was considered significant.

RESULTS

pQCT

Metaphysis

The results from the metaphyseal measurements on femurs from rams are presented in table 1. Sewage sludge exposure significantly increased the total BMD (10.5 %, p<0.05), reduced total CSA (11.5 %, p<0.05), trab CSA (17.1 %, p<0.05), and PERIC (5.7 %, p<0.05).

Table 1. Results obtained from pQCT (peripheral quantitative computed tomography) analysis of the rams at the metaphyseal measurements at 3.6 %, of the total bone length from the reference point towards the diaphysis. The dams of the treated rams had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Dams of control rams had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the rams started *in utero*, from conception, and after birth during period of lactation to weaning at approximately 4 months of age. Thereafter the ram lambs were moved from the treated or control pastures and kept on control pastures until slaughter. The rams were slaughtered at the age of 18 months. n=number of individuals; BMC=bone mineral content; BMD=bone mineral density; trab=trabecular; CSA=cross sectional area; PERIC=periosteal circumference.

RAM	**Control**	**Treated**	**p-value**	
Metaphysis	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
Weight (kg)	77.9 ± 1.0	80.3 ± 1.8	0.2506	**-**
Total BMC (mg/mm)	432.9 ± 14.0	427.6 ± 9.4	0.5872	0.4207
Total BMD (mg/cm³)	459.7 ± 15.7	508.0 ± 11.1	< 0.0001	**0.0089**
Trab BMC (mg/mm)	152.2 ± 9.8	140.0 ± 8.1	0.1152	0.1742
Trab BMD (mg/cm³)	264.6 ± 18.4	281.6 ± 10.1	0.1630	0.3620
Total CSA (mm²)	945.2 ± 21.1	847.8 ± 29.6	< 0.0001	**0.0002**
Trab CSA (mm²)	583.8 ± 24.7	498.6 ± 26.5	0.0001	**0.0017**
PERIC (mm)	108.9 ± 1.2	103.0 ± 1.8	< 0.0001	**0.0002**

The results from the metaphyseal measurements on femurs from ewes are presented in table 2. Sewage sludge exposure significantly increased the trab BMC (41.6 %, $p<0.05$).

Table 2. Results obtained from pQCT (peripheral quantitative computed tomography) analysis of the ewes at the metaphyseal measurements at 3.6 % of the total bone length from the reference point towards the diaphysis. The treated ewes had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the treated ewes began *in utero* and continued until approximately 18 months of age. The ewe lambs were slaughtered at the age of 18 months. n=number of individuals; BMC=bone mineral content; BMD=bone mineral density; trab=trabecular; CSA=cross sectional area; PERIC=periosteal circumference.

EWE	**Control**	**Treated**	**p-value**	
Metaphysis	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
Weight (kg)	63.6 ± 1.0	61.2 ± 1.2	0.1306	-
Total BMC (mg/mm)	363.7 ± 7.2	346.0 ± 9.6	0.0135	0.4812
Total BMD (mg/cm³)	510.7 ± 15.5	473.7 ± 13.6	0.0024	0.2729
Trab BMC (mg/mm)	74.6 ± 4.9	105.6 ± 9.9	< 0.0001	**0.0366**
Trab BMD (mg/cm³)	201.3 ± 8.7	234.6 ± 12.2	0.0004	0.0886
Total CSA (mm²)	719.3 ± 25.2	736.5 ± 27.3	0.4308	0.7439
Trab CSA (mm²)	371.9 ± 22.8	442.7 ± 27.2	0.0009	0.1615
PERIC (mm)	94.9 ± 1.6	96.0 ± 1.8	0.4294	0.7394

Diaphysis

The results from the diaphyseal measurements on femurs from rams are presented in table 3. Sewage sludge exposure significantly increased the total BMD (13.8 %, $p<0.05$) and cortTHKC (11.8 % $p<0.05$). Analysis of the diaphyseal measure point furthermore revealed significantly reduced total CSA (12.1 %, $p<0.05$), PERIC (5.9 %, $p<0.05$), ENDOC (12.3 %, $p<0.05$), RPCMW (9.4 %, $p<0.05$), IPCMW (14.3 %, $p<0.05$), and marrow cavity (25.8 %, $p<0.05$).

Table 3. Results obtained from pQCT (peripheral quantitative computed tomography) analysis of the rams at the diaphyseal measurements at 50 % of the total bone length. The dams of the treated rams had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Dams of control rams had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the rams started *in utero*, from conception, and after birth during period of lactation to weaning at approximately 4 months of age. Thereafter the ram lambs were moved from the treated or control pastures and kept on control pastures until slaughter. The sheep were slaughtered at the age of 18 months. n=number of individuals; BMC=bone mineral content; BMD=bone mineral density; CSA=cross sectional area; cort=cortical; THKC=thickness circular ring model; PERIC=periosteal circumference; ENDOC=endosteal circumference; RPCMW=moment of resistance; IPCMW=polar moment of inertia.

RAM	**Control**	**Treated**	**p-value**	
Diaphysis, 50 %	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
Weight (g)	77.9 ± 1.0	80.3 ± 1.8	0.2506	**-**
Total BMC (mg/mm)	380.4 ± 10.0	384.6 ± 8.5	0.7498	0.8033
Total BMD (mg/cm³)	643.6 ± 16.9	732.3 ± 15.9	0.0009	**0.0001**
Total CSA (mm²)	592.9 ± 14.2	528.8 ± 19.1	0.0134	**<0.0001**
Cort BMC (mg/mm)	338.7 ± 10.0	346.3 ± 7.8	0.5560	0.9119
Cort BMD (mg/cm³)	1315.7 ± 3.9	1321.6 ± 4.0	0.2974	0.0731
Cort CSA (mm²)	257.4 ± 7.4	262.1 ± 6.2	0.6292	0.9415
CortTHKC (mm)	3.4 ± 0.1	3.8 ± 0.1	0.0079	**0.0107**
PERIC (mm)	86.2 ± 1.0	81.4 ± 1.4	0.0124	**<0.0001**
ENDOC (mm)	64.8 ± 1.3	57.7 ± 1.6	0.0017	**<0.0001**
RPCMW (mm³)	2516.8 ± 84.3	2299.5 ± 110.7	0.1325	**0.0090**
IPCMW (mm^4)	39493.3 ± 1728.7	34550.9 ± 2170.5	0.0887	**0.0025**
Marrow cavity (mm²)	335.6 ± 12.9	266.7 ± 14.7	0.0019	<**0.0001**

The results from the diaphyseal measurements on femurs from ewes are presented in table 4. Sewage sludge exposure caused no significant differences between the two groups of ewes.

Table 4. Results obtained from pQCT (peripheral quantitative computed tomography) analysis of the ewes at the diaphyseal measurements at 50 % of the total bone length. The treated ewes had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the treated ewes began *in utero* and continued until approximately 18 months of age. The ewes were slaughtered at the age of 18 months. n=number of individuals; BMC=bone mineral content; BMD=bone mineral density; CSA=cross sectional area; cort=cortical; THKC=thickness circular ring model; PERIC=periosteal circumference; ENDOC=endosteal circumference; RPCMW=moment of resistance; IPCMW=polar moment of inertia.

EWE	**Control**	**Treated**	**p-value**	
Diaphysis, 50 %	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
Weight (g)	63.6 ± 1.0	61.2 ± 1.2	0.1306	-
Total BMC (mg/mm)	305.2 ± 6.7	285.5 ± 6.8	0.0512	0.2217
Total BMD (mg/cm³)	671.1 ± 14.7	659.0 ± 17.4	0.6018	0.9031
Total CSA (mm²)	456.5 ± 11.7	434.7 ± 9.1	0.1545	0.2876
Cort BMC (mg/mm)	271.5 ± 6.7	254.0 ± 6.8	0.0796	0.3437
Cort BMD (mg/cm³)	1322.3 ± 8.8	1316.2 ± 5.9	0.5704	0.9996
Cort CSA (mm²)	205.2 ± 4.6	192.9 ± 4.8	0.0738	0.3144
CortTHKC (mm)	3.1 ± 0.1	3.0 ± 0.1	0.2582	0.8029
PERIC (mm)	75.7 ± 1.0	73.9 ± 0.8	0.1618	0.2985
ENDOC (mm)	56.1 ± 1.1	55.0 ± 1.0	0.4857	0.4362
RPCMW (mm³)	1757.4 ± 61.9	1572.6 ± 49.4	0.0291	0.2516
IPCMW (mm^4)	23979.2 ± 1056.0	21456.6 ± 813.7	0.0717	0.2516
Marrow cavity (mm²)	251.3 ± 9.7	241.8 ± 8.9	0.4802	0.4205

THREE-POINT BENDING TEST

The results from the three-point bending test on femurs from rams are presented in table 5. Sewage sludge exposure significantly increased the femoral stiffness (6.4 %, $p<0.05$) in the rams.

Table 5. Results obtained from the three-point bending test conducted in a MTS 858 Mini Bionix with hydraulic grip control of rams at the diaphyseal measure point at 50 % of the total bone length. The dams of the treated rams had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Dams of control rams had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the rams started *in utero*, from conception, and after birth during period of lactation to weaning at approximately 4 months of age. Thereafter the ram lambs were moved from the treated or control pastures and kept on control pastures until slaughter. The rams were slaughtered at the age of 18 months. n=number of individuals.

RAM	**Control**	**Treated**	**p-value**	
Diaphysis, 50 %	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
Weight (kg)	77.9 ± 3.4	80.3 ± 6.2	0.2506	-
Displacement (mm)	4.2 ± 0.4	4.4 ± 0.2	0.8953	0.5213
Load (N)	5440.7 ± 320.3	6396.7 ± 153.6	0.0240	0.5806
Energy (N×mm)	15823.9 ± 1744.6	16663.1 ± 1316.6	0.9929	0.1206
Stiffness (N/mm)	2570.4 ± 84.9	2734.4 ± 124.2	0.6085	**0.0094**

The results from the three-point bending test on femurs from ewes are presented in table 6. Sewage sludge exposure significantly decreased the load at failure (17.3 %, $p<0.05$) and the stiffness (10.7 %, $p<0.05$).

Table 6. Results obtained from the three-point bending test conducted in a MTS 858 Mini Bionix with hydraulic grip control of ewes at the diaphyseal measure point at 50 % of the total bone length. The treated ewes had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the treated ewes began *in utero* and continued until approximately 18 months of age. The ewe lambs were slaughtered at the age of 18 months. n=number of individuals.

EWE	**Control**	**Treated**	**p-value**	
Diaphysis, 50 %	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
Weight (kg)	63.6 ± 3.5	61.2 ± 4.0	0.1306	-
Displacement (mm)	3.5 ± 0.2	4.0 ± 0.3	0.1854	0.9536
Load (N)	4851.4 ± 139.3	4134.5 ± 194.0	0.0271	**0.0043**
Energy (N×mm)	10236.9 ± 583.2	11884.9 ± 970.3	0.0690	0.1357
Stiffness (N/mm)	2183.1 ± 125.4	1971.8 ± 110.7	0.5430	**0.0468**

BONE MARKERS

The results from the analysis of bone-specific alkaline phosphatase (BAP), carboxyterminal telopeptide of type 1 collagen (CTX) and 25-hydroxy vitamin D (25-OH D) in serum from rams and ewes are presented in table 7-8. Sewage sludge exposure caused no significant difference between exposed and control animals, neither between rams nor between ewes.

Table 7. Results obtained from the serum analysis of rams. The dams of the treated rams had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Dams of control rams had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the rams started *in utero*, from conception, and after birth during period of lactation to weaning at approximately 4 months of age. Thereafter the ram lambs were moved from the treated or control pastures and kept on control pastures until slaughter. The rams were slaughtered at the age of 18 months. n=number of individuals; BAP=bone-specific alkaline phosphatase; CTX=carboxyterminal telopeptide of type 1 collagen; 25-OH D=25-hydroxy vitamin D.

BONE MARKER	**Control**	**Treated**	**p-value**	
	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
BAP (ng/ml)	14.5 ± 1.1	17.7 ± 2.4	0.2289	0.7061
CTX (ng/ml)	1.2 ± 0.2	1.1 ± 0.1	0.5113	0.6597
25-OH D (nmol/l)	19.4 ± 2.1	19.6 ± 2.1	0.9138	0.4715

Table 8. Results obtained from the serum analysis of ewes. The sheep had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). The treated ewes had been bred on a pasture fertilized with sewage sludge twice annually (2.25 tonnes of dry matter/ha). Control ewes had been grazing on pasture fertilized with conventional, inorganic fertilizer. The exposure of the treated ewes began *in utero* and continued until approximately 18 months of age. The ewes were slaughtered at the age of 18 months. n=number of individuals; BAP=bone-specific alkaline phosphatase; CTX=carboxyterminal telopeptide of type 1 collagen; 25-OH D=25-hydroxy vitamin D.

BONE MARKER	**Control**	**Treated**	**p-value**	
	Mean ± SE (n=12)	Mean ± SE (n=12)	t-test	ANCOVA
BAP (ng/ml)	15.1 ± 1.4	18.1 ± 1.9	0.3134	0.7171
CTX (ng/ml)	0.6 ± 0.1	0.6 ± 0.1	0.1728	0.6928
25-OH D (nmol/l)	24.3 ± 2.8	25.0 ± 3.4	0.9333	0.8610

DISCUSSION

The exposed rams exhibited multiple effects in the diaphysis of femur: *e.g.* decreased CSA, reduced marrow cavity, increased cortical thickness (fig 3), and increased stiffness. The increased cortical thickness and decreased marrow cavity indicate a reduced bone resorption at the endosteum (fig 3).

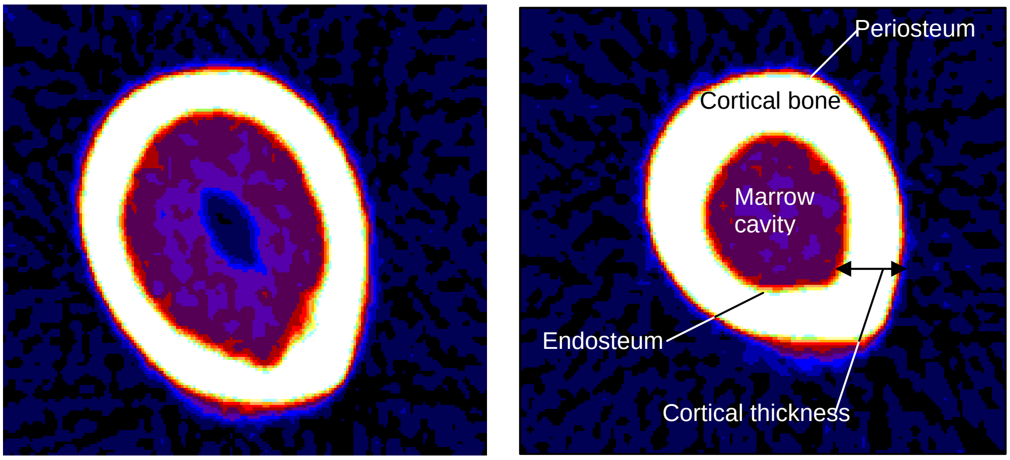

Fig. 3. Representative images from the pQCT scan at the 50 % measure point from male sheep. The white outer part is cortical bone and the inner darker part is the marrow cavity. To the left control (22059) and to the right treated (22049). Note the small area of the marrow cavity and the shorter endosteal circumference and periosteal circumference in the treated specimen. Also note the increased cortical thickness in the treated specimen.

This is the first study on adult rams within this project. In a previous experiment Öberg investigated effects of sewage sludge on ram foetuses (110 days post mating), exposed *in utero*, and whose dams had been reared on sewage sludge treated pastures since the age of 18 months. The exposure resulted in increased diaphyseal total BMC (Öberg 2008).

The ewes in the present study exhibited no effects at the diaphysis in the pQCT-measurements. These results differ from the ones presented by Broman which indicated an estrogenic effect by compounds present in the sewage sludge manifested by increased cort CSA at the diaphysis and significantly increased maximal energy absorption (energy to failure) (Broman 2006). The three-point bending test showed that the exposed ewes of the present study had more fragile bone (decreased load at failure and decreased stiffness). Similar effects have been revealed by Lind *et al.* in a study were PCB 126 exposure reduced the maximum torque and stiffness of the humerus in exposed female rats (Lind *et al.* 2000). In another study, rats were exposed to the PCB containing Arochlor 1254 and the exposure caused weaker bone (Andrews 1989). Other agents that may impair mechanical characteristics in ewes include dioxins. Studies on rats exposed to TCDD have shown a lowering of tibial breaking force (Jamsa *et al.* 2001; Miettinen *et al.* 2005).

Evaluation of the pQCT scans on metaphyseal bone showed an increase in trab BMC in the ewes. This resembles an estrogenic effect similar to that found in female goat offspring exposed perinatally to PCB 153 (Lundberg *et al.* 2006).

The differences in responses between rams and ewes in the present study might be caused by the deviating exposure scenarios. During development *in utero,* and during lactation, the ram and ewe lambs were exposed to the same batch of sewage sludge. After weaning at approximately 4 months of age however, the rams were no longer exposed to the contaminants contained in sewage sludge, whereas the ewes were maintained on the treated pastures until 18 months of age.

A large number of anthropogenic compounds are present in the sewage sludge (Brunner *et al.* 1988) and some of them might have synergistic effects. Based on the results of the present study it is not possible to identify the agent(s) causing the effects on bone tissues. The sewage sludge used in this study was not chemically analysed, but its content is most likely similar to sludge used in earlier studies *e.g.* (Paul *et al.* 2005; Rhind *et al.* 2005). Earlier analyses of sewage sludge used as fertilizer have revealed the presence of several PCB congeners (28, 52, 101, 118, 138, 153, 180), with the highest concentration of 24.3 µg/kg dry matter in sewage sludge of the treated plots compared to 0.25 µg/kg dry matter in inorganic fertilizer of the control plots (Rhind, personal communication) The composition of sewage sludge used as fertilizer is likely to differ between batches due to coincidental fluctuations in emissions. Furthermore, the functioning of the sewage treatment plants regarding *e.g.* capacity and efficiency in the cleaning process may affect the content in the sewage sludge.

Among the compounds analysed in sewage sludge, PCB 153, NP and DEHP might induce the effects on bone tissue that were observed. In a recent study Arvidsson discovered increased cortical thickness in ewe foetuses exposed to PCB 153 (Arvidsson 2008). NP can cause estrogenic effects (Soto *et al.* 1991) and has been found in sewage sludge in concentrations between 50 and 250 mg/kg (Rhind *et al.* 2005). DEHP can cause antiandrogenic effects (Gray *et al.* 2000; Mylchreest *et al.* 1998) and has been found in sewage sludge in concentrations between 50 and 250 mg/kg (Rhind *et al.* 2005) .

A part of the stimulation of bone formation is due to mechanical stress exerted on the bone by the individual body weight. Thus, a heavier animal is more likely to attain thicker and denser bone due to mechanical stress. Even though the treated rams were slightly heavier than the controls they had generally lower values in the pQCT variables, which is somewhat unexpected.

The serum levels of the bone markers (BAP, CTX, and 25-OH D) did not differ between exposed and control animals, neither between rams nor between ewes. The information obtained from the serum analyses is difficult to interpret. The blood samples were taken on the day of slaughter. Regarding the rams, this single blood sample was taken about 14 months after the end of the exposure. The levels and variations over time (diurnal, seasonal) of the bone markers in sheep are not known. However, further studies on sheep bred on pastures treated with sewage sludge should consider using urine, if possible, instead of blood for analyses of bone markers. These urine samples should be taken several times during the exposure period. This would decrease the amount of variation and allow monitoring of bone marker production over time.

The results presented in this report support earlier findings within this project, revealing effects on bone tissue in grazing animals, suggesting that human health might be at risk since we consume meat from animals and crops grown on fields fertilized with sewage sludge and that may have accumulated inorganic and organic pollutants present in the sludge. A continuous use of sewage sludge in agriculture requires further knowledge on routes of exposure and accumulation properties of pollutants commonly found in sewage sludge (Rhind *et al.* 2005). Also, additional knowledge about effects of these compounds is needed.

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