Survey of Hormone Activities in Municipal Biosolids and Animal Manures

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ABSTRACT: The potential exists for natural or synthetic hormonal chemicals present in agricultural fertilizers to be transferred to adjacent aquatic environments in order to alter endocrine function in exposed wildlife. Recombinant yeast and mammalian cell line (BG1Luc4E$_2$) assays were used to screen crude organic extracts of municipal biosolids and animal manures for estrogen-, androgen-, and progesterone receptor gene transcription activities. Of the biosolid extracts, those samples that had undergone aerobic digestion had no or minimal estrogen- and no androgen receptor gene transcription activities. In contrast, those biosolid samples that had undergone anaerobic digestion had much higher estrogen- and, for all but one site, androgen receptor gene transcription activities. Extracts prepared from animal manure samples had variable levels of androgen- and estrogen receptor gene transcription activities, which may be related to the type, sex, age, and reproductive status of the animals. The diet and treatment of animals with hormone implants also appeared to be factors influencing hormone activity in animal manure. Progesterone receptor gene transcription activity was observed for only one chicken litter sample. Overall, results of this study suggest that *in vitro* bioassays can be used to survey and detect hormone activity in municipal biosolids and animal manures. Furthermore, results of these assays can be used to develop

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practices that will minimize the potential environmental endocrine-disrupting effects of these substances. Environ Toxicol 19: 216–225, 2004.
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INTRODUCTION

The potential endocrine-disrupting effects of municipal sewage biosolids and animal manures used as agricultural fertilizers have become an environmental concern. The impact of runoff contaminated with biosolids or animal manures on aquatic and terrestrial ecosystems or on the health of wildlife is largely unknown. However, exposure of various organisms to exogenous natural and synthetic hormones has been shown to have a variety of developmental and physiological effects (reviewed in Lange et al., 2002). For example, studies conducted by Irwin et al. (2001) detected significantly elevated levels of vitellogenin in female turtles residing in ponds in cattle pastures. It was suggested that this may lead to impairment of the turtles' reproductive fitness by changing energy allocation, physiology, or egg production. In another study, approximately 3- to 4-fold-higher levels of estrogens were detected in treated sewage water from an agricultural settlement compared to an urban settlement (Shore et al., 1993a). Therefore, further studies to characterize the potential endocrine-disrupting activities of municipal biosolids and animal manures are warranted.

Little is known about the concentrations and activities of hormones and hormonally active substances in municipal biosolids. Using recombinant yeast estrogen receptor gene transcription assays, Holbrook et al. (2002) determined that biosolids contributed between 5% and 10% of the estrogenic activity of influent wastewater delivered to a municipal sewage treatment plant. In contrast, treated liquid effluent contained between 25% and 43% of the estrogenic activity, and 51%–67% of the estrogenic activity was biodegraded during the wastewater or biosolid treatment processes. Using chemical analyses, Andersen et al. (2003) confirmed that only ~5% of wastewater estrogens are sorbed by digested sewage sludge. The persistence of the estrogenic activity contained in the biosolid fraction when used as an agricultural fertilizer is unknown.

To our knowledge, only one study has thoroughly examined the fate of androgens during wastewater treatment. Thomas et al. (2002) used a yeast-based androgen receptor gene transcription assay to detect androgenic activity in sewage treatment plant effluents and receiving estuaries. The highest androgenic activity was associated with plants that only used primary treatment of the sewage prior to discharge, whereas the lowest activity was observed for plants where the sewage received more advanced treatment. Using a toxicity identification evaluation (TIE) approach, it was determined that essentially all the androgenic compounds identified were naturally produced steroids or steroid metabolites.

Much more information is available regarding the concentrations of hormones and hormonally active substances in livestock manures. It has been known for decades that farm animals excrete large amounts of hormones that for some species are in the range of milligrams per animal per day (Lange et al., 2002, reviewed in Hanselman et al., 2003). In addition to naturally produced hormones, many farm animals are also treated with exogenous sources of hormones or synthetic substances to synchronize reproductive cycles and improve feed efficiency. In particular, the widely used growth promoter trenbolone acetate (TB) has potent androgenic and antiglucocorticoid activities (Wilson et al., 2002; reviewed in Schiffer et al., 2001). Because the current trend is toward the use of exogenous hormones and intensive farming practices, the potential for adverse effects from the use of animal manure on agricultural lands adjacent to environmentally sensitive areas cannot be discounted.

The objective of the present study was to use existing hormone receptor gene transcription assays to conduct a representative survey of municipal biosolid and animal manure samples for estrogen-, androgen-, and progesterone receptor gene transcription activities. Biosolid samples from sewage treatment plants using different processes and manure samples from a variety of livestock classes representative of typical farm practices were studied. Results from this study will help to ascertain whether these substances when used as agricultural fertilizers potentially may have endocrine-disrupting effects in the environment. Furthermore, practices that could be used to reduce or eliminate these sources may be identified.

MATERIALS AND METHODS

Chemicals

Oxalyticase was obtained from Enzogenetics (Corvallis, OR, USA). Sodium dodecyl sulfate (SDS) and β-mercaptoethanol came from BioRad (Mississauga, Ontario, Canada). Minimal essential medium (MEM), L-glutamine, and phosphate-buffered saline were obtained from Invitrogen (Burlington, Ontario, Canada). Charcoal dextran–stripped and research-grade fetal bovine sera (FBS) came from Gemini Bio-Products (Woodland, CA, USA). Passive lysis buffer and luciferase assay reagent were from Promega (Madison, WI, USA). The Coomassie protein assay kit was

from Pierce (Rockford, IL, USA). Ethyl acetate was from Caledon (Georgetown, Ontario, Canada) and absolute ethanol from Commercial Alcohols, Inc. (Brampton, Ontario, Canada). Radiolabeled hormones were from Perkin Elmer Life and Analytical Sciences Inc., Woodbridge, Ontario, Canada). All other chemicals were obtained from Sigma-Aldrich Canada Inc. (Oakville, Ontario, Canada).

Sample Collection and Extraction

Biosolid and animal manure samples were collected in polypropylene containers, frozen immediately and shipped by overnight delivery to the Southern Crop Protection and Food Research Centre (London, Ontario, Canada). For both manure and biosolid samples, one 5-g (wet weight) portion was weighed into a clean glass vial for extraction and a second 5-g portion was dried overnight (16–20 h, 103°C) and reweighed to determine the dry weight of the sample. For all samples, except chicken litter, 10 mL of ethyl acetate was added, and the mixture was shaken vigorously by hand for 20 sec. The vial was transferred to a Burrell wrist action shaker (Burrell Corp., Pittsburgh, PA, USA) and vigorously shaken automatically for 10 min. The sample was then centrifuged [Sorvall Kendro Laboratory Products, Asheville, NC, USA, GLC-1, 900 rpm (164 g)], for 10 min after which the supernatant was transferred into a clean glass vial. The extraction process was repeated for a total of 3 times. Because of the large volume of material, chicken litter samples, were initially extracted with 20 mL of ethyl acetate, with 15 mL of ethyl acetate used for the last two extractions. For all samples, the pooled supernatant was dried under nitrogen in a 37°C water bath. The dried extracts were stored at −7°C. For use in the bioassays, the extracts were resuspended in 1 mL of absolute ethanol, and serial dilutions were prepared.

Extraction recovery of 17β-estradiol, progesterone, and testosterone from three representative samples of each type of manure or biosolid was determined as follows. Known concentrations of radiolabeled hormone standards were added to triplicate 5- to 10-g portions (wet weight) of each matrix and mixed thoroughly. The samples were immediately extracted as described above. For biosolid, swine manure, and chicken litter samples, the dried extract was resuspended in 1 mL of absolute ethanol, and 10 mL of UniverSol scintillation cocktail (ICN, Cosa Mesa, CA, USA) was added to each sample. Radioactivity was measured with a Model LS 6500 liquid scintillation counter (Beckman Coulter, Irvine, CA, USA). Because of the high levels of background luminescence, analysis by liquid scintillation counting of beef and dairy manure extracts was impossible. It was necessary to analyze these extracts by reverse-phase high-performance liquid chromatography with radioactivity detection (HPLC-RD; EG&G Berthold LB509 Radioflow Detector, Berthold GMBH & Co. KG., Bad Wildbad, Germany). Mean percent recoveries (± standard deviation) for 17β-estradiol, progesterone, and testosterone were, respectively: biosolids—60.8 ± 7.6, 80.2 ± 1.2, 66.7 ± 9.1; swine manure—71.2 ± 1.2, 94.9 ± 2.8, 86.7 ± 5; chicken litter—54.0 ± 7.5, 64.7 ± 1.9, 61.7 ± 21.8; beef cattle manure—38.0 ± 4.9, 78.6 ± 4.0, 88.8 ± 4.4; dairy cow manure—53.5 ± 3.6, 92.9 ± 4.8, 90.1 ± 2.7.

Bioassays

Recombinant Yeast Assays

Recombinant yeast strains BJ3505 (estrogen) and YPH500 (androgen and progesterone) were transformed as described previously and kindly provided by Dr. K .W. Gaido and Dr. D. P. McDonnell (Gaido et al., 1997). The yeast were maintained and used for hormone receptor gene transcription assays in a modified version (described below) of that described previously (Gaido et al., 1997) to allow the use of 96-well plates for both extract exposure and end-point spectrophotometer measurements. Briefly, 10 μL of each serial dilution of the hormone standards (17β-estradiol, testosterone, or progesterone—included on each plate) or sample extract was pipetted into duplicate wells of 96-well plates. The plates were allowed to dry for 30 min. Copper sulfate (0.1 mM, final) was added to yeast grown in their respective selective media ($OD_{600\ nm}$ = 0.8–1.0), and 200 μL was pipetted into each well. The plates were shaken for 2 min, then incubated in a humidified container overnight at 30°C without shaking. After ~19–20 h, the yeast in each well were resuspended by pipetting, and for estrogen and androgen assays, 100 μL was transferred to each well of a second 96-well plate. Because the color development of the progesterone assays proceeded more quickly, the yeast were diluted 1:1 by transferring 50 μL of the yeast suspension to a second 96-well plate containing 50 μL of progesterone receptor selective medium per well. The colorimetric reaction was started by the addition of 100 μL of assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2 mg/mL 2-nitrophenyl- β-D-galactopyranoside (ONPG), 0.1% SDS, 50 mM β-mercaptoethanol, 200 U/mL oxalyticase). The plates were shaken for 2 min, then left at room temperature. After 40 min, absorbance at 415 and 595 nm was measured using a microtiter plate reader (Bio-Rad, Mississauga, Ontario, Canada).

Human Ovarian Carcinoma Cell Line (BG1Luc4E$_2$) Assay

BG1Luc4E$_2$ cells were kindly provided by Dr. M. S. Denison and Dr. P. A. Harper. Cells were maintained and exposed to test substances as described previously (Rogers and Denison, 2002) with minor modifications. Cells were plated in 12-well plates at 6×10^4 cells/well in MEM supplemented with 10% FBS. After 24 h the medium was

removed and replaced with estrogen-stripped medium (ESM, MEM without phenol red supplemented with 2 mM glutamine and 10% charcoal-stripped FBS). ESM was removed from the wells 24 h later, and the cells were exposed to fresh ESM treated with serial dilutions of a 17β-estradiol standard (included in each assay) or sample extracts. After an exposure of 24 h, the medium was removed, and the wells were washed twice with phosphate buffered saline. Passive lysis buffer (250 μL) was added to each well, and the plates were rocked at room temperature for 20 min. The cell lysates were transferred to microcentrifuge tubes on ice, then stored at -80°C until analyzed. Just prior to analysis the samples were thawed rapidly in a room-temperature water bath and then mixed for 10–15 s with a vortex mixer. The samples were centrifuged at 12,000g for 15 s, and the supernatants were transferred to clean tubes. Luciferase activities of the cleared lysates (50 μL) were determined after injection of 50 μL luciferase assay reagent using a 96-well luminometer plate reader (Dynex Technologies, Chantilly, VA, USA, MLX luminometer, delay time 2 s, integration time 10 s). Protein concentrations of cleared lysates were determined using a Coomassie assay.

Data Calculations

Full concentration–response data were obtained for each extract tested in the bioassays. For the recombinant yeast assays, the data for each well were corrected by dividing the absorbance measured at 415 nm for the substrate (ONPG) by the absorbance measured at 595 nm for yeast density. A logistic curve was used to fit the data and to determine curve parameters. Standard EC_{20} values, corresponding to the concentration of the standard required to elicit a response equal to 20% of the maximal response, were calculated for each 96-well plate. Because the extracts had variable maximal responses, their potencies were calculated by determining the dilution at which their responses were equal to 20% of the maximum of the standard for that plate (extract EC_{20}), as described previously (Metcalfe et al., 2001). Although the responses observed represent the combined activity of all hormone receptor agonists (and possibly antagonists) present in the biosolid or manure samples, the hormone receptor gene transcription activities were expressed as the concentration of pure standard hormone required to elicit the same response. Calculation of these hormone "equivalents" involved the following equation:

$$\begin{aligned}\text{Hormone equivalents (ng/g)} &= [\text{standard } EC_{20}\ (\text{ng/mL})/\text{extract } EC_{20}] \\ &\times [\text{volume of assay medium (mL)}/ \\ &\quad \text{volume of extract tested } (\mu\text{L})] \\ &\times [\text{volume of stock extract} \\ &\quad (\mu\text{L})/\text{dry weight of sample (g)}].\end{aligned}$$

Luciferase activity data from the BG1Luc4E_2 cell culture bioassays were corrected for sample protein concentrations and handled in a manner similar to that described for the recombinant yeast bioassays.

The detection limits of the bioassays were arbitrarily set to the EC_{20} value for each standard. For the recombinant yeast estrogen, androgen, and progesterone receptor gene transcription assays, these were 0.05 $\pm$ 0.01 ng/mL 17β-estradiol (n = 46), 0.53 $\pm$ 0.12 ng/mL testosterone (n = 51), and 0.17 $\pm$ 0.03 ng/mL progesterone (n = 28). For the BG1Luc4E_2 estrogen receptor gene transcription cell culture bioassay, the detection limit was 0.00067 $\pm$ 0.00016 ng/mL 17β-estradiol (n = 5). Any extract that either showed no response or a response not exceeding 20% of the maximum response of the standard is indicated as $<$20%.

RESULTS

Municipal Biosolids

Municipal biosolid samples were collected from 19 sewage treatment plants across the province of Ontario, Canada, and some sites were sampled at various stages of the treatment process. All samples were analyzed using the estrogen, androgen, and progesterone receptor gene transcription assays. The type of digestion process (aerobic or anaerobic) that the sewage had undergone, regardless of the stage at which the samples were collected, appeared to influence the estrogen- and androgen receptor gene transcription activities of the sample extracts (Fig. 1). Those samples that had undergone aerobic digestion had very low or undetectable estrogen (mean: 11.3 ng/g dry wt) and undetectable androgen receptor gene transcription activities, whereas those samples that had undergone anaerobic digestion usually showed both estrogen (mean: 1233 ng/g dry wt) and androgen (mean: 543 ng/g dry wt) receptor gene transcription activities. No progesterone receptor gene transcription activity was detected in any biosolid sample (data not shown).

Seventeen of the biosolid samples were also analyzed using the BG1Luc4E_2 assay for estrogen receptor gene transcription activity. The graph in Figure 2 shows the results of this assay plotted against the results obtained for the same samples from the recombinant yeast estrogen receptor gene transcription assay. Regression analysis indicated that the relationship between the assays was significant (r^2 = 0.50, $P < 0.001$), suggesting that both assays gave similar values for the relative potencies of the extracts. However, the absolute estradiol equivalent values obtained were approximately 25 times higher using the recombinant yeast assay. The reasons for this were not determined but may include differences between the yeast strain and the BG1Luc4E_2 cells in cell membrane permeability and receptor induction and/or binding kinetics. Because the recombi-

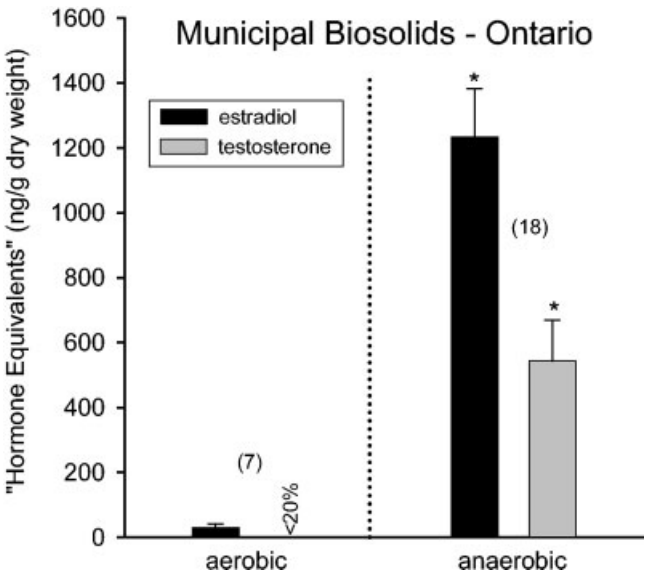

Fig. 1. Hormone-equivalent values for estrogen- and androgen receptor gene transcription activities in Ontario municipal biosolids obtained after aerobic or anaerobic treatment. The number of samples is indicated in brackets and standard errors are shown. Asterisk indicates a significant difference between treatments ($P < 0.05$); responses below the detection limit are indicated as <20%.

nant yeast system is a much more efficient screening assay, all remaining samples were analyzed only with the recombinant yeast.

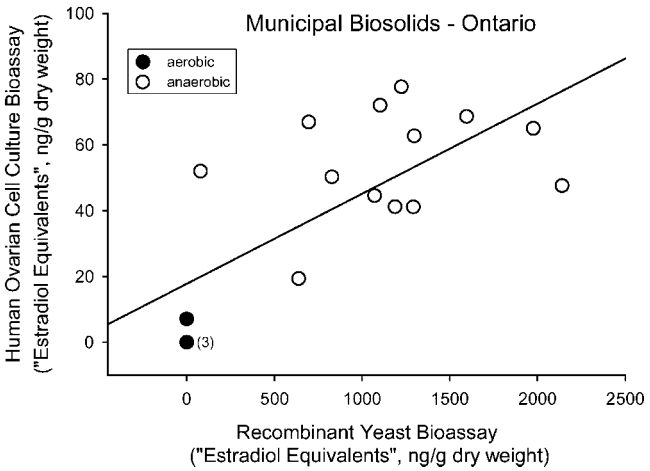

Fig. 2. Linear regression of estradiol equivalents obtained for 17 Ontario municipal biosolid samples assayed in a human ovarian cell culture bioassay (y axis) and a recombinant yeast assay (x axis) The equation for the regression line is $y = 0.0274x + 17.785$ ($r^2 = 0.50$, $P < 0.001$). Each symbol represents data for one sample unless another number is listed in brackets.

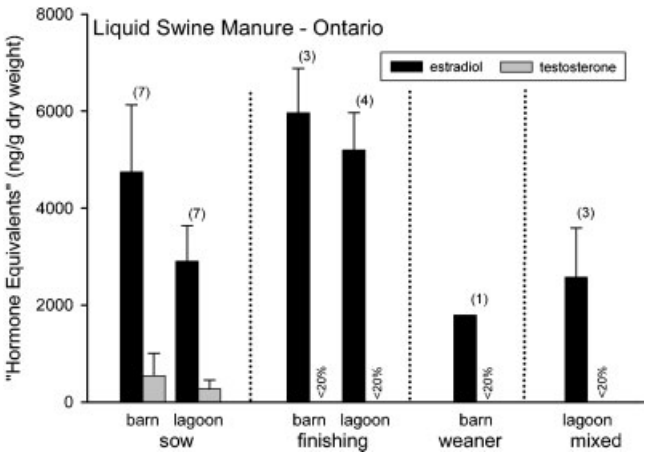

Fig. 3. Hormone-equivalent values for estrogen- and androgen receptor gene transcription activities in liquid swine manure samples from southwestern Ontario. The type of hog operation and the collection location are indicated on the x axis. The number of farms sampled (in brackets) and the standard errors are shown.

Animal Manures

Swine

Liquid swine manure samples were collected from a number of farms in southwestern Ontario. The samples were either collected fresh from the barns or were obtained from farm storage lagoons. Figure 3 shows the data categorized according to the type of hog operation from which the manure came and according to the type of collection. Estrogen receptor gene transcription activity was detected in all swine manure samples. Although differences were not significant ($P > 0.05$), the rank order of the manure for estrogen receptor gene transcription activity was finishing pigs (pigs being prepared for market) > sows > weaners (weaned pigs less than 1 year old). Only a few sow manure samples had detectable androgen receptor gene transcription activity. Stored manure samples tended to have slightly lower estrogen- and androgen receptor gene transcription activities, but these differences were not significant ($P > 0.05$). None of the swine manure samples had detectable progesterone receptor gene transcription activity.

Dairy Cows and Veal Calves

Fresh composite (feces and urine) manure samples were collected from six groups of five dairy cows each kept at the Agriculture and Agri-Food Canada research farm in Lennoxville, Quebec. Three groups consisted of cows in early gestation that were lactating, and three groups consisted of pregnant cows that were not lactating. A sample of mixed manure (nonlactating pregnant cows and veal calves) that had been stored for about 8–9 months and collected during a barn wash also was analyzed. All samples had detectable estrogen receptor gene transcription activity, but there were

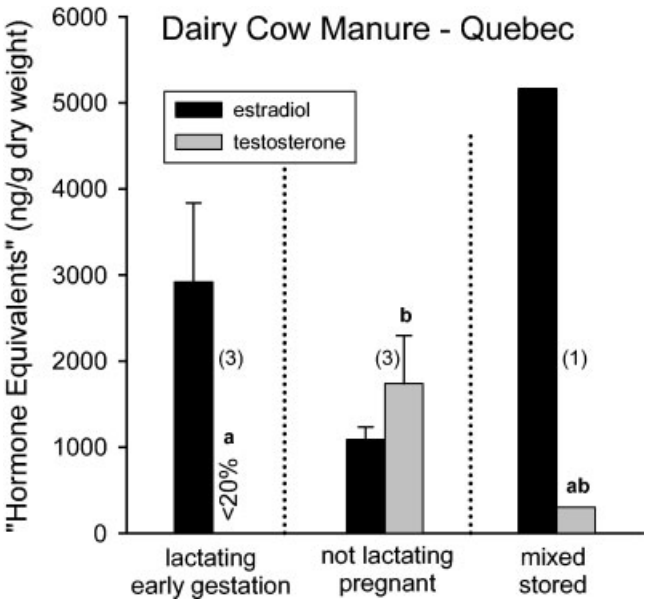

Fig. 4. Hormone-equivalent values for estrogen- and androgen receptor gene transcription activities in dairy cow composite or mixed dairy cow and veal barn wash manure samples from Lennoxville, Quebec. The reproductive status of the cows or the manure type is indicated on the *x* axis. The number of samples for each group (in brackets) and the standard errors are shown. Letters *a* and *b* indicate significant differences among groups for testosterone equivalents ($P < 0.05$).

no significant differences among means when the data were grouped according to whether the cows were lactating or according to whether the manure was fresh or had been stored mixed with veal calf manure (Fig. 4). No androgen receptor gene transcription activity was detected in manure samples from lactating, early-gestation cow manure. Greater androgen gene transcription activity was detected in pregnant, lactating cow manure ($P < 0.05$). The stored mixed-manure sample also had detectable androgen receptor gene transcription activity.

Dairy cow manure samples were also collected from six commercial farms in Newfoundland. Both fresh fecal pats and manure that had been stored under various conditions (liquid, solid, composted) were obtained. All cows had been treated with the commercial synthetic prostaglandin formulations Estrumate (cloprostenol sodium; Schering Canada Inc., Pointe Claire, Quebec, Canada) or Lutalyse (dinoprost tromethamine; Pfizer Canada Inc., Kirkland, Quebec, Canada) to synchronize the estrous cycles of the cows and to improve breeding efficiency. This treatment and the diets fed to the cows on each farm are indicated in Figure 5. Estrogen receptor gene transcription activity varied greatly among the farms (range: 22–3048 ng/g dry wt). Androgen receptor gene transcription activity tended to be lower and for two samples was not detected (range: 0–383 ng/g dry wt). Storage of the manure did not consistently change either the estradiol or testosterone equivalents compared to its effect on fresh manure for each farm. Mean estradiol equivalents obtained for combined fresh and stored manure samples were higher for those farms that used a feed containing soy than for those farms that did not use such a feed ($P < 0.05$). No progesterone receptor gene transcription activity was observed in manure from any dairy operation.

Beef Cattle

Fecal pats were collected and pooled for groups of 10 cattle from three feedlots in southern Alberta. Each group of cattle consisted of either heifers or steers which had been treated with commercial hormone implants on the same day. Heifers were implanted with either Component TE-H (14 mg of estrogen, 140 mg of trenbolone acetate; Elanco, Guelph, Ontario, Canada) or Synovex Plus (20 mg of estrogen, 200 mg of trenbolone acetate; Wyeth, Markham, Ontario, Canada). Heifers from one feedlot were also fed 0.4 mg of melengestrol acetate (MGA)/head/day (Pfizer Canada Inc., Kirkland, Quebec, Canada). Steers were implanted with Component TE-S (14 mg of estrogen, 140 mg of trenbolone acetate; Elanco, Guelph, Ontario, Canada). The hormone-equivalent values for beef cattle fecal pat samples are shown in Figure 6. The data have been categorized by sex, type of treatment, and number of days since the last hormone implant. For those heifers treated with TE-H, there appeared to be a time-dependent trend, such that fecal pats from those

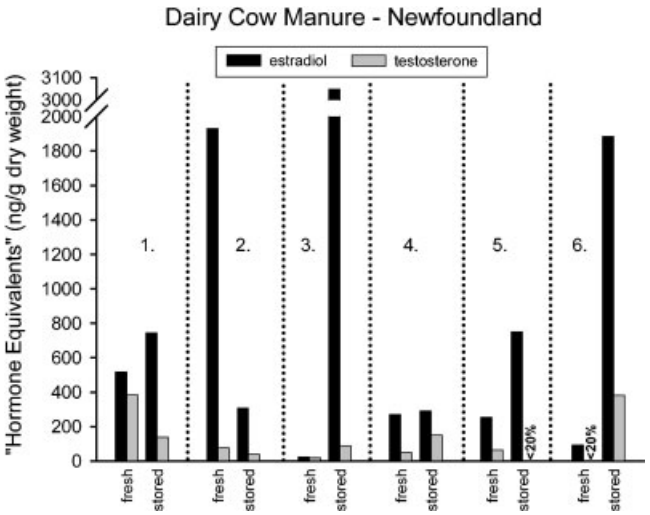

Fig. 5. Hormone-equivalent values for estrogen- and androgen receptor gene transcription activities in dairy cow manure samples from Newfoundland. The type of manure collection (fresh or stored) is indicated on the *x* axis. Numbers 1–6 represent the hormone treatment and feed that each group of cows received as follows: 1. Lutalyse, corn (n = 210 cows); 2. Lutalyse, soy/corn (n = 110 cows); 3. Estrumate, soy/corn/brewer's grain (n = 100 cows); 4. Lutalyse, corn/brewer's grain (n = 110 cows); 5. Lutalyse, corn (n = 45 cows); 6. Lutalyse; soy/corn/brewer's grain (n = 130 cows).

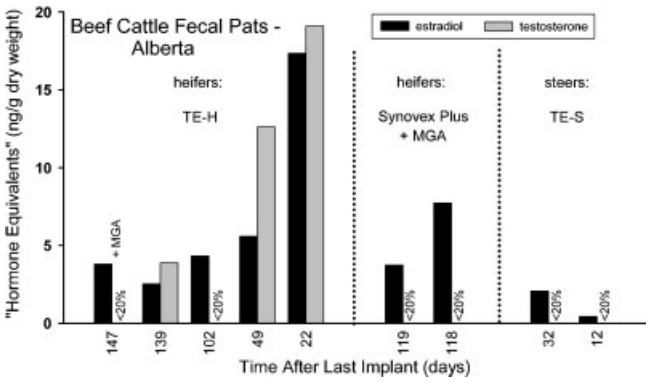

Fig. 6. Hormone-equivalent values for estrogen- and androgen receptor gene transcription activities in beef cattle fecal pats from southern Alberta. The *x* axis is number of days since last implant of TE-H, Synovex Plus, or TE-S; cattle are grouped based on sex (n = 10 heifers or steers/group) and treatment.

animals most recently implanted had the highest estrogen- and androgen receptor gene transcription activities. Fecal pats from heifers treated with Synovex Plus and MGA had detectable estrogen but no androgen receptor gene transcription activities. Steers treated with TE-S had very low estrogen receptor gene transcription activity and no detectable androgen receptor gene transcription activity despite recent implantation. No progesterone receptor gene transcription activity was detected in any beef cattle fecal pat samples.

Chickens

Litter samples, consisting of a mixture of excrement and wood shavings, were collected from 24 chicken farms in the Fraser Valley, British Columbia, Canada. Five farms raised breeder laying hens that produced fertilized eggs, seven farms raised commercial layers that produced unfertilized eggs, and 12 farms raised broiler chickens (immature) for meat. Of the commercial layer operations, three flocks consisted of pullets (hens less than 1 year old). Hormone-equivalent data obtained for each class of chicken are shown in Figure 7. Although all flocks produced litter with detectable estrogen receptor gene transcription activity, only the breeder layer flocks consistently produced litter that had androgen receptor gene transcription activity. Only 1 of 4 commercial layer flocks, 1 of 3 commercial layer pullet flocks, and 2 of 12 broiler flocks produced litter with detectable androgen receptor gene transcription activity. Of the commercial layers, there was a tendency for those flocks comprised of pullets to have litter with the highest estrogen receptor gene transcription activity. However, this difference was not significant ($P > 0.05$). Progesterone receptor gene transcription activity was observed for litter from one breeder layer flock (67 ng/g dry wt, data not shown).

DISCUSSION

Municipal Biosolids

Results of this and previous studies suggest that different types of processes used in sewage treatment plants influence the hormone activities of the resulting biosolids or liquid effluent. In the current study biosolids obtained from sewage that had been treated aerobically exhibited little or no estrogen and androgen receptor gene transcription activities. In contrast, biosolids obtained from sewage treated anaerobically usually showed detectable androgen and estrogen receptor gene transcription activities. In previous studies extended biological treatments, including percolating filter bed and activated sludge systems, were found to be more effective than effluents receiving only primary treatment in reducing the estrogenic or androgenic activities of wastewater (Thomas et al., 2002; Svenson et al., 2003). Another study showed that recirculation of the sewage sludge, which caused nitrification and denitrification, resulted in significant reductions in natural and synthetic estrogens detected in the effluent (Andersen et al., 2003). More specifically, estrogenic activity associated with the biosolid fraction was shown to decrease during mesophilic (30°C–40°C) aerobic digestion (Holbrook et al., 2002). Thus, the current results support previous findings for both municipal sewage biosolids and wastewater effluents.

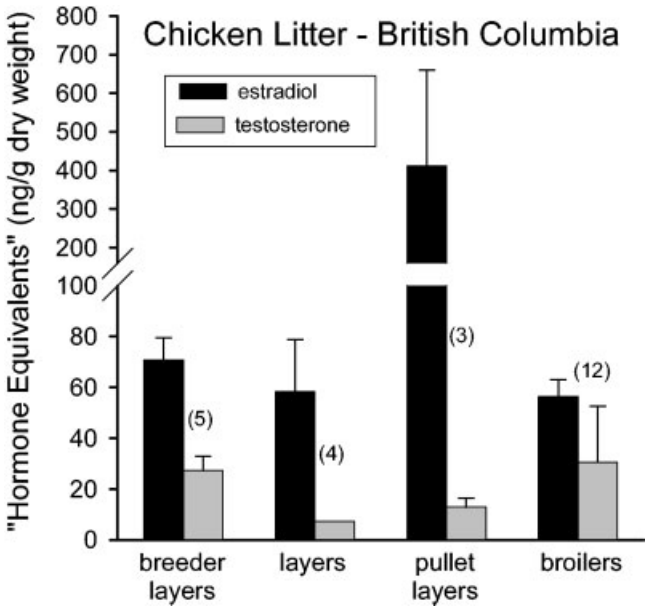

Fig. 7. Hormone-equivalent values for estrogen- and androgen receptor gene transcription activities in chicken litter from British Columbia. Type of flock is shown on the *x* axis; number of flocks in each group is shown in brackets; standard errors are indicated by bars.

Animal Manures

The levels of hormone activities detected in animal manures in the present study appeared to vary according to the species, sex, age, and reproductive status of the animals. Diet and treatment of animals with hormone implants also appeared to be factors. The effect of manure storage on hormone activity was not clear. Overall, estrogen receptor gene transcription activity was detected in all manure samples. The highest level was detected in manure from finishing pigs (5965 ng/g dry wt) and the lowest level in manure from steers (0.43 ng/g dry wt). Androgen receptor gene transcription activity was not detected in manure from at least one animal or group of animals of each species investigated. The highest level of androgen receptor gene transcription activity was detected in manure from nonlactating, pregnant dairy cows (1737 ng/g dry wt).

Daily and yearly steroid hormone excretion rates were previously estimated for a number of farm animals (Lange et al., 2002, reviewed in Hanselman et al., 2003). Although the data are not complete, the highest yearly amounts of estrogen and progestin excretion were typically associated with pregnant or egg-laying animals, and the highest androgen excretion rates were observed for mature male animals. Boars also have high estrogen excretion rates, exceeding those of sows. This finding may help to explain why manure obtained from finishing pigs had the highest estrogen receptor gene transcription activity in the present study. However, if this were the case, being able to detect elevated androgen receptor gene transcription activities in these samples also would have been expected. To our knowledge, high androgen excretion rates for pregnant dairy cows have not been reported previously.

The metabolism and route of excretion of estrogens and androgens varies among types of farm animals (reviewed in Velle, 1976). Ruminants metabolize 17β-estradiol and estrone to 17α-estradiol, which has low estrogenic activity. In contrast, the major estrogen metabolite in pigs is estrone. For androgens, epitestosterone is the major metabolite in ruminants, whereas 11-deoxy-17-ketosteroids are the major metabolites in boars. For both estrogen and androgen metabolites, feces is the major route of excretion in ruminants, whereas urine is the major route of excretion in pigs. Therefore, it is unlikely that the very low levels of estrogen- and androgen receptor gene transcription activities observed in beef cattle fecal pats observed in the present study can be attributed to urinary excretion of the steroid metabolites. The low levels are more likely a result of the cattle not being reproductively active and of circulating hormone levels being very low. It should also be noted that the concentration of trenbolone acetate (140–200 mg/head) implanted in the cattle is less than the yearly amount of androgens (390 mg) estimated to be excreted by a mature bull (Lange et al., 2002). Finally, it must also be stressed that recombinant yeast assays measure hormonal potencies (ability to bind to hormone receptor and activate gene transcription), not the actual quantities of the excreted steroid metabolites, which usually are considerably less active than the parent compounds.

Diet also appears to be a factor contributing to the level of hormone activity detected in animal manures. In the present study it was observed that manure from dairy cows that had been fed a diet containing soybean meal had significantly higher estrogen receptor gene transcription activities than manure from cows not fed soy. It was previously shown that soy-based foods contain the phytoestrogens daidzein and genistein and the daidzein metabolite equol (Axelson et al., 2003; Lintelmann et al., 2003). Equol also has been detected and identified as a major phytoestrogen in swine manure (Burnison et al., 2003). In humans, concentrations of urinary equol after ingestion of a single soy-based meal were 100–1000 times higher than the concentrations of naturally produced estrogens (Axelson et al., 2003). However, the potency of equol was reported to be 1000- and 200-fold less than estradiol for binding to sex steroid–binding protein and estrogen receptor, respectively, and 2200-fold less than testosterone for binding to the androgen receptor (Burnison et al., 2003). Based on these findings, it is likely that the elevated estrogen receptor gene transcription activity detected in dairy cow manure in the present study was a result of the presence of equol or other phytoestrogens in the soy-containing diet. Because high concentrations of these compounds may be present in manure, further studies are warranted to assess their persistence and fate after application to agricultural land.

Nondietary exogenous chemicals also may influence hormone activity levels in animal manures. Of these, trenbolone acetate (TB) and melengestrol acetate (MGA) are likely the most important, as they are widely used as growth promoters for cattle in the United States and Canada. TB is usually administered in combination with estrogen as a subcutaneous implant and MGA is provided as a feed additive. TB has potent androgenic and antiglucocorticoid activities, whereas MGA acts as a progestin (Wilson et al., 2002; reviewed in Schiffer et al., 2001). Bile, and hence feces, is the major route of excretion of TB in cattle (Pottier et al., 1981). Metabolites of TB and parent MGA can be detected in both manure and manure-amended soil and are relatively stable (Schiffer et al., 2001).

Few studies have been conducted to assess the potential environmental impact of TB and MGA. However, under controlled laboratory conditions, it was observed that low concentrations of TB significantly reduced spawning behavior and egg production in female fathead minnows (Ankley et al., 2003). These fish also gained weight and grew dorsal tubercles normally seen only in male fish. Furthermore, plasma estradiol, testosterone, and vitellogenin levels were decreased. Male fathead minnows exposed to higher concentrations of TB exhibited decreased plasma testosterone metabolites and increased estradiol and vitellogenin concen-

trations (Ankley et al., 2003). In the present study a time-dependent trend was observed for both androgen and estrogen receptor gene transcription activities in fecal pats collected from heifers treated with TB, estrogen, and, in some cases, MGA. Those heifers most recently implanted with TE-H or Synovex Plus had fecal pats with the highest levels of estrogen and/or androgen receptor gene transcription activities compared to those implanted at earlier times. Overall, although the hormone-equivalent values obtained for the beef cattle fecal pats in the present study were very low in comparison to manures from other farm animals (see above), the combination of these results with the observed effects on fathead minnows, the widespread use of TB, and the stability of its metabolites suggest that further studies are required to assess the potential ecological risk from the use of this and other growth-promoting chemicals.

A review of the literature indicates that chickens have lower circulating levels of progesterone, testosterone, and estrogens than many mammalian farm animals (reviewed in Velle, 1976). Pregnant horses, cattle, sheep, goats, and pigs all have considerably higher levels of plasma estrogens than do ovulating domestic fowl. Similarly, yearly excretion rates of progestins, androgens, and estrogens were reported to be substantially lower for chickens than for either cows or pigs, regardless of the reproductive status of the animals (reviewed in Lange et al., 2002). However, despite the lower excretion rates of hormones by chickens, chicken litter deserves special consideration because it is used as a protein source in cattle feed in a number of countries. Adverse effects, such as development of premature udders or delayed puberty, have been reported in cattle fed chicken litter (reviewed in Shore et al., 1993b, 1995). In the present study only litter from breeder laying chickens consistently exhibited both androgen and estrogen receptor gene transcription activities. However, young commercial laying hens (pullets) had the highest estrogen receptor gene transcription activities. When compared to previously reported values obtained using radioimmunoassay techniques, the estrogen equivalents calculated for chicken litter in the present study compare reasonably well, but the testosterone equivalents were considerably lower in this study (Shore et al., 1993b). The reasons for this discrepancy are not known. Overall, although the actual hormone-equivalent values of the chicken litter samples were not as high as most of the swine or cow manure samples, the use of chicken litter as a protein supplement should be monitored to ensure that animal feeds are not a significant source of estrogenic or androgenic compounds.

Progestins

In the present study progesterone receptor gene transcription activity assays were conducted on all biosolid and manure samples. However, only one sample (chicken litter) had detectable activity. As the method was optimized with progesterone standards before use, it is unlikely that performance of the assay was an issue. Furthermore, strong progesterone receptor gene transcription activity was detected using this assay for the synthetic progestins, norethindrone and norgestrol, which are commonly used in oral contraceptives (data not shown).

The most likely reason for not detecting progesterone receptor gene transcription activity in the biosolid and manure samples is that progesterone is metabolized and deactivated to several reduced pregnanediones, prenanolones, and prenanediols prior to fecal excretion (Jewgenow and Meyer, 1998; reviewed in Lange et al., 2002). Unmetabolized progesterone is barely detectable, if at all, in fecal samples (Schwarzenberger et al., 1996). This is in contrast to estrogens and androgens, which are end products of steroid metabolism such that circulating and excreted compounds are the same. Furthermore, the reported quantification of "progesterone" in feces is misleading, because the assays used to measure fecal progesterone did not detect parent compound concentrations but rather values for cross-reacting, reduced pregnane metabolites containing a common 20-oxo group (Schwarzenberger et al., 1996).

It is unclear why one chicken litter sample in the present study had detectable progesterone receptor gene transcription activity. Based on our findings that metabolically stable synthetic progestins such as norgestrol and norethindrone could be detected using the progesterone receptor gene transcription activity assay, detection of activity only in the municipal biosolid samples would be expected. However, it is possible that the concentrations of these compounds in the biosolid fraction were too low.

CONCLUSIONS

The results from this study present a representative survey of hormone activities in municipal biosolid and animal manure samples used as agricultural fertilizers. Yeast-based hormone receptor gene transcription assays provided a robust and rapid method of screening a wide variety of samples obtained after different treatments or farming practices. Although most samples had detectable activities, further studies are underway to assess the persistence and fate of these substances when applied as fertilizers to agricultural fields.

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