

Animal Manure Separation Technologies Diminish the Environmental Burden of Steroid Hormones

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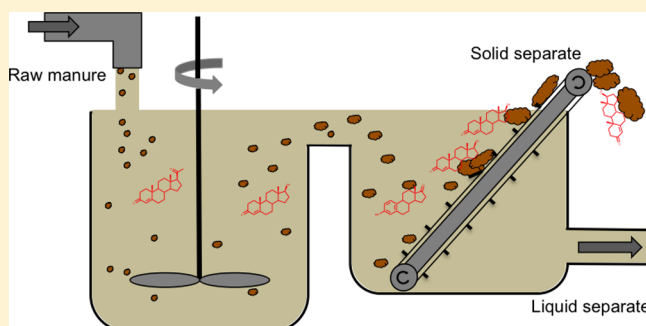
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Supporting Information

ABSTRACT: Newly developed treatment technologies are capable of separating livestock manure into a liquid fraction and a solid fraction using sedimentation, mechanical, and/or chemical methods. These technologies offer a potential means of distributing nutrients to agricultural lands without the unwanted environmental risks associated with the release of steroid hormones to adjacent waterways. To assess the potential benefit of these technologies in reducing the level of release of steroid hormones to adjacent waterways, distribution profiles of nine steroid hormones (pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, testosterone, dihydrotestosterone, estrone, 17 α -estradiol, and 17 β -estradiol) were determined in raw swine manure, and in solid and liquid fractions separated from ten full-scale manure separation systems. Steroid hormone concentrations, normalized for nitrogen content, were significantly higher in separated solids than in liquids. If separated liquids are applied instead of raw manure, steroid hormone loading can be reduced by a factor of 2 at a constant nitrogen fertilization level.



INTRODUCTION

Steroid hormones are biologically active at trace concentrations. As result, their release to surface waters following land application of animal manure is a serious concern.^{1–6} Much of the previous research on steroid hormones has focused on the release of steroid hormones by municipal wastewater treatment plants^{1,7–12} and combined sewer systems.¹³ Over the past decade, it has been demonstrated that agricultural practices, utilizing land application of animal manure, also serve as a significant source of steroid hormones.^{5,14,15} For example, steroid hormones in swine manure leached from manure-amended soil to soil drainage water systems, resulting in concentrations in surface waters exceeding the threshold that leads to vitellogenin induction in sensitive fish species.^{14,16}

Because of its nutrient content, it is a common agricultural practice to use livestock manure as an agricultural soil fertilizer. Depending on the agricultural practice, prior to or just after storage, the raw manure may be divided into a liquid fraction and a solid fraction using newly developed manure separation technologies by sedimentation, mechanical, and/or chemical methods.^{17,18} The separation process simplifies handling of the

manure and may provide a means for applying plant available nutrients in the liquid fraction to the land with less coincidental application of particle-associated contaminants in the solids, such as trace metals and surplus nutrients, but also potentially steroid hormones, which may be transported to adjacent waterways.^{17–19}

To assess the potential benefit of applying manure separation, the masses of progestagens (pregnenolone and progesterone), androgens (dehydroepiandrosterone, androstenedione, testosterone, and dihydrotestosterone), and estrogens (estrone, 17 α -estradiol, and 17 β -estradiol) were quantified in both the raw state and solid and liquid fractions of separated manure collected from ten Danish pig farms.

METHODOLOGY

Materials and Methods. Grab samples were collected from ten different Danish farm sites as raw material and

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separated solid and liquid fractions of pig slurry. Farms 1–7 used chemical–mechanical separation technology with polyacrylamide polymer flocculation followed by mechanical separation with a belt sieve (0.2 mm) and a screw press; farms 8 and 9 used mechanical separation technology with a rotating filter drum and screw press, and farm 10 used a mechanical screw press (Table S1 and Figure S1 of the Supporting Information). The ten farms were producing both fattening pigs and sows with piglets, and manures were a combination of slurry from these, the proportion varying from 1:3 to 3:1 between farms.

All samples were collected between December 2008 and March 2009 at the farms during separator operation, which took place every one to two weeks; thus, samples included manure that accumulated in the pretank of the animal houses over that period. Raw slurries were sampled from a pretank equipped with a mixer, at a depth of approximately 1 m, and then stirred thoroughly by being pumped before and during separation. Samples were taken by subsampling approximately equal volumes of raw slurry or solid or liquid fractions every 10 min over a period of 30 min during separator operation, to ensure samples were representative of the manure tank. The composite samples were stored in 10 L airtight buckets and transported at ambient temperature (0–10 °C) to the laboratory within 24 h. The samples were then carefully mixed to ensure homogeneity, split into subsamples stored in 250 mL polypropylene plastic or glass containers, and frozen at –18 °C for further analyses. The pH values of the different fractions were measured after a 5-fold dilution in deionized water.¹⁷ The total nitrogen and phosphorus contents were determined using the Kjeldahl method and inductively coupled plasma optical emission spectrometry, respectively, as described previously.²⁰ Dry weights were determined by loss on drying at 105 °C.²¹ The steroid hormone extraction and analysis by isotope dilution gas chromatography tandem mass spectrometry (GC–MS/MS) is described elsewhere.^{1,22} Briefly, the fresh sample was lyophilized to reach a water content of <10% (by mass). A subsample of approximately 0.5 g was spiked with deuterated internal standards [*d*₇-androstenedione, *d*₄-estrone, *d*₃-17 β -estradiol, and *d*₄-ethynyl-estradiol (CDN isotopes, Pointe-Claire, QC) and *d*₅-progesterone, *d*₃-testosterone, and *d*₃-dihydrotestosterone (Toronto Research Chemicals, Toronto, ON)]. Thereafter, the sample was extracted using a sequential extraction approach with pressurized liquid extraction and an integrated in-cell cleanup (ASE200, Dionex, Sunnyvale, CA).¹ The sample extract was purified by a two-step solid-phase extraction (amino-propyl and silica gel) before derivatization and analysis by GC–MS/MS (Varian CP-3800 with PTV and Varian 1200 triple-quadrupole, Varian).²² The method limits of detection (LODs) were in the ranges of 7.2–8.6 ng [g of dry weight (dw)]^{–1} for the progestagens, 4.0–7.2 ng (g of dw)^{–1} for the androgens, and 1.6–3.2 ng (g of dw)^{–1} for the estrogens¹ (Table S2 of the Supporting Information). Steroid hormone recoveries for manure samples ranged from 77 to 100%.¹ For statistical evaluations, a Kruskal–Wallis one-way analysis of variance (nonparametric method) followed by Dunn's multiple-comparison test was performed using GraphPad Prism 6.0b.²³

RESULTS AND DISCUSSION

To assess the potential for release of steroid hormones from manure-amended agricultural fields, concentration data were normalized with respect to the mass of the nutrient, because the

fertilizer is field-amended on the basis of total nitrogen or total phosphorus content depending on which is the higher in relation to regulations.^{18,24,25}

Characteristics of Raw Manure and Solid and Liquid Fractions. Solids typically accounted for 1–5% of the material in the raw manure on a weight basis. As a result, when separated, the relatively small amounts of separated solids were depleted of nitrogen ($p < 0.01$ compared to raw manure), which is mainly present as ammonium in the raw manure and therefore ends up in the liquid fraction, and the nitrogen in the solid fraction is mainly organic-bound (Figure 1 and Table S1 of the Supporting Information). As expected, the nitrogen content of the liquid fraction was not significantly different

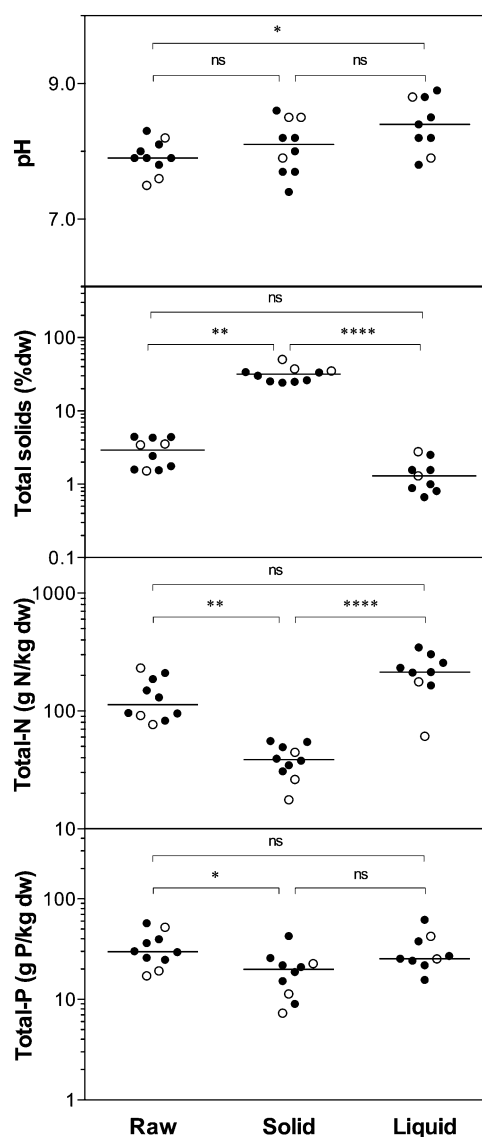


Figure 1. Physicochemical properties of the investigated manure and its two separates from ten Danish pig farms. Filled circles are data for farms 1–7, facilities that use chemical–mechanical separation technology. Empty circles are data for farms 8–10, facilities that use mechanical separation technology (a screw press with or without a rotating filter drum). The horizontal lines indicate the group median. Detailed data can be found in the Table S1 of the Supporting Information. One sample (liquid fraction from farm 9) was not analyzed because of access limitations. * $p < 0.05$. ** $p < 0.01$. **** $p < 0.0001$. ns, not significant.

from that of the raw manure when expressed relative to dry weight. A similar trend was observed for the total phosphorus content, which is mainly present as inorganic P,^{26,27} with a significantly ($p < 0.05$) lower concentration of phosphorus in the solid separate compared to the raw manure, and no statistical difference between the liquid and raw manure (Figure 1 and Table S1 of the Supporting Information). However, because of the significantly higher dry matter content of the solid fractions, these are enriched in phosphorus and nitrogen when expressed per fresh weight. No statistical differences between technologies were observed, as it was possible to obtain samples from only three mechanical manure separators (and seven chemical–mechanical separators).

Steroid Hormones. Concentrations of steroid hormones were grouped according to their mode of action, progestagens (PRO and PRE), androgens (AN, DHEA, TS, and DHT), and estrogens (E1, α E2, and β E2). A complete list of the individual steroid hormone levels in all investigated manure samples is found in Table S2 of the Supporting Information. All three steroid hormone classes were detected in separated solids. The relative values of progestagen concentration to total nitrogen content (milligrams per kilogram of N) were higher in the solid separates than in raw manure or liquid fractions ($p < 0.001$ and $p < 0.01$, respectively), while values for estrogens were different only at $p < 0.05$ and $p < 0.01$, respectively, and those of androgens only different at $p < 0.01$ between solid and liquid fractions (Figure 2). The observed low, or below LODs, progestagen levels in raw manure could be caused by progestagens that were mainly available as conjugated metabolites in raw manure and deconjugated by microorganisms during or after separation; however, more research is needed to clarify. Moreover, the androgen DHEA was not detected in any sample. The relative steroid hormone concentration to total phosphorus instead of total nitrogen yielded a similar picture (Figure S2 of the Supporting Information), though not as distinct.

Because of the ability of steroid hormones to absorb to organic material, no clear differences were observed when relating hormone concentrations to the dry matter content (Table S2 and Figure S3 of the Supporting Information), which is consistent with other findings.^{10,14–16,28,29} Consequently, a major fraction of the total amount of steroid hormones is expected in the solid separates. A recent study investigating steroid hormone runoff from a municipal biosolid-amended agricultural soil indicated that steroid hormones were mainly present in $0.7 \mu\text{m}$ filtered runoff and not in the suspended particle phase.³⁰ These findings could have been explained by steroid hormones associated with $<0.7 \mu\text{m}$ (biosolid) particles. It is also possible that the findings were related to more complex sorption kinetics (e.g., hysteresis) and association with new and smaller particles ($<0.7 \mu\text{m}$) as shown for testosterone in soils.³¹ As we lyophilized all samples (including the liquid fraction), our measurements of individual hormones represent the sum of aqueous and particle-associated steroids (including $<0.7 \mu\text{m}$ particles). Via comparison of the separated fractions to the raw manure, only the progestagens was significantly ($p < 0.01$) higher in the solid separate (Figure S3 of the Supporting Information). Such data have not been published previously, and this difference between progestagens and the other two steroid subgroups could be caused by the progestagens being slightly more lipophilic than the androgens and estrogens, an observation we have made previously.^{1,22}

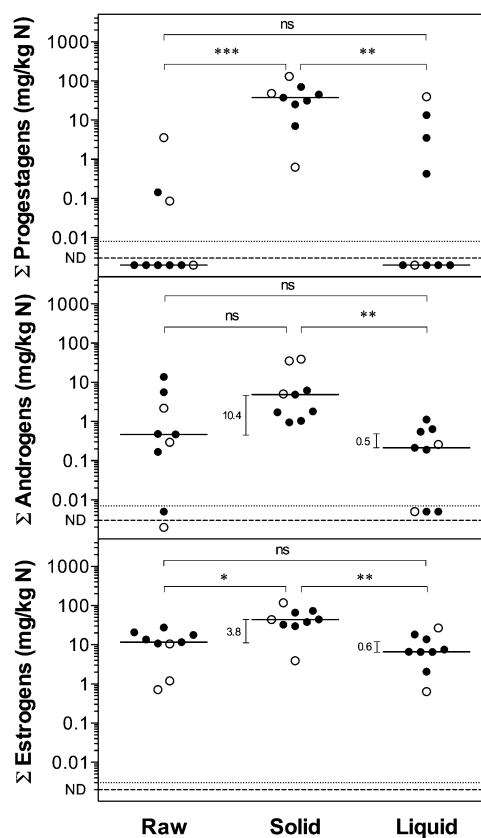


Figure 2. Cumulative concentrations of steroid hormones in the different manure fractions relative to the measured total nitrogen content. Filled circles are data for farms 1–7, facilities using chemical–mechanical separation technology. Empty circles are data for farms 8–10, facilities using mechanical separation technology (a screw press with or without a rotating filter drum). The horizontal lines depict the group median. Samples between the dotted line and dashed line contain identified (but not quantifiable) steroid hormones. Steroid hormones were not detected in samples below the dashed line but are included at the displayed value for statistical purposes. Detailed data reported in Table S2 of the Supporting Information. Progestagens are comprised of progesterone and pregnenolone. Androgens are comprised of dehydroepiandrosterone, androstenedione, testosterone, and dihydrotestosterone. Estrogens are comprised of estrone, 17α -estradiol, and 17β -estradiol. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. ns, not significant. Vertical lines with values display the median factor between raw manure and separated.

Among the estrogens, estrone (E1) is found at the highest concentrations (Table S2 of the Supporting Information), which might be caused by a higher level of excretion of E1 or conditions favoring oxidation, as α E2 and β E2 are readily oxidized into E1. Another more likely reason is the presence of microorganisms with $3\beta/17\beta$ -hydroxysteroid dehydrogenase (e.g., the Gram-negative bacterium *Comamonas testosteroni*^{32–34}). A recent review of bacterial metabolism of sex steroids is nicely summarized elsewhere.³⁵ Similarly for the androgens, AN, the oxidized form of TS is typically present at the highest concentrations. Again, the same trend is evident for progestagens, where PRO, the oxidized form of PRE, is dominant (Table S2 of the Supporting Information). Despite the manure being from fattening pigs and sows with piglets, they still excrete androgens. This manure separator study is the first of its kind, and clearly more data are needed to establish

mass balances to produce more accurate descriptions of the fate of steroid hormones and other contaminants.

Implications. Available manure separation technologies can separate raw manure into a solid fraction and a liquid fraction. The solid fraction contains higher concentrations of steroid hormones when related to total nitrogen or phosphorus content because nitrogen and phosphorus stay in the water fraction. Therefore, use of liquid separates from manure as an agricultural fertilizer could reduce the loading of steroid hormones to fields by approximately 50% (Figure 2). Veterinary drugs with similar physicochemical properties, especially substances such as trenbolone acetate,⁶ but also antibiotics and antiparasitics,³⁶ are expected to associate with solids. Therefore, the environmental burden of such contaminants would likely also be reduced by this practice. Steroid hormones are expected to be more bioavailable in the liquid fraction; however, recent studies have shown particle-associated hormones are partially bioavailable.³⁷

The reduced volume of manure solid fractions provides opportunities for further treatment and improved waste management strategies.³⁸ For example, composting and anaerobic digestion (e.g., biogas production)³⁹ of the separated solids may lower the concentration of steroid hormones, allowing the residuals to be applied to agricultural lands after treatment. The presence and transformation of conjugated steroid hormones (i.e., glucuronides and sulfates) in the raw manure and separates should be investigated, although the conversion of conjugated steroids into biologically active steroids is expected to occur at a fast rate.^{40–42} In addition, other (unknown) steroid hormone metabolites, not measured in this study, may not behave in the same manner as the measured hormones in the separation technologies.⁴³ Additional research is needed to assess the effect of treatment on the overall endocrine-disrupting potential of manure. This study investigated nine endogenous steroid hormones, yet a great number of livestock animals are treated prophylactically with steroid-mimicking medicines. It is very likely that these medicine residues also will contribute to the overall environmental endocrine load and risk.^{2,3,44}

■ ASSOCIATED CONTENT

⑤ Supporting Information

Detailed chemical characterization and steroid hormone levels in manure and its separates (Tables S1 and S2), schematics of manure separation technologies (Figure S1), and grouped steroid hormone levels in manure and its separates related to total phosphorus and total solids (Figures S2 and S3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PRE, pregnenolone; PRO, progesterone; DHEA, dehydroepiandrosterone; AN, androstenedione; TS, testosterone; DHT, dihydrotestosterone; E1, estrone; α E2, 17 α -estradiol; β E2, 17 β -estradiol; EE2, 17 α -ethynyl-estradiol; dw, dry weight.

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