17 B-Estradiol mineralization in human waste products and soil in the presence and absence of antimicrobials

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ABSTRACT

Natural steroidal estrogens such as 17 β-estradiol (E2), as well as antimicrobials such as doxycycline and norfloxacin, are excreted by humans and hence detected in sewage sludge and biosolid. The disposal of human waste products on agricultural land results in estrogens and antibiotics being detected as mixtures in soils. The objective of this study was to examine microbial respiration and E2 mineralization in sewage sludge, biosolid and soil in the presence and absence of doxycycline and norfloxacin. The antimicrobials were applied to the media either alone or in combination at total rates of 4 and 40 mg kg^{-1}, with the 4 mg kg^{-1} rate being an environmentally relevant concentration. The calculated time that half of the applied E2 was mineralized ranged from 294 to 418 d in sewage sludge, from 721 to 869 d in soil and from 2,258 to 14,146 d in biosolid. E2 mineralization followed first-order and the presence of antimicrobials had no significant effect on mineralization half-lives, except for some antimicrobial applications to the human waste products. At 189 d, total E2 mineralization was significantly greater in sewage sludge (38 ±0.7%) > soil (23 ±0.7%) > biosolid (3 ±0.7%), while total respiration was significantly greater in biosolid (1,258 mg CO_2) > sewage sludge (253 mg CO_2) ≥ soil (131 mg CO_2). Strong sorption of E2 to the organic fraction in biosolid may have resulted in reduced E2 mineralization despite the high microbial activity in this media. Total E2 mineralization at 189 d was not significantly influenced by the presence of doxycycline and/or norfloxacin in the media. Antimicrobial additions also did not significantly influence total respiration in media, except that total CO_2 respiration at 189 d was significantly greater for biosolid with 40 mg kg^{-1} doxycycline added, relative to biosolid without antimicrobials. We conclude that it is unlikely for doxycycline and norfloxacin, or their mixtures, to have a significant effect on E2 mineralization in human waste products and soil. However, the potential for E2 to be persistent in biosolids, with and without the presence of antimicrobials, is posing a challenge for biosolid disposal to agricultural lands.

Keywords: 17 β-estradiol, antimicrobial, doxycycline, norfloxacin, sewage sludge, biosolid, soil, mineralization, respiration.

INTRODUCTION

17 β-estradiol (E2) is a natural steroidal estrogen produced by vertebrates. Estrogens are excreted by vertebrates in both urine and feces with an estimated global rate of 29,500 kg of natural estrogens being excreted by humans each year. [1] 17 β-estradiol has been frequently detected in human waste products such as in sewage sludge and biosolid at rates ranging up to 230 μg kg^{-1}. [2-5] The disposal of such human waste products to agricultural land enhances soil nutrients and carbon content, but also introduces estrogens that can contaminate surface waters via runoff [6,7] and groundwater via leaching. [8,9] Contamination of surface waters with estrogens at low concentrations has been shown to induce vitellogenesis and other abnormal secondary sexual characteristics in male and juvenile female fish [10,11], for example the production of an egg yolk

protein precursor has been observed in male fish exposed to E2 concentrations as low as 25 ng L^{-1}. [12] Drinking water contaminated with estrogens could pose health risk to humans. [7,12]

Bacteria that can degrade E2 in soil and human waste products include the genera *Aminobacter, Escherichia, Pseudomonas, Sphingomonas* (Proteobacteria), *Corynebacterium, Microbacterium, Nocardioides*, *Rhodococcus* (Actinobacteria), and *Flavobacterium* (Bacteroidetes). [13-19] Microbial degradation is a main path of estrogen degradation [20-22], hence antimicrobial toxicity towards these bacteria may result in increased E2 persistence in soil and human waste products. For example, chlortetracycline, sulfamethazine and tylosin applied to soil at concentrations as low as 2 mg kg^{-1} significantly reduced the transformation of E2 to estrone (E1). [23] Antimicrobials affect bacterial compositions with the impact on specific species being depended on the types and concentrations of antimicrobial present, as well as the duration of exposure and the level of bacterial resistance to the antibiotics. [24]

E2 mineralization can be quantified by using radiolabeled [4-^{14}C] E2 in microcosm experiments [21,25,26], whereby the recovery of $^{14}CO_2$ indicates that the steroid molecule has been inactivated because of ring cleavage. [27] Antimicrobial additions to soil and other media has shown to reduce ~~reduced~~ E2 mineralization in some cases. [20,23,26] For example, the addition of 40 mg kg^{-1} tetracycline to manure decreased total E2 mineralization by 14% relatively to manure without antibiotics added, but E2 mineralization in soils was not impacted. [26]

In this study, we examine the impact of the antimicrobials doxycycline and norfloxacin on E2 mineralization in media. Doxycycline, which selectively inhibits the 30S ribosomal subunit in bacteria, belongs to the tetracycline group of antimicrobials. Doxycycline is commonly used to treat wide array of infections in humans (urinary tract, intestine and eye) and is also increasingly used as an anti-cancer therapeutic. [28] Norfloxacin belongs to the fluoroquinolone group of antimicrobials and is primarily used to treat urinary tract infections and respiratory diseases in humans by selectively inhibiting DNA replication in target bacteria. [29] Both doxycycline and norfloxacin are readily excreted by humans and have been detected in sewage sludge and biosolid at concentrations of up to 1.5 mg kg^{-1} for doxycycline and 11 mg kg^{-1} for norfloxacin [30-34], and in agricultural soils following biosolid application at concentrations of up to 15 μg kg^{-1} for doxycycline and 45 μg kg^{-1} for norfloxacin. [32,35] Both antimicrobials have been detected in surface water following wastewater effluent discharge and following surface runoff from soils amended with biosolid. [31,32,36,37] Tetracycline and fluoroquinolone compounds, either alone or in combination, have reported toxicity to photosynthetic aquatic organisms such cyanobacteria. [38] Tetracycline has shown to decrease soil bacterial diversity following its application to a plant-soil system. [24] Fluoroquinolones have shown to inhibit the growth of *Azopirillum brasilense*, a nitrogen-fixing bacteria in soil. [39]

The objective of this study was to assess the individual and combined effects of doxycycline and norfloxacin on microbial respiration and E2 mineralization in sewage sludge, biosolid and soil. We tested the hypothesis that when present at an environmentally relevant concentration of 4 mg kg^{-1} doxycycline and norfloxacin individually or combined will not inhibit microbial activity to the extent of significantly reducing E2 mineralization in media, but that at a ten times greater concentration rate, E2 mineralization will be reduced in one or more of the media tested.

MATERIALS AND METHODS

Chemicals and Media

Analytical grade 17 β-estradiol (≥98% pure), doxycycline (≥98% pure) and norfloxacin (≥98% pure) were purchased from Sigma-Aldrich Chemical Company (Saint Louis, MO, USA). 17 β-estradiol [4-^{14}C] (99% radiochemical purity, specific activity 45-60 mCi mmol^{-1}) was purchased from American Radiolabeled Chemicals Inc. (Saint Louis, MO, USA).

Media were sewage sludge, biosolid and soil samples (Table 1). Sewage sludge was obtained from facultative lagoon-based sewage treatment facility in southern Manitoba, Canada that services approximately 8000 people. Grab samples were taken from the outer edge of a lagoon and mixed thoroughly. The lagoon consisted of a primary stabilization cell that receives raw sewage while undergoing simultaneous treatment processes that include sedimentation, bioflocculation, chemical precipitation, biochemical oxidation, fermentation and disinfection. Grab samples of biosolid were obtained from a wastewater treatment plant in the province of Alberta, Canada. The plant treats more than 100,000 million litres of wastewater annually and the treatment processes to transform untreated sewage sludge into biosolid includes pre-treatment screening, enhanced primary treatment, primary treatment clarifying, solids settling, fermentation, and anaerobic digestion. The soil was sampled (0-15 cm) from the Ap-horizon of a Newdale Clay Loam, which is recognized as the provincial soil of Manitoba and represents a large area of agricultural land in this province. The soil is classified as an Orthic Black Chernozem (nearest equivalent to the U.S. Soil Taxonomy are the Udic Boroll subgroups).[40]

Both sewage sludge and biosolid media were analyzed for selected hormones (E2, EE2, estriol, estrone, progesterone and testosterone) and pharmaceuticals (acetaminophen, benzoylecgonine, chloramphenicol, ciprofloxacin, clindamycin, cocaine, codeine, cotinine, enrofloxacin, erythromycin, fluoxetine, lincomycin, methamphetamine, norfloxacin, norfluoxetine, ofloxacin, oxolinic acid, pentoxifylline, pipemidic acid, sulfabenzamide, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfaquinoxaline, sulfathiazole and trimethoprim) by Alberta Innovates Technology Futures, Edmonton, AB. Detection limits ranged from 0.01 to 0.10 ng g^{-1} for hormones and from 0.2 to 5.0 ng g^{-1} for pharmaceuticals. Hormones were below detection limits in both samples. Cotinine was the only pharmaceutical detected but in both biosolid (26.8 ng g^{-1}) and sewage sludge (7.3 ng g^{-1}). Cotinine is a metabolite of nicotine. In a study of river sediments, nicotine inhibited microbial respiration [41], but the impact of cotinine on soil microbial communities is unknown.

Microcosm Experiments

The impact of antibiotics on E2 mineralization in these media was quantified using a factorial design of antimicrobial additions plus controls without antimicrobials. Both radiolabeled and analytical grade E2 was applied and the E2 experiment was terminated at 189 days at which time E2 mineralization had slowed to <0.1% per day for all treatments. In a parallel experiment, only analytical E2 was applied, but the same factorial design plus control was utilized to quantify the impact of antibiotics on microbial respiration (CO_2 production) over 189 days.

Microcosms consisted of a 500 mL Mason jar containing a 90 mL glass beaker with 25 g media (wet weight), a 20 mL scintillation vial with 8 mL of 0.5 M NaOH to trap CO_2 (as well as $^{14}CO_2$ in case of the E2 experiment) and a 15 mL test tube with 3 mL of acidified water (pH≤3) to preserve a humid environment without trapping CO_2. Microcosms were pre-incubated at 20˚C for seven days prior to E2 applications. Antibiotics were added on day 5. Antibiotic stock solutions were prepared in milli-Q water that was acidified with 0.5 M HCl (pH 4) to help dissolve doxycycline and/or norfloxacin. Antibiotic concentrations were 0.1 g L^{-1} doxycycline or norfloxacin, 1.0 g L^{-1} doxycycline or norfloxacin, 0.05 g L^{-1} doxycycline plus 0.05 g L^{-1} norfloxacin, and 0.5 g L^{-1} doxycycline plus 0.5 g L^{-1} norfloxacin. Flasks were wrapped in tin foil to minimize photolysis and solutions were used within 2 hours after preparation. Antibiotic solutions (1 mL) were added to achieve a total antimicrobial concentration of 4 mg kg^{-1}, which is within the range reported for sewage sludge and biosolid [30-34], and at a ten times greater concentration (40 mg kg^{-1}). For the treatments not receiving antimicrobials (i.e. control), 1 mL of acidified water (pH 4) was added to the media to ensure a similar decrease in pH of ~ 0.2 to 0.3, depending on the media.

E2 stock solutions (0.5 mL) were mixed with media in microcosms to achieve an E2 concentration of 50 µg kg^{-1}. For the E2 mineralization experiments, E2 stock solutions were prepared to also include radiolabeled E2 so that each microcosm contained 1,667 Bq of [4-^{14}C] E2. NaOH traps were removed and replaced every 2 to 4 days for the first two weeks, then every

4 to 7 days until week 13, and finally every 10 to 15 days until completion of the experiment at 189 d. For the E2 experiment, scintillation cocktail (8 mL, 30% Scintisafe scintillation cocktail; Fisher Scientific, Fairlawn, NJ) was added to the removed NaOH traps and the evolved radioactivity was measured by Liquid Scintillation Counting (LSC) using an LS 6500 (Beckman Instruments, Fullerton, CA) with automated quench correction (#C Method) and a maximum counting time of 10 min. For the respiration experiment, removed NaOH traps were titrated with 0.05 M HCl to determine CO_2 (g) evolved.

Statistical Analyses

The effect of media and antibacterial treatment on total E2 mineralization or total respiration was analyzed by ANOVA using PROC MIXED in SAS ver. 9.4 (SAS Institute Inc., 2013). All data respected normality based on Shapiro-Wilks statistic ≥ 0.9. Dissipation kinetics of E2 for each media was generated and compared using PROC NLIN in SAS. Dissipation data for E2 was described by the first-order kinetic model $C_t = C_0e^{-kt}$, where C_t is the amount of radioactivity left in the media at time t [Bq.], C_0 is the initial amount of radioactivity in the media [Bq], and k is the first-order rate constant [d^{-1}]. Half-lives of radioactivity in media [d^{-1}] was calculated by ln2/k. The calculated half-life is equivalent to the time that 50% of the radioactivity would have been mineralized in a media. The Tukey multiple comparison procedure was used for pairwise comparisons of treatment means. Treatment differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Total E2 mineralization at 189 d significantly decreased in the order of sewage sludge > soil > biosolid (Table 2) and ranged from 33 to 42% for sewage sludge, 21 to 24% for soil, and 1 to 5% for biosolid. Antimicrobial treatment had no significant effect on total E2 mineralization (Table 2). E2 mineralization followed first-order kinetics (Fig. 1) with the calculated time that half of the applied E2 was mineralized (E2-½) ranging from 294 to 418 d in sewage sludge, from 721 to 869 d in soil and from 2,258 to 14,146 d in biosolid. E2-½ significantly increased in the order of sewage sludge < soil < biosolid (Table 3). The presence of antimicrobials had no significant effect on E2-½ in soil. Similarly, in a previous microcosm study of 133 d [42], 40 and 200 mg kg^{-1} tetracycline had no significant effect on E2 mineralization when applied to the Newdale clay loam soil, but the same amounts of tetracycline applied to manure inhibited E2 mineralization. In the human waste products, the combined application of 2 mg kg^{-1} doxycycline plus 2 mg kg^{-1} norfloxacin significantly increased E2-½ in sewage sludge, relative to sewage sludge without antimicrobials added (Table 3). Likewise, single applications of 4 or 40 mg kg^{-1} norfloxacin and single application of 40 mg kg^{-1} doxycycline increased E2-½ in biosolid, relative to all other treatments (Table 3). In another laboratory experiment of shorter duration (7 d) [23], the presence of a single antimicrobial (chlortetracycline, sulfamethazine or tylosin) in a loam soil reduced the transformation of E2 to estrone (E1) when the antimicrobial was applied at 2 or 200 mg kg^{-1}.

Respiration (mg CO_2) was always numerically greater in biosolid than sewage sludge or soil, including at the onset of the experiment (Fig. 2) and, regardless of the antimicrobial treatment, cumulative respiration at 189 d was significantly greater in biosolid than sewage sludge or soil

(Fig. 3). Cumulative respiration was significantly smaller in biosolid when norfloxacin and doxycycline were applied together, relative to biosolid receiving single applications of 4 or 40 mg kg^{-1} norfloxacin or 40 mg kg^{-1} doxycycline. Cumulative respiration was significantly greater in biosolid with 40 mg kg^{-1} doxycycline than in biosolid free of antibiotics. Total CO_2 respiration at 189 d was significantly (P=< 0.0001) influenced by media × antimicrobial treatment interactions. Specifically, when norfloxacin or doxycycline were applied together, or when no antimicrobials were added, cumulative respiration was significantly greater in the sewage sludge than soil; however, when either norfloxacin or doxycycline were applied alone, sewage sludge and soil had a similar cumulative respiration (Fig. 3). Antimicrobial treatment did not show a significant effect on cumulative respiration in sewage sludge or soil.

Microbial degradation is a main path of E2 mineralization. [20-22] This study applied antibiotics to human waste products and soil at an environmentally relevant concentration and at a rate that was ten times greater. Regardless of whether norfloxacin or doxycycline was applied alone or in combination, the antimicrobials did not supress microbial respiration and, consistently, antimicrobial applications had no pronounced effect on E2 mineralization. Biosolid showed a very small total E2 mineralization relative to sewage sludge and soil, yet this medium had a much greater microbial respiration. E2 is hydrophobic with a low water solubility (5.4 - 13.3 mg L^{-1}) and high octanol-water partitioning coefficient (3.8 - 4) [43], and hence has a strong affinity for organic matter substances in biosolid. [44] Desorption of E2 from biosolid has been reported to be low (e.g., 0.4% [43]), further suggesting reduced availability of E2 for bacterial degradation. In a previous microcosm study, Rose et al. [42] reported much larger CO_2 respirations for biosolid samples than primary and secondary sewage sludge samples. In that study [42], a substantial 17 αethinylestradiol (EE2) mineralization was observed in sewage sludge, but EE2 mineralization was negligible in biosolid because of strong sorption and reduced bioavailability of EE2. Biosolid is a valuable agricultural amendment which can increase soil organic matter content and nutrient levels. Based on our findings, doxycycline and norfloxacing alone or in combination at environmentally relevant concentrations are not expected to suppress E2 mineralization in biosolids or soil, reassuring biosolid field application. However, the observed persistence of E2 in biosolid will increase the risk of soil contamination by estrogens and further the risk of surface and ground water contamination [8,9] and crop uptake [8,45]. Monitoring of E2 concentration in biosolid combined with management strategies such as composting or extended storage to reduce E2 concentration prior to land application can be employed to reduce the environmental dispersal of E2. [46]

CONCLUSION

E2 mineralization in human waste products and soil followed first-order kinetics. Total E2 mineralization at 186 d significantly decreased in the order of sewage sludge > soil > biosolid, yet cumulative respiration was significantly larger in biosolid than sewage sludge or soil. Biosolid has a large total C content and strong E2 sorption to organic matter constituents could have reduced E2 availability of microbial degradation. Regardless of whether norfloxacin or doxycycline were applied alone or in combination, the antimicrobials did not supress microbial respiration and antimicrobial applications had no pronounced effect on E2 mineralization.

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REFERENCES

[1] Combalbert, S.; Hernandez-Raquet, G. Occurrence, fate, and biodegradation of estrogens in sewage and manure. Appl. Microbiol. Biotechnol. **2010,** 86 (6), 1671-1692.

[2] Braga, O.; Smythe, G.A.; Schafer, A.I.; Feitz, A.J. Fate of steroid estrogens in Australian inland and coastal wastewater treatment plants. Environ. Sci. Technol. **2005,** 39 (9), 3351-3358.

[3] Andersen, H.; Siegrist, H.; Halling-Sorensen, B.; Ternes, T.A. Fate of estrogens in a municipal sewage treatment plant. Environ. Sci. Technol. **2003,** 37 (18), 4021-4026.

[4] Ternes, T.A.; Andersen, H.; Gilberg, D.; Bonerz, M. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal. Chem. **2002,** 74 (14), 3498-3504.

[5] Muller, M.; Rabenoelina, F.; Balaguer, P.; Patureau, D.; Lemenach, K.; Budzinski, H.; Barcelo, D.; De Alda, M.L.; Kuster, M.; Delgenes, J.; Hernandez-Raquet, G. Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. Environ. Toxicol. Chem. **2008,** 27 (8), 1649-1658.

[6] Finlay-Moore, O.; Hartel, P.G.; Cabrera, M.L. 17 Beta-Estradiol and Testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual. **2000,** 29 (5), 1604-1611.

[7] Rahman, M.F.; Yanful, E.K.; Jasim, S.Y. Endocrine disrupting compounds (EDCs) and pharmaceuticals and personal care products (PPCPs) in the aquatic environment: implications for the drinking water industry and global environmental health. J. Water Health **2009,** 7 (2), 224-243.

[8] Gottschall, N.; Topp, E.; Metcalfe, C.; Edwards, M.; Payne, M.; Kleywegt, S.; Russell, P.; Lapen, D.R. Pharmaceutical and personal care products in groundwater, subsurface drainage, soil, and wheat grain, following a high single application of municipal biosolids to a field. Chemosphere **2012,** 87 (2), 194-203.

[9] Kjaer, J.; Olsen, P.; Bach, K.; Barlebo, H.C.; Ingerslev, F.; Hansen, M.; Sorensen, B.H. Leaching of estrogenic hormones from manure-treated structured soils. Environ. Sci. Technol. **2007,** 41 (11), 3911-3917.

[10] Routledge, E.J.; Sheahan, D.; Desbrow, C.; Brighty, G.C.; Waldock, M.; Sumpter, J.P. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environ. Sci. Technol. **1998,** 32 (11), 1559-1565.

[11] Fenske, M.; Maack, G.; Schafers, C.; Segner, H. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, Danio rerio. Environ. Toxicol. Chem. **2005,** 24 (5), 1088-1098.

[12] Lai, K.M.; Scrimshaw, M.D.; Lester, J.N. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. **2002,** 32 (2), 113-132.

[13] Yoshimoto, T.; Nagai, F.; Fujimoto, J.; Watanabe, K.; Mizukoshi, H.; Makino, T.; Kimura, K.; Saino, H.; Sawada, H.; Omura, H. Degradation of estrogens by Rhodococcus zopfii and Rhodococcus equi isolates from activated sludge in wastewater treatment plants. Appl. Environ. Microbiol. **2004,** 70 (9), 5283-5289.

[14] Khanal, S.K.; Xie, B.; Thompson, M.L.; Sung, S.; Ong, S.; Van Leeuwen, J. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. Environ. Sci. Technol. **2006,** 40 (21), 6537-6546.

[15] Yu, C.; Roh, H.; Chu, K. 17 beta-estradiol-degrading bacteria isolated from activated sludge. Environ. Sci. Technol. **2007,** 41 (2), 486-492.

[16] Kurisu, F.; Ogura, M.; Saitoh, S.; Yamazoe, A.; Yagi, O. Degradation of natural estrogen and identification of the metabolites produced by soil isolates of Rhodococcus sp and Sphingomonas sp. J. Biosci. Bioeng. **2010,** 109 (6), 576-582.

[17] Duong, C.N.; Lee, J.H.; Lim, B.J.; Kim, S.D. Biodegradation of estrogen conjugates by bacteria isolated from river sediments. Water Sci. Tech. **2011,** 64 (8), 1750-1758.

[18] Isabelle, M.; Villemur, R.; Juteau, P.; Lepine, F. Isolation of estrogen-degrading bacteria from an activated sludge bioreactor treating swine waste, including a strain that converts estrone to beta-estradiol. Can. J. Microbiol. **2011,** 57 (7), 559-568.

[19] Liang, R.; Liu, H.; Tao, F.; Liu, Y.; Ma, C.; Liu, X.; Liu, J. Genome sequence of Pseudomonas putida strain SJTE-1, a bacterium capable of degrading estrogens and persistent organic pollutants. J. Bacteriol. **2012,** 194 (17), 4781-4782.

[20] Shareef, A.; Egerer, S.; Kookana, R. Effect of triclosan and triclocarban biocides on biodegradation of estrogens in soils. Chemosphere **2009,** 77 (10), 1381-1386.

[21] Stumpe, B.; Marschner, B. Factors controlling the biodegradation of 17 beta-estradiol, estrone and 17 alpha-ethinylestradiol in different natural soils. Chemosphere **2009,** 74 (4), 556-562.

[22] Stumpe, B.; Marschner, B. Organic waste effects on the behavior of 17 beta-estradiol, estrone, and 17 alpha-ethinylestradiol in agricultural soils in long- and short-term setups. J. Environ. Qual. **2010,** 39 (3), 907-916.

[23] Chun, S.; Lee, J.; Geyer, R.; White, D.C.; Raman, D.R. Effect of agricultural antibiotics on the persistence and transformation of 17 beta-estradiol in a Sequatchie loam. J. Environ. Sci. Heal. B **2005,** 40 (5), 741-751.

[24] Lin, H.; Jin, D.; Freitag, T.E.; Sun, W.; Yu, Q.; Fu, J.; Ma, J. A compositional shift in the soil microbiome induced by tetracycline, sulfamonomethoxine and ciprofloxacin entering a plant-soil system. Environ. Pollut. **2016,** 212, 440-448.

[25] Colucci, M.S.; Bork, H.; Topp, E. Persistence of estrogenic hormones in agricultural soils: 17 beta-estradiol and estrone. J. Environ. Qual. **2001,** 30 (6), 2070-2076.

[26] Rose, K.P.; Farenhorst, A. Estrone and 17 beta-estradiol mineralization in liquid swine manure and soil in the presence and absence of penicillin or tetracycline. J. Environ. Sci. Heal. B **2014,** 49 (5), 331-337.

[27] Bradley, P.M.; Barber, L.B.; Chapelle, F.H.; Gray, J.L.; Kolpin, D.W.; McMahon, P.B. Biodegradation of 17 beta-estradiol, estrone and testosterone in stream sediments. Environ. Sci. Technol. **2009,** 43 (6), 1902-1910.

[28] Barbie, D.A.; Kennedy, B.K. Doxycycline: new tricks for an old drug. Oncotarget **2015,** 6 (23), 19336-19337.

[29] Saleh, G.A.; Askal, H.F.; Refaat, I.H.; Abdel-aal, F.A.M. Review on recent separation methods for determination of some fluoroquinolones. J. Liq. Chromatogr. Rel. Technol. **2013,** 36 (10), 1401-1420.

[30] He, K.; Soares, A.D.; Adejumo, H.; McDiarmid, M.; Squibb, K.; Blaney, L. Detection of a wide variety of human and veterinary fluoroquinolone antibiotics in municipal wastewater and wastewater-impacted surface water. J. Pharm. Biomed. Anal. **2015,** 106, 136-143.

[31] Jia, A.; Wan, Y.; Xiao, Y.; Hu, J. Occurrence and fate of quinolone and fluoroquinolone antibiotics in a municipal sewage treatment plant. Water Res. **2012,** 46 (2), 387-394.

[32] Langdon, K.A.; Warne, M.S.U.; Kookana, R.S. Aquatic hazard assessment for pharmaceuticals, personal care products, and endocrine-disrupting compounds from biosolids-amended land. Integr. Environ. Assess. Manag. **2010,** 6 (4), 663-676.

[33] Lindberg, R.H.; Wennberg, P.; Johansson, M.I.; Tysklind, M.; Andersson, B.A.V. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. Environ. Sci. Technol. **2005,** 39 (10), 3421-3429.

[34] Lindberg, R.H.; Bjorklund, K.; Rendahl, P.; Johansson, M.I.; Tysklind, M.; Andersson, B.A.V. Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. Water Res. **2007,** 41 (3), 613-619.

[35] Walters, E.; McClellan, K.; Halden, R.U. Occurrence and loss over three years of 72 pharmaceuticals and personal care products from biosolids-soil mixtures in outdoor mesocosms. Water Res. **2010,** 44 (20), 6011-6020.

[36] Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environ. Sci. Technol. **2002,** 36 (6), 1202-1211.

[37] Fatta-Kassinos, D.; Meric, S.; Nikolaou, A. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal. Bioanal. Chem. **2011,** 399 (1), 251-275.

[38] Gonzalez-Pleiter, M.; Gonzalo, S.; Rodea-Palomares, I.; Leganes, F.; Rosal, R.; Boltes, K.; Marco, E.; Fernandez-Pinas, F. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment. Water Res. **2013,** 47 (6), 2050-2064.

[39] Lewis, G.; Juhasz, A.; Smith, E. Environmental metabolites of fluoroquinolones: synthesis, fractionation and toxicological assessment of some biologically active metabolites of ciprofloxacin. Environ. Sci. Pollut. Res. Int. **2011,** 19 (7), 2697-707.

[40] . Soi1 Classification Working Group.The Canadian system of soil classification. Agriculture and Agri-Food Canada Publ. 1646, Ottawa, ON **1998,** Agriculture and Agri-Food Canada Publ.1646.

[41] Bunch, A.R.; Bernot, M.J. Distribution of nonprescription pharmaceuticals in central Indiana streams and effects on sediment microbial activity. Ecotoxicology **2011,** 20 (1), 97-109.

[42] Rose, K.P.; Farenhorst, A.; Claeys, A.; Ascef, B. 17 beta-estradiol and 17 alpha-ethinylestradiol mineralization in sewage sludge and biosolids. J. Environ. Sci. Heal. B **2014,** 49 (11), 871-879.

[43] Andaluri, G.; Suri, R.P.S.; Kumar, K. Occurrence of estrogen hormones in biosolids, animal manure and mushroom compost. Environ. Monit. Assess. **2012,** 184 (2), 1197-1205.

[44] Bedard, M.; Giffear, K.A.; Ponton, L.; Sienerth, K.D.; Moore, V.D.G. Characterization of binding between 17 beta-estradiol and estriol with humic acid via NMR and biochemical analysis. Biophys. Chem. **2014,** 189, 1-7.

[45] Jacobsen, A.M.; Lorenzen, A.; Chapman, R.; Topp, E. Persistence of testosterone and 17 beta-estradiol in soils receiving swine manure or municipal biosolids. J. Environ. Qual. **2005,** 34 (3), 861-871.

[46] Xia, K.; Bhandari, A.; Das, K.; Pillar, G. Occurrence and fate of pharmaceuticals and personal care products (PPCPs) in biosolids. J. Environ. Qual. **2005,** 34 (1), 91-104.

FIGURE CAPTIONS

Fig 1. Cumulative 17 β-estradiol mineralization in human waste products and soil with and without antimicrobials added. Symbols refer to: solid box = 4 mg kg^{-1} doxycycline, open box = 40 mg kg^{-1} doxycycline, solid circle = 4 mg kg^{-1} norfloxacin; open circle = 40 mg kg^{-1} norfloxacin, solid triangle = 2 mg kg^{-1} doxycycline + 2 mg kg^{-1} norfloxacin, open triangle = 20 mg kg^{-1} doxycycline + 20 mg kg^{-1} norfloxacin, and cross (x) in an open box = control.

Fig 2. Cumulative respiration in human waste products and soil with and without antimicrobials added. Symbols refer to: solid box = 4 mg kg^{-1} doxycycline, open box = 40 mg kg^{-1} doxycycline, solid circle = 4 mg kg^{-1} norfloxacin; open circle = 40 mg kg^{-1} norfloxacin, solid triangle = 2 mg kg^{-1} doxycycline + 2 mg kg^{-1} norfloxacin, open triangle = 20 mg kg^{-1} doxycycline + 20 mg kg^{-1} norfloxacin, and cross (x) in an open box = control.

Fig 3. Cumulative respiration at 189 days in human waste products and soil with and without antimicrobials added. Control, no antimicrobial in the medium; Doxy4 and 40, doxycycline at 4 and 40 mg kg^{-1}; Norflox4 and 40, norfloxacin at 4 and 40 mg kg^{-1}; DoxyNor4 and 40, doxycycline and norfloxacin 1:1 mixture each at 2 and 20 mg kg^{-1}. Different letters indicate significant differences in cumulative CO_2 emission.

Table 1. Selected physical and chemical properties of sewage sludge, biosolid and soil

Parameter	Sewage sludge	Biosolid	Soil
Moisture (%)[1]	63	77	31
pH[2]	8.0	7.4	7.5
Total N[3] (mg kg^{-1})	4,400	11,050	6,800
Total C[4] (mg kg^{-1})	46,000	64,500	3.5%[5]
NH_4-N[6]	595	4460	13[7]
Cu (mg kg^{-1})[8]	0.82	1.39	1.03
Zn (mg kg^{-1})[8]	1.30	1.94	0.78

[1]Determined gravimetrically. [2]1:1 solution. [3]LECO model CHN 600 carbon-hydrogen-nitrogen determinator. [4]Loss on ignition method. [5]Soil organic carbon content. [6]Steam distillation into boric acid followed by titration with a standard acid. [7]NO_3^--N; Cadmium reduction method followed by modified Griess–Ilosvay method. [8]Aqua regia digestion followed by Inductively Coupled Plasma Optical Emission Spectroscopy.

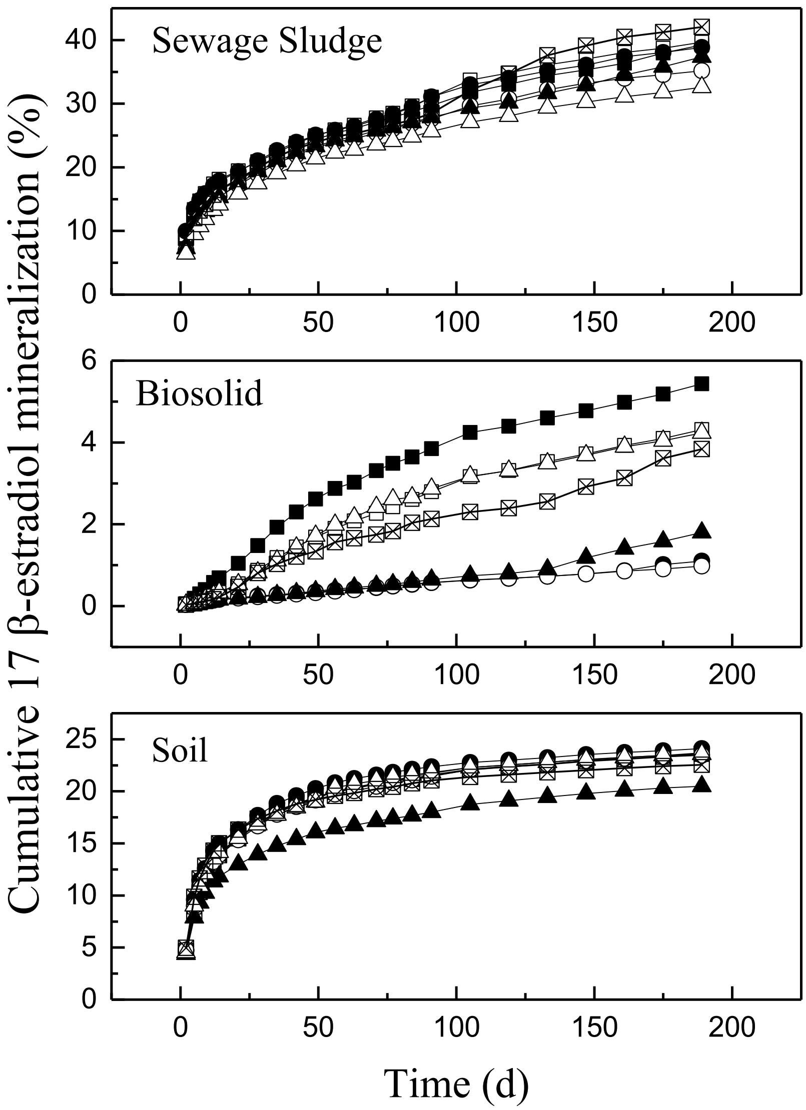
Table 2. Treatment effects of media and antimicrobials on total 17 β-estradiol mineralization at 189 d.

Treatment effect	Mineralization (%)
Media, P < 0.0001	
Sewage sludge	38a
Soil	23b
Biosolid	3c
Antimicrobials, P = 0.14	
Antimicrobial×Media, P = 0.26	
Control	23a
4 mg kg^{-1} doxycycline	23a
40 mg kg^{-1} doxycycline	21a
4 mg kg^{-1} norfloxacin	20a
40 mg kg^{-1} norfloxacin	20a
2 mg kg^{-1} doxycycline + 2 mg kg^{-1} norfloxacin	20a
20 mg kg^{-1} doxycycline + 20 mg kg^{-1} norfloxacin	23a

Table 3. First-order dissipation models of 17 β-estradiol radioactivity in human waste products and soil, and the calculated days after application that half of initial radioactivity is mineralized in these media in the presence and absence of antimicrobials. Different letters indicate significantly different mineralization half-lives (HL).

Media	Antimicrobials (in mg kg^{-1})	Model	HL*	RMSE	CV
Sewage sludge	Control	$C_t = 112167e^{-0.0021t}$	332ab	11872	12.2
Sewage sludge	4 doxycycline	$C_t = 110429e^{-0.0021t}$	329ab	12903	13.5
Sewage sludge	40 doxycycline	$C_t = 110087e^{-0.0020t}$	345ab	12095	12.6
Sewage sludge	4 norfloxacin	$C_t = 112963e^{-0.0019t}$	373bc	10835	10.9
Sewage sludge	40 norfloxacin	$C_t = 112929e^{-0.0019t}$	363bc	11285	11.4
Sewage sludge	2 doxycycline + 2 norfloxacin	$C_t = 114202e^{-0.0017t}$	418c	10220	10.0
Sewage sludge	20 doxycycline + 20 norfloxacin	$C_t = 113171e^{-0.0024t}$	294a	12825	13.3
Soil	Control	$C_t = 113537e^{-0.00096t}$	721d	6855	6.45
Soil	4 doxycycline	$C_t = 113342e^{-0.00096t}$	723d	6680	6.30
Soil	40 doxycycline	$C_t = 112309e^{-0.00095t}$	733d	6697	6.37
Soil	4 norfloxacin	$C_t = 113483e^{-0.00095t}$	731d	6560	6.17
Soil	40 norfloxacin	$C_t = 116055e^{-0.00080t}$	869d	7870	7.17
Soil	2 doxycycline + 2 norfloxacin	$C_t = 113238e^{-0.00095t}$	727d	6773	6.39
Soil	20 doxycycline + 20 norfloxacin	$C_t = 112349e^{-0.00080t}$	869d	5961	5.61
Biosolid	Control	$C_t = 128997e^{-0.00031t}$	2258e	6147	4.87
Biosolid	4 doxycycline	$C_t = 129510e^{-0.00025t}$	2773e	2762	2.17
Biosolid	40 doxycycline	$C_t = 129689e^{-0.00005t}$	13330f	409	0.32
Biosolid	4 norfloxacin	$C_t = 129682e^{-0.00005t}$	14146f	373	0.29
Biosolid	40 norfloxacin	$C_t = 129829e^{-0.00008t}$	8252f	733	0.57
Biosolid	2 doxycycline + 2 norfloxacin	$C_t = 129472e^{-0.00025t}$	2795e	3138	2.47
Biosolid	20 doxycycline + 20 norfloxacin	$C_t = 129605e^{-0.00020t}$	3431e	2518	1.97

*HL=ln2/k. RMSE = Root Mean Square Error. CV = Coefficient of variation of the RMSE calculated by RMSE/Mean × 100.



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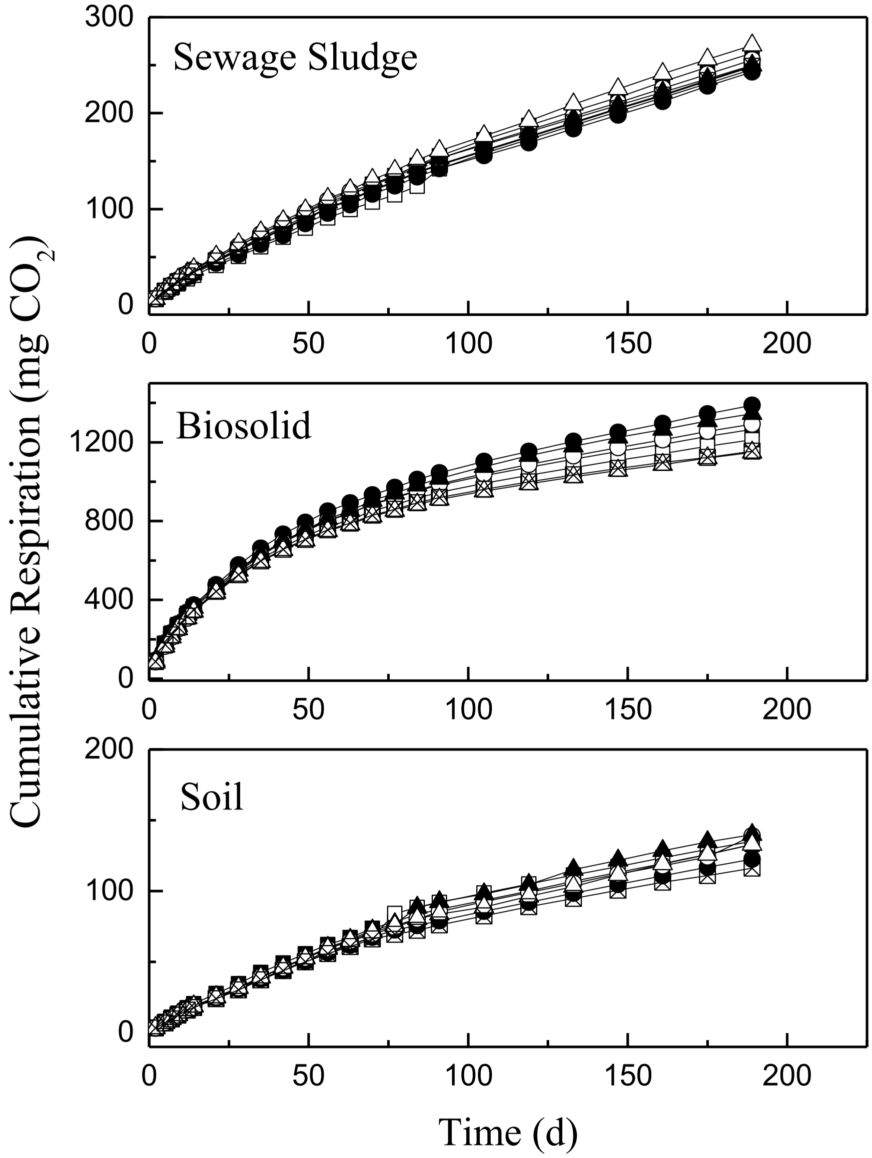
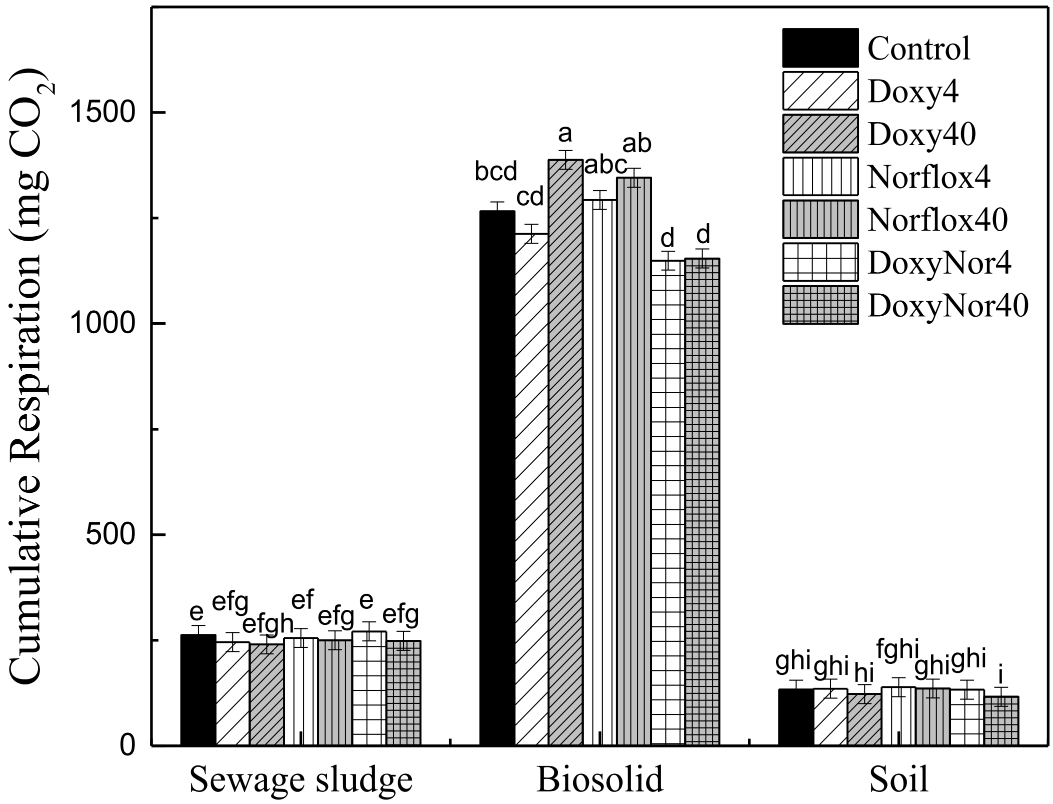


Fig 2. Cumulative respiration in human waste products and soil with and without antimicrobials added. Symbols refer to: solid box = 4 mg kg^{-1} doxycycline, open box = 40 mg kg^{-1} doxycycline, solid circle = 4 mg kg^{-1} norfloxacin; open circle = 40 mg kg^{-1} norfloxacin, solid triangle = 2 mg kg^{-1} doxycycline + 2 mg kg^{-1} norfloxacin, open triangle = 20 mg kg^{-1} doxycycline + 20 mg kg^{-1} norfloxacin, and cross (x) in an open box = control.



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