Accelerated Biodegradation of Veterinary Antibiotics in Agricultural Soil following Long-Term Exposure, and Isolation of a Sulfamethazine-degrading *Microbacterium* sp.

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The World Health Organization has identified antibiotic resistance as one of the top three threats to global health. There is concern that the use of antibiotics as growth promoting agents in livestock production contributes to the increasingly problematic development of antibiotic resistance. Many antibiotics are excreted at high rates, and the land application of animal manures represents a significant source of environmental exposure to these agents. To evaluate the long-term effects of antibiotic exposure on soil microbial populations, a series of field plots were established in 1999 that have since received annual applications of a mixture of sulfamethazine (SMZ), tylosin (TYL), and chlortetracycline (CTC). During the first 6 yr (1999–2004) soils were treated at concentrations of 0, 0.01 0.1, and 1.0 mg kg^{-1} soil, in subsequent years at concentrations of 0, 0.1, 1.0, and 10 mg kg^{-1} soil. The lower end of this concentration range is within that which would result from an annual application of manure from medicated swine. Following ten annual applications, the fate of the drugs in the soil was evaluated. Residues of SMZ and TYL, but not CTC were removed much more rapidly in soil with a history of exposure to 10 mg/kg drugs than in untreated control soil. Residues of ^{14}C-SMZ were rapidly and thoroughly mineralized to $^{14}CO_2$ in the historically treated soils, but not in the untreated soil. A SMZ-degrading *Microbacterium* sp. was isolated from the treated soil. Overall, these results indicate that soil bacteria adapt to long-term exposure to some veterinary antibiotics resulting in sharply reduced persistence. Accelerated biodegradation of antibiotics in matrices exposed to agricultural, wastewater, or pharmaceutical manufacturing effluents would attenuate environmental exposure to antibiotics, and merits investigation in the context of assessing potential risks of antibiotic resistance development in environmental matrices.

Antibiotics are widely used in North American and Asian livestock and poultry production for growth promotion, prophylaxis, and treatment of illness (Sarmah et al., 2006). There is a widespread concern that this practice promotes resistance to antibiotics used in human medicine, a crucially important public health challenge (World Health Organization, 2001; Aarestrup, 2005; McEwen, 2006). Many antibiotics are not metabolized and excreted largely unchanged (Pope et al., 2009). Consequently antibiotic residues are detected in water adjacent to livestock production areas, in soils treated with animal manures, and now very broadly in the environment (Kolpin et al., 2002; Batt et al., 2006; Aust et al., 2008). It is unknown if concentrations of antibiotics released through livestock production are sufficient to promote resistance in environmental bacteria, but given the apparent increase in abundance of antibiotic resistance genes detected in soil during the last 70 yr it is an issue of concern (Knapp et al., 2010). Soils fertilized with manure from medicated animals and water subjected to agricultural runoff are enriched in genes encoding resistance to specific antibiotics (Storteboom et al., 2010; Heuer et al., 2011b). A reservoir of antibiotic resistance genes in the environment that is made larger through contamination with agricultural wastes may represent an enhanced threat to human health (Wright, 2010).

Within this context we have been exploring the long-term effects of selected veterinary antibiotics on soil microbial populations. A series of replicated plots were established in London, Ontario, Canada, in 1999 that have since been treated once annually every spring with a mixture of sulfamethazine (SMZ), tylosin (TYL), and chlortetracycline (CTC) (Fig. 1). The drug mixture was applied to achieve a range of soil concentrations (0, 0.1, 1.0, and 10.0 mg of each drug per kilogram of soil) encompassing that which would be expected from a typical rate of annual application of manure from a commercial

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Abbreviations: AAFC, Agriculture and Agri-Food Canada; ADMP, 2-amino-3,4-dimethylpyrimidine, CTC, chlortetracycline; HPLC, high-performance liquid chromatography; DT_{50}, number of days required for 50% dissipation of a compound; LC-MS, liquid chromatography–mass spectrometry; PCR, polymerase chain reaction; SMZ, sulfamethazine, TYL, tylosin.

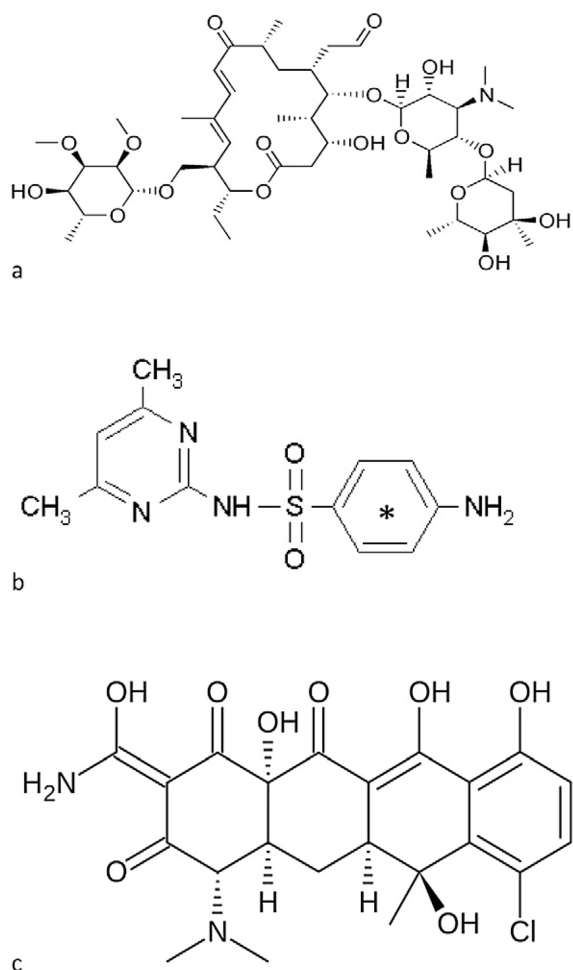

Fig. 1. Structures of (a) tylosin, (b) sulfamethazine, and (c) chlortetracycline. The asterisk in the benzylic portion of the sulfamethazine molecule indicates that the ring is uniformly ^{14}C-labeled.

swine farm. The antibiotics were added to the soil without the addition of manure to directly evaluate the effects of the drugs on soil microorganisms. In the present study, we evaluated the effect of long-term exposure to antibiotics on their soil persistence.

Materials and Methods

Chemicals and Media

Sodium SMZ, CTC, TYL tartrate, and 2-amino-3,4-dimethylpyrimidine (ADMP) were purchased from Sigma Chemical Co. (Toronto, ON). [Phenyl-ring-UL-^{14}C]-Sulfamethazine (sp. act. 55 mCi mmol, radioactive purity 99%) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Antibiotics were dissolved in sterile distilled water, filter sterilized (Millex-GV, 0.22 μm, Millipore, Toronto, ON), and frozen at −20°C until ready for use.

Description of Long-Term Soil Treatments

The experiment was undertaken at the Agriculture and Agri-Food Canada experimental farm in London, Canada. The soil is a silt loam (gray brown Luvisol) with the following properties: pH of 7.5, cation exchange capacity of 13.2, sand/silt/clay (%) of 18/67/15, and organic matter content of 3.4%. Before the experiment the soil had not been treated with manure or biosolids, materials that could have carried drugs. It had not been treated with pesticides or fertilizers since 1986. Twelve 2-m^2 plots isolated by fiberglass frames (Harris et al., 1971) were established and received annually from 1999 to 2004 a mixture of TYL, SMZ, and CTC to achieve concentrations for each drug of 0.01, 0.1 and 1 mg kg^{-1} soil to a depth of 15 cm. Each treatment was randomly applied in triplicate and three plots were left untreated as controls. The control plots were otherwise managed exactly as described for the treated. Starting in 2005, the annual applications were increased 10-fold to achieve concentrations of 0.1, 1, and 10 mg kg^{-1}. The increase was done because no effects of the lower concentrations were detected on various soil functional assays, results to be reported elsewhere. Drugs were applied each June by mixing in aqueous mixtures of the antibiotics to 1 kg of soil from each plot, followed by incorporation of the amended soil into the corresponding microplot to a depth of 15 cm using a mechanized rototiller. Plots were cropped continuously to soybeans (*Glycine max* var. Harrosoy) during each growing season, and received no further management other than manual weeding. Each microplot was sampled immediately before antibiotic application, 7 and 30 d post-application by collecting and pooling six 20-cm soil cores. These were sieved to a maximum particle size of 2 mm, and frozen at −7°C until ready for analysis.

Evaluation of Sulfamethazine, Chlortetracycline, and Tylosin Persistence in Soil

Fifty-gram (dry weight equivalent) portions of sieved soil adjusted to a soil moisture content of 15% were amended with 100 ng g^{-1} of unlabeled SMZ and 10,000 dpm g^{-1} [U-phenyl-^{14}C]-SMZ and incubated at 30°C in laboratory microcosms. Incubation conditions, radioisotope analytical methods and instrumentation, and calculations are described in detail in Al-Rajab et al. (2009) and Sabourin et al. (2011). Chlortetracycline and TYL were not available radiolabeled, and thus their persistence was evaluated by liquid chromatography–mass spectrometry (LC–MS/MS) as described below. Twenty-gram portions of soil were each spiked with 10 mg kg^{-1} of the respective drug (Sigma-Aldrich Canada Ltd.) and incubated as described for SMZ. One-gram portions were removed daily for a week, and extracted according to Carlson and Mabury (2006) for TYL and CTC. Briefly, 1 g of soil was weighed into a 15 mL Falcon tube and 1.2 mL of 1 M ammonium citrate buffer with ammonium hydroxide and 6 mL of ethyl acetate were added. The tube was vortexed for 15 s, shaken at 400 rpm for 1 h using a wrist action shaker and centrifuged at 1380 *g* for 10 min. The ethyl acetate layer was removed and placed in a clean glass labeled tube. Following this, another 6 mL were added to the tube containing the soil slurry and the vortex-shake-centrifuge sequence was repeated. The ethyl acetate layer was removed and added to the previously removed ethyl acetate. The ethyl acetate was dried down under a gentle stream of N_2 in a 40°C water bath. Sulfamethazine was extracted as described above, except that 100% methanol was the solvent.

Enrichment and Isolation of Sulfamethazine-Degrading Bacteria

Bacteria with the ability to grow at the expense of SMZ as sole nitrogen or carbon source with citrate as an additional source of carbon were enriched and isolated from soil as follows. A mineral salts medium consisted of the following components (per liter): K_2HPO_4, 1.6 g; KH_2PO_4, 0.4 g; NaCl, 0.1 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $CaCl_2{\cdot}2H_2O$, 25 mg; H_3BO_3, 2 mg; $MnSO_4{\cdot}H_2O$, 1.8 mg; $ZnSO_4{\cdot}7H_2O$, 0.2 mg; $CuSO_4$ 0.1 mg; $NaMoO_4{\cdot}2H_2O$ 0.25 mg. The pH was adjusted to 7.2 ± 0.2. The medium was autoclaved at 120°C for 20 min and then supplemented with the following sterilized (0.22 μm pore size cellulose acetate filter; SteriCup, Millipore, Mississauga, ON) solutions: Biotin, 0.1 mg; thiamin, 0.04 mg; $FeSO_4{\cdot}7H_2O$, 10 mg; SMZ, 50 mg; trisodium citrate, 250 mg. Portions of soil treated annually with 10 mg kg^{-1} drugs (the high rate treatment) were inoculated into flasks containing 15 mL of the SMZ/citrate medium and incubated statically at 30°C. Removal of the drug and accumulation of putative transformation product was evaluated weekly by UV (200–500 nm) spectroscopic analysis and by high-performance liquid chromatography (HPLC). Enrichment flasks showing SMZ degradation by UV scan and HPLC were selected for isolation of bacteria responsible for degrading SMZ. Briefly, 100 μL of enrichment was plated onto solid mineral salts medium containing 50 mg L^{-1} SMZ, and 250 mg L^{-1} citrate (SMZ mineral salts). Plates were incubated at 30°C for up to 1 wk, and were observed daily for growth. Individual isolated colonies were picked, streaked to purity and screened for SMZ degradation by inoculating them into 2 mL of sterile mineral salts medium containing 50 mg L^{-1} SMZ with or without 250 mg L^{-1} citrate. Cultures were incubated statically at 30°C and observed on days 0, 1, 4, 7, 11, and 20 by UV scan as described above. Following purification, tentative identity was established on the basis of the 16S rRNA gene sequence following polymerase chain reaction (PCR) amplification of DNA directly from a purified colony as follows. Genomic DNA was extracted from plated cells using the Promega Wizard kit (Thermo Fisher Scientific, Ottawa, ON) according to the manufacturer instructions. Genomic DNA template and primers p1–34 (5′-CCTACGGGAGGCAGCAG-3′) (Muyzer et al., 1993) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Lane et al., 1985) were used in the PCR to generate a partial 16S rRNA gene product. The PCR program consisted of 94°C for 10 min then 30 cycles of 94°C for 20 sec, 55°C for 30 s and 72°C for 45 s with a final elongation step at 72°C for 5 min. PCR products were visualized on 1.5% agarose gel stained with GelRed to check the expected size of 535 base pairs. Polymerase chain reaction product was purified using the Qiagen PCR purification kit (Qiagen, Mississauga, ON). Sequencing was performed on a 3130xl Genetic Analyzer (Applied Biosystems, Woodbridge, ON) using an ABI Prism BigDye terminator cycle sequencing kit using the same primers used for PCR amplification. Sequence was analyzed by the BioEdit software, forward and reverse sequences were aligned to obtain the whole sequence and submitted to sequence match analysis of Ribosomal Data Project II to see the phylotype linkage. The 16S rRNA gene sequence was deposited in the GenBank database under accession number JQ804844.

Analytical Methods

Ultraviolet absorbance spectra of culture supernatants were determined using a PowerWave XS microplate reader and Gen5 microplate reader software (Biotek, Winooski, VT). Baseline corrected UV scan curves were exported to Microsoft Excel 2010 (Microsoft Corp., Mississauga, ON) for further data analysis and formatting. Removal of parent and production of transformation products in soil extracts or culture supernatants was evaluated by HPLC-UV (Varian 9050 Variable Wavelength UV/Vis Detector; Varian, Palo Alto, CA) and HPLC-RD (EG&G Berthold LB509 Radioflow Detector, Berthold GMBH & Co. KG., Bad Wildbad, Germany) equipped with an Agilent Zorbax Eclipse XDB C-18 column (4.6 mm × 250 mm, 5-μm pore size; Santa Clara, CA). The UV detector was set at 264 nm. The mobile phase consisted of MeOH:40 mM ammonium acetate (35:65) delivered at 1 mL min^{-1}. Retention times were 12 min (SMZ), 5.6 min (sulfanilic acid), and 15 min (ADMP). Liquid chromatography–MS/MS analysis of extractable CTC and TYL A was performed in electrospray ionization-positive mode on a Waters Quatro Ultima triple quadrupole mass spectrometer at a flow rate of 1 mL min^{-1} using a Kinetex C18 column with a 4:1 post column split. The HPLC analysis was performed on a Waters Alliance 2795 HPLC system using a 15-min acetonitrile/water gradient with 0.1% formic acid (initial 5% acetonitrile, held for 1 min, then increased to 100% acetonitrile for 11 min, held at 100% for 2 min and returned to the initial conditions for 1 min). Each drug was analyzed individually. Chlortetracycline eluted at 5.6 min and TYL at 6.4 min. The cone was set to 20 V for both and the collision was 50 and 60 eV, respectively, the transitions were as described by Carlson and Mabury (2006). Statistically significant differences were established at the 95% confidence level. Dissipation rates for parent compounds were estimated on the basis of removal from the extractable phase, and expressed here as the number of days to dissipate 50% of the added parent compound (DT_{50}). Dissipation curves were plotted using SigmaPlot (Version 10, Systat Software Inc., Chicago). Data in figures represent the mean and standard deviation of triplicate samples. Dissipation rate constants were calculated based on parameters obtained from dynamic curve fitting (SigmaPlot) of replicates within each treatments. Curves were fitted to a first-order equation (exponential decay).

Results and Discussion

Fate of Antibiotics in Soil

The persistence in soil of CTC and TYL was evaluated in 2011 following 12 annual applications in the field. Soil samples from the high rate treatment and the nontreated control plots were supplemented with 10 mg kg^{-1} of either CTC or TYL. Chlortetracycline was dissipated at comparable rates in the soil that had been treated annually with the highest concentration of mixed antibiotics (DT_{50} of 3.3 ± 0.5 d) compared with untreated (2.8 ± 0.4 d) control soil (Table 1). In contrast, TYL was dissipated significantly more rapidly (DT_{50} of 2.0 ± 1.0 d) in soils exposed annually to the high concentration antibiotic mixture than in the control (10.2 ± 5.4 d) treatment (Table 1).

Table 1. Days required to dissipate 50% (DT_{50}) of the indicated antibiotic in field soil that had received five annual applications of 10 mg kg^{-1} and five annual applications of 1 mg kg^{-1} mixture of the three drugs, and control soil that had not been previously exposed to the drugs.

Antibiotic	DT_{50}	
	With history of exposure	With no history of exposure
	———— d ————	
Sulfamethazine	1.3 ± 0.3†	5.3 ± 2.0*
Tylosin	2.0 ± 1.0	10.2 ± 5.4*
Chlortetracycline	3.3 ± 0.5	2.8 ± 0.4

* Indicates a statistically significant Itreatment effect ($p < 0.05$).

† Data are presented as mean ± SD.

The persistence in soil of SMZ was evaluated in June 2009 following 10 annual applications in the field. The availability of ^{14}C-SMZ made a detailed analysis of dissipation pathways and kinetics possible. Soil was sampled immediately before the 11th annual application (i.e., twelve months since the preceding application), 7 d post-application to evaluate any acute effect of the treatment, and 30 d post application to evaluate recovery from the treatment. In the pre-application sampling, ^{14}C-SMZ was mineralized to $^{14}CO_2$ in the soil that had been treated with the highest concentration of drugs, whereas there was no significant mineralization in soils from the other treatments (Fig. 2). Likewise, 7 d after the 11th annual application, the high rate treated soil rapidly mineralized ^{14}C-SMZ, whereas the other soils did not. Thirty days post-application, the high rate treatment was again the most active; however the 1 mg kg^{-1} treatment was also significantly more active than the control soils. As determined by HPLC analysis of soil extracts, residues of ^{14}C-SMZ were removed from the high-rate treated soil (DT_{50} of 1.3 ± 0.3 d) significantly ($p < 0.05$) more rapidly than the untreated (5.3 ± 2 d) soils (Table 1).

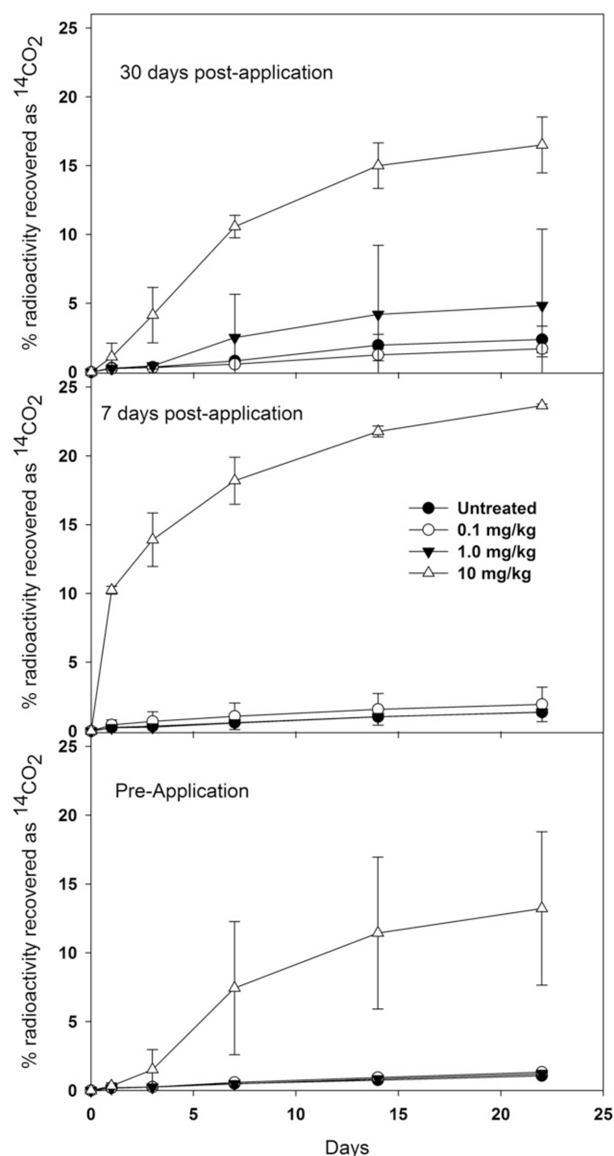

Fig. 2. Mineralization of ^{14}C-sulfamethazine in agricultural soil exposed to the antibiotic at the indicated concentrations. Field plots were sampled immediately before (bottom panel), 7 (center panel), or 30 d after the 11th antibiotic application.

Microbial Basis for Sulfamethazine Dissipation

To explore a potential microbial basis for the degradation of SMZ, samples of the high-rate treated and the untreated control soils were used to inoculate SMZ mineral salts medium. Attempts to enrich SMZ-degrading bacteria from untreated control soils were completely unsuccessful; soil slurries did not degrade SMZ. In contrast, the historically high-rate treated soils yielded an enrichment culture that completely removed SMZ, and that accumulated stoichiometric amounts (on a molar basis) of a product with a UV spectrum (maximum absorbance 228 and 286 nm) and HPLC retention time (15 min) corresponding to a standard of 2-amino-4,6-dimethylpyrimidine. The identity of the metabolite was further confirmed by LC-MS analysis, *m/z* 124 $[M+1]^+$.

Solid SMZ mineral salts medium was inoculated with the enrichment culture, and following 5 d of growth two distinct colony types were observed, a small yellow colony and a larger buff colored. When resuspended in liquid SMZ mineral salts medium the buff colony type did not degrade SMZ, whereas the yellow one did. The latter was therefore retained for further investigation.

A partial 16S rRNA gene sequence was compared with sequences in GenBank, shared 100% similarity with the genus *Microbacterium*, and on this basis the organism was tentatively identified as a *Microbacterium* sp., now designated strain C448. On SMZ mineral salts medium following 5 d of growth, colonies were pinpoint, yellow tinged, glistening, circular, and convex with an entire edge. Microscopic examination of Gram-stained cells revealed clustered or single Gram positive short rods with no branching. In liquid medium, *Microbacterium* sp. strain C448 mineralized ^{14}C–SMZ and excreted stoichiometric amounts of 2-amino-4,6-dimethylpyrimidine, completely consistent with the enrichment culture (data not shown). Taken together, these results indicate that the bacterium mineralized the benzylic portion of the molecule, and that the pyrimidine portion of the molecule was excreted as an end product.

Significance of Enhanced Degradation of Antibiotics

The present study has established for the first time that repeated long-term exposure of soil to a mixture of antibiotics resulted in accelerated biodegradation and reduced soil persistence of SMZ and TYL. There was no effect of treatment history

on CTC persistence, but this antibiotic was so rapidly dissipated that any enhancement of biodegradation would have been difficult to detect. Sulfamethazine and other sulfonamide antibiotics are amenable to biodegradation during wastewater treatment (Pérez et al., 2005; Yang et al., 2012). In soil, the DT_{50} for SMZ dissipation was from 2 to 21 d (Accinelli et al., 2007 Lin et al., 2010), for CTC from 20 to 42 d (Halling-Sørensen et al., 2005; Carlson and Mabury, 2006) and TYL from 4.5 to 86 d (Halling-Sørensen et al., 2005; Carlson and Mabury, 2006). The DT_{50} for TYL and SMZ dissipation in the present study were within the lower values of published values, whereas CTC was removed much more rapidly (Table 1) than previously published values. The extraction method was that of Carlson and Mabury (2006), such that variance between the two studies was caused by a factor other than extraction method. Chlortetracycline is rapidly removed from manure during composting (DT_{50} of 1 d; Dolliver et al., 2008) and is readily removed during sewage treatment (Li and Zhang, 2011) indicating that it is readily amenable to biodegradation.

The accelerated degradation reported here was explored after annual exposure to 10 mg antibiotic kg^{-1} soil, although SMZ mineralization following exposure to 1 mg kg^{-1} also occurred (Fig. 2). Future studies will examine the effect of lower exposure concentrations on stimulation of degradation. To generalize the significance of these results to other antibiotics and other exposure scenarios (e.g., wastewater, agricultural effluents), dose–response relationships at lower environmentally relevant concentrations will have to be established.

Environmental pollution with organic chemicals is a potent selective pressure for the evolution of novel biodegradative pathways (Copley, 2009; van der Meer, 2006). Accelerated biodegradation of several herbicides and insecticides occurs in soils following repeated application in normal farming practice (Chapman and Harris, 1990; Houot et al., 2000; Walker and Suett, 1986). By analogy, we propose that in response to repeated or chronic exposure to environmental concentrations of some antibiotics, enrichment of bacteria able to derive nutritional benefit from these medicines takes place, resulting in accelerated biodegradation. The resulting decreased persistence of antibiotic residues, and consequently reduced exposure of bacteria to these residues, would attenuate the selection pressure for resistance development in the environment. Furthermore, decreased persistence will result in reduced transfer of residues to other compartments. Decreased exposure of aquatic or terrestrial micro-, meso-, and macrofauna to antibiotics will mitigate nontarget effects that are of regulatory concern (Brooks et al., 2009; Barrett et al., 2009).

The persistence of antibiotics following release into the environment from agriculture, municipal wastewater treatment, and pharmaceutical manufacturing is a critical factor affecting selection pressure for resistance development. Results from the present study do not imply that effluents, manures, and other materials that contain antibiotic residues or genetic determinants of antibiotic resistance pose no risk. Particularly in light of the fact that antibiotics are detected in water adjacent to land receiving manure (Aust et al., 2008), and that antibiotic-resistance determinants excreted by medicated swine are transferable to other bacteria in manured soils (Heuer et al., 2011a). Furthermore, municipal wastewater treatment effluents are rich in bacteria and plasmids carrying multiple resistance genes (Szczepanowski et al., 2009; Munir et al., 2011), and aquatic sediments chronically exposed to elevated fluoroquinolone residues in manufacturing effluents are enriched in resistance genes (Kristiansson et al., 2011). All environmental inputs containing antibiotic residues or resistance determinants that could potentially exacerbate the environmental reservoir of antibiotic resistance must be managed responsibly.

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