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Fate of Chlorate Present in Cattle Wastes and Its Impact on *Salmonella* Typhimurium and *Escherichia coli* O157:H7

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Chlorate salts are being developed as a feed additive to reduce the numbers of pathogens in feedlot cattle. A series of studies was conducted to determine whether chlorate, at concentrations expected to be excreted in urine of dosed cattle, would also reduce the populations of pathogens in cattle wastes (a mixture of urine and feces) and to determine the fate of chlorate in cattle wastes. Chlorate salts present in a urine−manure−soil mixture at 0, 17, 33, and 67 ppm had no significant effect on the rates of *Escherichia coli* O157:H7 or *Salmonella* Typhimurium inactivation from batch cultures. Chlorate was rapidly degraded when incubated at 20 and 30 °C with half-lives of 0.1 to 4 days. Chlorate degradation in batch cultures was slowest at 5 °C with half-lives of 2.9 to 30 days. The half-life of 100 ppm chlorate in an artificial lagoon system charged with slurry from a feedlot lagoon was 88 h. From an environmental standpoint, chlorate use in feedlot cattle would likely have minimal impacts because any chlorate that escaped degradation on the feedlot floor would be degraded in lagoon systems. Collectively, these results suggest that chlorate administered to cattle and excreted in wastes would have no significant secondary effects on pathogens present in mixed wastes on pen floors. Lack of chlorate efficacy was likely due to low chlorate concentrations in mixed wastes relative to chlorate levels shown to be active in live animals, and the rapid degradation of chlorate to chloride at temperatures of 20 °C and above.

KEYWORDS: Chlorate; cattle waste; *Salmonella*; *E. coli* 0157:H7; feed addtivite; pathogens

INTRODUCTION

Contamination of beef carcasses with human pathogens including *Escherichia coli* O157:H7 during slaughter and processing has been implicated in numerous incidents of human illness and has been a financial burden to the beef industry for years. Although beef producers, packers, and retailers are actively seeking pre- and postharvest solutions to eliminate pathogens, and though progress has been made toward producing pathogen-free meat products (*1*), continued problems with carcass contamination by *E. coli* O157:H7 are demonstrated by USDA mandated recalls of 13.8 million kg of beef during 2007 (*2*).

A preharvest technology has been developed that greatly reduces or eliminates the numbers of pathogens inhabiting the gastrointestinal tracts of ruminant animals (*3*–*8*). This technology is based on the feeding of an experimental chlorate-containing product (ECP) 24−72 h prior to an animal's slaughter. During the chlorate exposure period it is hypothesized that bacterial species containing intracellular respiratory nitrate reductase metabolize chlorate (ClO_3^-) to chlorite (ClO_2^-), which is toxic to bacteria (*3, 9*). Chlorate toxicity is specific to nitrate reductase-containing bacteria that have the ability to intracellularly convert chlorate to chlorite, but which lack chlorite dismutase, an enzyme capable of rapidly metabolizing chlorite to chloride (*10, 11*). An additional advantage of chlorate use in ruminant animals is that adverse affects on commensal microbiota of gastrointestinal tracts (*3, 5*) have not been documented

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Table 1. Outline of Sample Treatments

bacterial population	atmosphere	temp (°C)	chlorate level (mg/kg)				harvest time (days)	vials (*n*)	analyses
fecal	aerobic	5	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate
		20	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate
		30	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate
	anaerobic	5	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate
		20	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate
		30	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate
fecal + pathogens	aerobic	5	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		20	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		30	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
	anaerobic	5	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		20	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		30	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
pathogens only	aerobic	5	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		20	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		30	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
	anaerobic	5	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		20	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*
		30	0	17	33	67	0, 0.5, 1, 3, 7, 14, 21, 28	96	chlorate,*E. coli*, *Salmonella*

in experiments to date. As with many antibacterial agents, development of chlorate resistance occurs readily in pure bacterial culture but does not persist in mixed culture (*12*).

In order for a new feed additive to be approved by regulatory agencies, a number of important criteria must be met. First, carcass and meat quality should not be adversely affected by the proposed feed additive. To this end, King et al. (*13*) demonstrated that preslaughter chlorate administration to beef cattle had no adverse effects on carcass or meat quality. Still another critical variable for consideration is the magnitude of residue remaining in edible tissues at slaughter. Should chlorate residues exceed estimated safe tissue concentrations, its use as a preharvest food safety tool would be precluded. In a preliminary study, Smith et al. (*14*) demonstrated that chlorate residues in edible tissues of beef cattle were below provisional safe tissue concentrations estimated by the U.S. FDA Center for Veterinary Medicine. Under the conditions of the study, concentrations of chlorate residues in edible tissues were always less than 60% of the FDA-estimated safe tissue concentration, regardless of the dose of chlorate used. In addition, the sole metabolic product of chlorate identified in tissues or excreta of cattle, swine, poultry, and rats was chloride ion (*14–18*). From a food safety perspective, the presence of chloride in edible tissues is inconsequential. Collectively, these data suggest that chlorate residues should not be a barrier for the development of a viable chlorate product, with the caveat that the estimated Safe Tissue Concentrations of chlorate are fairly accurate. Thus, chlorate use in cattle appears to have numerous favorable characteristics from the standpoint of efficacy, product quality, and food safety.

Chlorate does not accumulate in beef tissues because it is rapidly transformed to chloride and(or) excreted in the urine (*15*). The cumulative urinary excretion of chlorate, when expressed as a percentage of the dose, was 7% to 28% representing urinary concentrations from 30 to 777 mg/kg. An average cumulative excretion of 1.3 g of chlorate was excreted in the urine of animals receiving a chlorate dose of 43 mg/kg of body weight (*14*). For feed yards marketing only a few animals, the excretion of 1.3 g of chlorate per animal would not likely represent a concern, but in feed yards marketing thousands of animals the cumulative burden of chlorate added to the soil surface could be significant.

The efficacy of chlorate present in mixed urine and feces against pathogens such as *E. coli* O157:H7 and *Salmonella* is unknown, however Tamási and Lantos (*19*), demonstrated that 10 mM (834 ppm) chlorate completely eliminated wild type *E. coli* from liquid cow manure. Gram-negative pathogens exist in both aerobic and anaerobic fecal environments (*20*) and can survive for variable lengths of time depending upon a number of environmental factors (*21, 22*). Pathogens shed from feedlot animals could be exposed to chlorate that was excreted in urine of chlorate treated animals. If so, treatment of cattle with chlorate could have a secondary benefit of decreasing pathogen burdens within feedlot pens.

Assuming that chlorate residues are active at killing pathogens in manure, numerous environmental questions surrounding chlorate use must be addressed. Specifically, chlorate is a strong oxidant that could represent a fire-hazard should it accumulate and dry onto organic matter (*23, 24*). Additionally, chlorate is an extremely water soluble anion (*25*) that could be transported from the feedlot to surface waters through runoff or into ground waters through leaching. The objectives of this study were 1) to determine the efficacy at which chlorate kills *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in mixed cattle urine-feces-soil and 2) to determine the stability of chlorate in a urine-feces-soil mixture and in a simulated beef cattle lagoon.

MATERIALS AND METHODS

Experiment 1. Fate of Chlorate, *E. coli* O157:H7, and *S. enterica* Serovar Typhimurium in a Urine−Feces−Soil Mixture. *General.* **Table 1** provides an overview of three sets of experiments used to assess the survival of pathogens and the stability of chlorate in the presence or absence of *E. coli* O157:H7 and *Salmonella* Typhimurium. Incubations were conducted with equal portions (1 g) of three main components: 1) a 3:1 mixture of air-dried cattle feces and soil; 2) cattle urine fortified with [^{36}Cl]chlorate; and 3) a freshly prepared extract of cattle feces containing: A) no *E. coli* 0157:H7 or *Salmonella* Typhimurium; B) both *E. coli* 0157:H7 and *Salmonella* Typhimurium; or C) autoclaved (killed) fecal bacterial extracts plus *E. coli* O157:H7 and *Salmonella* Typhimurium. Chlorate stability was measured under all incubation conditions, but the survival of *E. coli* O157:H7 and *Salmonella* were measured only in the incubation sets in which the pathogens had been fortified (i.e., with and without competition from fresh fecal bacteria). For each set of incubation conditions, triplicate vials were incubated with 0, 17, 33, and 66 ppm of [^{36}Cl]chlorate (final chlorate concentration) at 5, 20, and 30 °C; triplicate vials were incubated for time periods of 0, 0.5, 1, 3, 7, 14, 21, and 28 days each.

Radiolabeled Sodium Chlorate. Sodium [^{36}Cl]chlorate was purified as described by Smith et al. (*16*). The radioactive chlorate used for the

study had a specific activity of 401 dpm/μg and a radiochemical purity of greater than 99% as assessed by ion chromatography with radiochemical detection.

Preparation of Dried Feces−Soil Mixture. Milnor soil (Hecla-Hamar loamy fine sand; 67% sand; 1.54 g cm^{-3}; porosity 0.42; 2.23% organic matter; 14.0% clay; 19.0% silt) was collected and dried at 70 °C to a constant weight. Cattle feces were collected from the North Dakota State University dairy, mixed in a Hobart bowl mixer, and spread evenly in a series of weighed aluminum pans. Fecal material was dried to a constant weight within a forced-air oven at 70 to 80 °C and subsequently ground in a Wiley Mill using a 2-mm screen resulting in a preparation that was 97.3% dry matter, 12.0% ash, and 15.7% protein (Kjeldahl method). Soil and feces were combined on a dry weight basis to form a mixture containing 25% soil and 75% feces. Soil was incorporated at 25% of the dry matter to simulate a composition that might occur after repeated trampling of fecal matter into a feedlot "floor". The soil−feces combination was mixed overnight in a 2-L jar (Spiramix 10; Denley, UK).

Preparation of [^{36}Cl]Chlorate-Fortified Urine. Approximately 6 L of cattle urine were collected from the North Dakota State University dairy farm. Urine was mixed in a bucket, distributed to 1 L glass bottles and autoclaved. Sodium [^{36}Cl]chlorate stock solutions (0, 50, 100, and 200 mg/L) were prepared by adding 500 mL of autoclaved urine to 1-L volumetric flasks, adding the appropriate amounts of stock [^{36}Cl]chlorate solutions, and diluting to volume with autoclaved control urine. Contents of each 1-L volumetric flask were transferred to 1-L Wheaton bottles and reautoclaved. The radiochemical composition of each fortified urine solution was reassessed after autoclaving to ensure that chlorate was not degraded during the autoclaving process. Radiochemical purity was >99% after autoclaving the formulated urine.

Preparation of Incubation Vials. For each of the three experiments, 576 20-mL Wheaton serum vials were labeled, 1.0 ± 0.1 g of the soil-feces mixture was added, and the weights recorded. Vials were sorted by atmospheric condition (aerobic or anaerobic) and incubation temperature (5, 20, and 30 °C) and stored in cardboard flats. Each flat was covered with aluminum foil until addition of urine and the appropriate bacterial consortium.

Preparation of E. coli O157:H7 and Salmonella Stock Suspensions. Tryptic soy broth (40 mL) was added to each of five 50-mL serum vials and autoclaved; each was subsequently inoculated with 100 μL of a thawed *E. coli* O157:H7 stock solution. The *E. coli* O157:H7 strain used was a novobiocin and nadalixic acid resistant strain (*3*). After inoculation, each serum vial was incubated for 24 h at 37 °C for propagation of the pathogen. *E. coli* O157:H7 was subsequently quantified on MacConkey agar after serial dilution and plating. *Salmonella enterica* serovar Typhimurium resistant to novobiocin and chloramphenicol (*3*) was propagated as described for *E. coli* O157:H7. *Salmonella* was quantified on XLT-4 agar after dilution and plating.

Preparation of Fecal Supernatant and Inoculation with E. coli O157:H7 and Salmonella enterica. Duplicate 200 g aliquots of fresh cattle feces were placed into Nalgene centrifuge bottles and 200 mL of autoclaved phosphate buffered saline (PBS; 0.68 g KH_2PO_4, 2.42 g K_2HPO_4, 16 g NaCl per L), cooled to room temperature, were added. Fecal solids were suspended by mixing and were then pelleted by centrifugation (3750*g*). Supernatants were removed from each bottle and combined. A 10-mL aliquot of the combined supernatant was reserved for plating to determine "background" counts of *Salmonella* and *E. coli* O157:H7 (none were detected). Subsequently, 288 mL of the fecal supernatant was inoculated with 36 mL each of the *E. coli* O157:H7 and *Salmonella* tryptic broth suspensions resulting in innocula containing approximately 2- to 9 × 10^9 cells of each species per mL. For the experiment designed to measure the effect of fecal bacteria without additions of pathogens on chlorate stability, fecal supernatants were not inoculated with pathogens. For the experiment designed to measure the effect of only *E. coli* O157:H7 and *Salmonella* on chlorate stability, the pathogens were inoculated into autoclaved fecal suspensions.

Inoculation of Soil−Feces Mixture. At the initiation of each experiment, 1 mL of urine containing either 0, 50, 100, or 200 mg/L of sodium [^{36}Cl]chlorate was added to each incubation vial; immediately thereafter, 1 mL of the appropriate fecal inoculum was added to each tube. The total weight of each vial was measured, aerobic vials were covered with aluminum foil, and anaerobic tubes were sealed with butyl caps. Flats of vials were stored in the dark at the proper temperature. Final concentrations of chlorate in the completed incubations were 0, 17, 33, and 67 μg/g, representing approximately 0, 6800, 13200, and 29000 dpm of [^{36}Cl]chlorate, respectively. Aerobic samples were weighed daily and weight losses due to evaporation were replaced by adding an equivalent weight of water; pH was not measured in any of the batch samples.

Sample Collection. Sample tubes were harvested at preparation (day 0), and on day 0.5, 1, 3, 7, 14, 21, and 28. At sample collection, 9 mL of sterile phosphate buffered saline was added to each vial, vials were vortex mixed, and subsequently centrifuged at low speed to remove soil sediments for 5 min on a Savant centrifugal evaporating unit (~283*g*; Savant Instruments, Holbrook, NY) without vacuum. A 1-mL sample was removed for serial dilution in preparation of quantification of *E. coli* O157:H7 and *Salmonella*. The remainder of the sample was stored frozen until chlorate analysis could be conducted.

Quantification of E. coli O157:H7 and Salmonella. MacConkey agar was prepared according to the manufacturer's instructions with the following modification. After autoclaving, 1 mL of a 20 mg/L filter-sterilized solution each of novobiocin and naladixic acid was added per liter of MacConkey agar. Dilution tubes were prepared by adding 9.0 mL of PBS in test tubes and autoclaving. Aliquots (1 mL) of sample incubations were removed at sample harvest and were serially diluted through 10^{-7} dilution. Plates were incubated at 35 °C for 24 h and *E. coli* O157:H7 colonies were enumerated.

Salmonella were quantified on XLT-4 agar prepared according to manufacturer's instructions except that filter sterilized solutions of novobiocin and chloramphenicol (25 μg/mL each) were added after the addition of the agar to water. Plates were poured, plated with diluted sample, and quantified as described for *E. coli* O157:H7.

Quantification of Chlorate. [^{36}Cl]Chlorate was analyzed in sample supernatants after precipitation of [^{36}Cl]chloride with silver nitrate (*26*). Briefly, samples were thawed, sonicated (10 min), and duplicate 0.25 mL aliquots were added to 7-mL LSC vials containg 6 mL of LSC fluid (Ultima Gold; Perkin-Elmer Life Sciences, Waltham, MA) for the determination of total radioactive residues. Additional 0.5 mL duplicate aliquots were added to microcentrifuge tubes, 0.5 mL aliquots of water were added, and chloride ions were precipitated with the addition of 0.25 M silver nitrate (0.25 mL), prepared in nanopure water. Tubes were vortexed and centrifuged and supernatants were filtered through 17 mm, 0.45 μm PTFE syringe filters (Alltech, Deerfield, IL). From each filtrate, a 0.75-mL aliquot was removed for determination of soluble radioactivity (i.e., [^{36}Cl]ClO_3^-). Background radioactivity was determined by counting aliquots removed from control samples. For each sample set, a set of duplicate tubes containing 0.5 mL of phosphate buffer was fortified with a solution containing 52.8% of [^{36}Cl]chloride and 47.2% [^{36}Cl]chlorate. Radioactivity in sample aliquots was determined by LSC for 10 min each. Analyzed chlorate composition of the fortified samples was 51.7 ± 1.3% (n = 127).

Statistical Analysis. Data were pooled so that the main effect of chlorate on *E. coli* O157:H7 and *Salmonella enterica* could be determined under aerobic or anaerobic conditions, with or without competition (live fecal bacteria vs pathogens only; see table **1**). To this end, rates of *E. coli* and *Salmonella* depletion were typically calculated using nonlinear regression analysis (single phase exponential decay; $Y = (Y_0 - Y_{28}) \times e^{-kx} - Y_{28}$, where Y is the log of the pathogen concentration, k is the decay rate constant, and x is the study day; GraphPad Prism 5.0, San Diego, CA). In instances in which populations of bacteria increased during the initial time points (typically when competition from fecal bacterial was absent), rate constants of depletion were calculated using single phase exponential decay from the time of peak bacterial numbers. In two instances, the inactivation of bacterial populations were poorly fit by single phase exponential decay; in these instances rates of bacterial populations were modeled using least-squares linear regression from the time of peak bacterial populations through day 28 of the study. Rate constants and slopes were compared using one-way analysis of variance (F test).

Chlorate degradation data were modeled using a single phase exponential decay curve and the resulting rate constants were used to calculate chlorate half-lives (half-life = ln(2)/K). Fitting chlorate

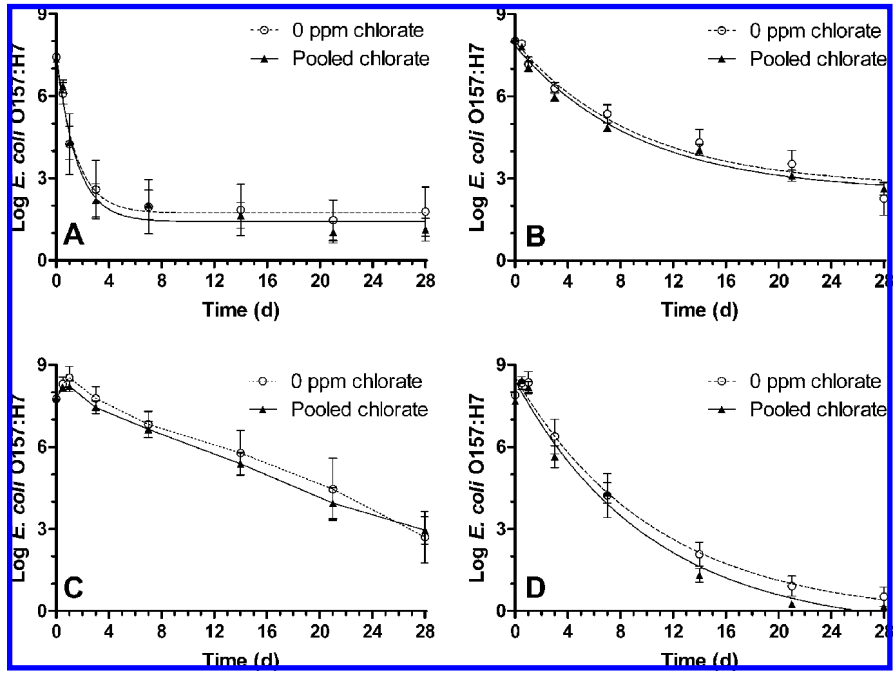

Figure 1. Effect of chlorate (solid lines) on the survival of *E. coli* O157:H7 relative to controls (hatched lines) in batch cultures containing live (**A**, **B**) or killed (**C**, **D**) fecal bacteria under aerobic (**A**, **C**) or anaerobic (**B**, **D**) conditions.

degradation data to first order exponential decay curves was inappropriate for the 5 °C anaerobic incubations of fecal bacteria, and the 5 °C incubations of aerobic and anaerobic pathogens with killed fecal bacteria. These data were fit by linear regression and half-life was calculated using the resulting slope (half-life = [0.5(starting chlorate percentage) ÷ slope]).

Experiment 2. Fate of [^{36}Cl]Chlorate in Simulated Beef Cattle Lagoons. Lagoon waste (96.6% water, 3.4% dry matter, 3.6 μS cm^{-1}, 0.08% N, 0.04% P_2O_5, 0.08% KO_2, 133.4 μg/mL NH_4^+, 8.7 μg/mL NO_3^-, 0.246% Ca, 33.0 μg/mL Mg, 28.3 μg/mL Zn, 3.2 μg/mL Cu, 519 μg/mL Fe, 14.9 μg/mL Mn) was collected from a feedlot lagoon located at the North Dakota State University Carrington Research Center, Carrington, ND. Liquid and sediment were collected into 5 gallon buckets using a polyvinyl chloride (PVC) sampling device and transported to the laboratory where they were stored at 5 °C until use (within 2 weeks of collection).

Four laboratory-scale lagoon simulators were constructed of 10-L Nalgene carboys modified with three polyethylene stopcocks (6.35 × 6.35 mm) placed 8 (stopcock A), 18 (stopcock B), and 25 (stopcock C) cm from the bottom of the carboy. An additional port (8 cm from bottom) was added to each carboy to accommodate a redox electrode (Phoenix Electrodes, Houston, TX). Holes were drilled into each carboy cap to accommodate stems through which headspace gases were diverted. Stems were connected to Tygon tubing tipped with Pasteur pipettes. Pasteur pipettes were submerged in 50 mL of 3 N NaOH housed within 250 mL Erlenmeyer flasks in order to trap carbon dioxide. After bubbling through NaOH traps, gases evolved from each lagoon were diverted into a water-filled 2-L polyethylene graduated cylinder inverted into a large pail of water. From these cylinders gas volumes were measured.

Each lagoon simulator was charged with 10 L of fresh lagoon slurry in which sediment material was suspended by stirring. Simulators were leak tested and equilibrated at 22 °C for 6 days to allow resedimentation of suspended particles. Redox potentials were measured daily throughout the equilibration period. Each day 20 mL of liquid slurry was removed through stopcock B and assayed for endogenous chloride content with a hand-held Oakton Ion 5 chloride ion meter (Cole-Parmer). Minutes prior to dosing, approximately 400 mL of liquid layer waste was removed from each lagoon through stopcock B to accommodate the volume of the subsequent dosing solution. Three simulators were dosed through stopcock C with ~400 mL of [^{36}Cl]sodium chlorate (41 dpm/μg; 18.4 μCi total) dissolved in cattle urine. The fourth simulator received the same volume of control cattle urine. The total mass of chlorate added to each lagoon (except the control) was 1 g so that the final concentration of chlorate in each lagoon was 100 mg/L.

Immediately after charging each lagoon with [^{36}Cl]chlorate, duplicate 10 mL samples (T0) were collected into 15-mL polypropylene tubes (Sarstedt, Newton, NC); samples were flash frozen in a dry ice-acetone bath. Thereafter duplicate aliquots were removed from each lagoon and flash frozen at 1, 2, 4, 8, 12, 24, 36, 48, 72, and 168 h. Chlorate was analyzed after precipitation of Cl^- using silver nitrate as described above for the bacterial incubations. With each sample set run, duplicate control lagoon aliquots were fortified with a standard containing a known composition of [^{36}Cl]Cl^- (52.8%) and [^{36}Cl]ClO_3^- (47.2%) for use as assay standards. Chlorate content of the analyzed fortified samples was 47.0 ± 0.8% (average ± standard deviation of 11 analytical sets).

RESULTS

Survival of *E. coli* O157:H7 in Mixed Urine−Feces−Soil. Effects of chlorate on survival of *E. coli* O157:H7 in batch incubations containing a mixture of soil, feces, and urine is shown in **Figure 1**. For clarity, only the main effects of chlorate are shown (dose and temperature data are pooled). There was no overall effect of chlorate on the rates of *E. coli* survival ($P \geq 0.15$; **Table 2**). *E. coli* O157:H7 numbers decreased rapidly as incubation length progressed regardless of whether conditions were aerobic or anaerobic or whether chlorate was present or not. In incubations containing pathogens only (graphs C and D of **Figure 1**), *E. coli* O157:H7 numbers increased during the first 24 h and gradually declined thereafter. In contrast, when competitive fecal bacteria were present in incubations, *E. coli* numbers never increased (graphs A and B of **Figure 1**). When competitive bacteria were present, *E. coli* O157:H7 decreased to 10^2 to 10^3 cells per mL within 3 days under aerobic conditions (graph A). Under anaerobic conditions, *E. coli* O157:H7 only dropped to 10^3 cells/mL at 20 to 28 days. The most rapid depletion of *E. coli* occurred under aerobic conditions in the presence of competitive fecal bacteria (**Figure 1A**). Under

Table 2. Effects of Chlorate on Rates Constants (k) or Slopes of *E. coli* O157:H7 and *Salmonella enterica* Elimination from Batch Cultures

pathogen	conditions	competitive bacteria[a]	rate constant control	rate constant chlorate	P
E. coli O157:H7	aerobic	yes	0.71 ± 0.27^b	0.64 ± 0.13^b	0.81
	anaerobic	yes	0.07 ± 0.02^b	0.12 ± 0.01^b	0.15
	aerobic	no	-0.19 ± 0.02^c	-0.19 ± 0.01^c	0.89
	anaerobic	no	0.11 ± 0.02^d	0.11 ± 0.01^d	0.85
Salmonella	aerobic	yes	0.06 ± 0.02^b	0.06 ± 0.01^b	0.71
	anaerobic	yes	0.07 ± 0.03^b	0.07 ± 0.02^b	0.86
	aerobic	no	-0.09 ± 0.01^c	-0.09 ± 0.004^c	0.99
	anaerobic	no	0.03 ± 0.02^e	0.04 ± 0.02^e	0.84

[a] Incubations with competitive bacteria were inoculated with 1 mL of a phosphate buffered saline extract of fresh feces; incubations without competitive bacteria were inoculated with an autoclaved phosphate buffered saline extract of fresh feces. All incubations were inoculated with approximately 1×10^8 of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Rates of inactivation were calculated using nonlinear regression analysis, and comparisons of rate constants were made to determine statistical differences. [b] Rate constants were calculated using $Y = (Y_0 - Y_{28}) \times e^{-kx} + Y_{28}$. [c] Slope calculated by least-squares linear regression of data from T1 to T28. [d] Rate constants were calculated using $Y = (Y_{0.5} - Y_{28}) \times e^{-kx} + Y_{28}$. [e] Rate constants were calculated using $Y = (Y_1 - Y_{28}) \times e^{-kx} + Y_{28}$.

anaerobic conditions with no competitive fecal bacteria present (**Figure 1D**), *E. coli* was nearly eliminated from the cultures, but only at 20 to 28 days.

Survival of *Salmonella* in Mixed Urine−Feces−Soil. Figure 2 shows the survival of *Salmonella enterica* serotype Typhimurium in mixed urine-feces-soil incubated under aerobic or anaerobic conditions in the presence or absence of live fecal bacteria. Briefly, there were no overall effects ($P < 0.65$) of chlorate on the rate of *Salmonella* survival (**Table 2**). In the absence of competitive fecal bacteria, *Salmonella* numbers increased during the first 24 h of the study (**Figure 2C**,**D**). *Salmonella* appeared to survive throughout the experiment under anaerobic conditions with competitive bacteria (**Figure 2B**) and in aerobic incubations without competitive fecal bacteria (**Figure 2C**).

Table 2 shows the effects of chlorate on the calculated rates of *E. coli* elimination in the urine−feces−soil mixtures. Although the levels of *E. coli* O157:H7 bacteria were almost always numerically lower in chlorate-treated incubations (**Figure 2**), the rates of pathogen elimination in chlorate containing mixtures were not different ($P > 0.15$), indicating that chlorate did not influence *E. coli* levels in this model system.

Chlorate Stability in Urine−Feces−Soil. Figure 3 shows the degradation of chlorate in incubations containing fecal bacteria, fecal bacteria fortified with *E. coli* O157:H7 and *Salmonella*, or killed fecal bacteria extracts and *E. coli* O157:H7 and *Salmonella*. For the clarity of presentation, chlorate doses (17, 33, and 67 ppm) were pooled within temperature so that the main effects of chlorate and temperature are easily observed. A quick review of **Figure 3** clearly reveals that chlorate degradation occurred at 5, 20, and 30 °C, regardless of whether anaerobic or aerobic conditions existed. Further scrutiny of **Figure 3** clearly shows that regardless of incubation condition, at 20 and 30 °C chlorate was rapidly degraded to nadir levels within 7 days. Chlorate degradation at 5 °C appeared to be slower than at 20−30 °C but generally continued throughout the 28-day experimental period. Chlorate degradation generally plateaued at about 20 to 30% of the starting chlorate level, likely reflecting the depletion of nutrients (aerobic conditions) or electron donor (anaerobic conditions) within incubations.

Table 3 shows the estimated half-lives of chlorate present in soil-manure-urine mixtures. Chlorate half-lives ranged from 2.9 to 30 days at 5 °C and from 0.1 to 1.3 days at 30 °C. Incubations inoculated only with fecal extracts, without supplemental pathogens, metabolized chlorate the slowest. In these incubations, chlorate half-lives ranged from 1 to 30 days, depending upon the temperature and atmosphere. Incubations with a live consortium of fecal bacteria and pathogens reduced chlorate the quickest, with chlorate half-lives ranging from 0.1 to 5.9 days. It should be noted that incubations containing only "native" fecal bacterial probably underestimated the actual rate of chlorate degradation in feces, due to the dilution of bacteria that occurred by virtue of extraction and centrifugation of fecal solids. Chlorate degradation was rapid in both anaerobic and aerobic incubations. The two greatest influences on chlorate degradation were temperature and the consortium of bacteria present. That is, incubations containing only fecal bacteria extracts or only *Salmonella* and *E. coli* O157:H7 degraded chlorate more slowly than incubations containing a mixture of both. These data demonstrated clearly that chlorate degradation was not dependent on pathogens, nor was chlorate degradation dependent upon the depletion of oxygen within the environment because chlorate was reduced to chloride ion in both aerobic and anaerobic environments.

Chlorate Stability in Simulated Lagoons. In the lagoon system chlorate was degraded to chloride at an essentially constant rate over the 168 h study period (**Figure 4**). At 168 h, chlorate represented $3.4 \pm 2.1\%$ of the total radioactivity in each sample. Using linear regression (y-intercept, 97.9 ± 1.0; slope -0.553 ± 0.016; r^2 0.978), it was estimated that the half-life of chlorate was 88 h and that complete reduction of chlorate would have occurred at 177 h with a 95% confidence interval of 168 to 187 h.

DISCUSSION

An obvious result of this study is that in contrast to numerous studies showing the efficacy of chlorate against *E. coli* O157:H7 and *Salmonella*, chlorate had no effects ($P > 0.05$) on the rates that these pathogens declined in the soil-urine-feces matrix relative to controls. A likely explanation is that previous studies that have established the efficacy of chlorate salts in live animals or in vitro models have typically used concentrations of sodium chlorate much greater than those used in the present set of experiments. For example, experimental chlorate has been applied to animals or in vitro models as aqueous solutions ranging from 1.25 to 200 mM (**Table 4**; 133−21280 ppm). When delivered as a feed supplement, chlorate levels have ranged from 0.01 to 2.4% of body weight (equivalent to 4800−120000 mg/kg dietary chlorate content). Although dilution in the test animal or system must be taken into account, chlorate levels typically used have been substantially greater than the levels investigated in this study. Chlorate levels selected for this study were based on average chlorate concentrations of 168 mg/L excreted in urine of cattle administered 43 mg/kg BW of chlorate (average over a 48-h study period; calculated from 15). The maximum concentration of urinary chlorate applied to the urine-fecal-soil matrix was 200 mg/L, resulting in a final chlorate concentration of 67 mg/L, a level substantially lower than chlorate level proven to have efficacy in either animal or in vitro studies. Thus it could be argued that concentrations of chlorate were not sufficient to kill pathogens tested in this study, and that had chlorate been provided at higher concentrations, it would possibly have had an inhibitory or killing effect. Such is suggested by results of Tamási and Lantos (*19*) and

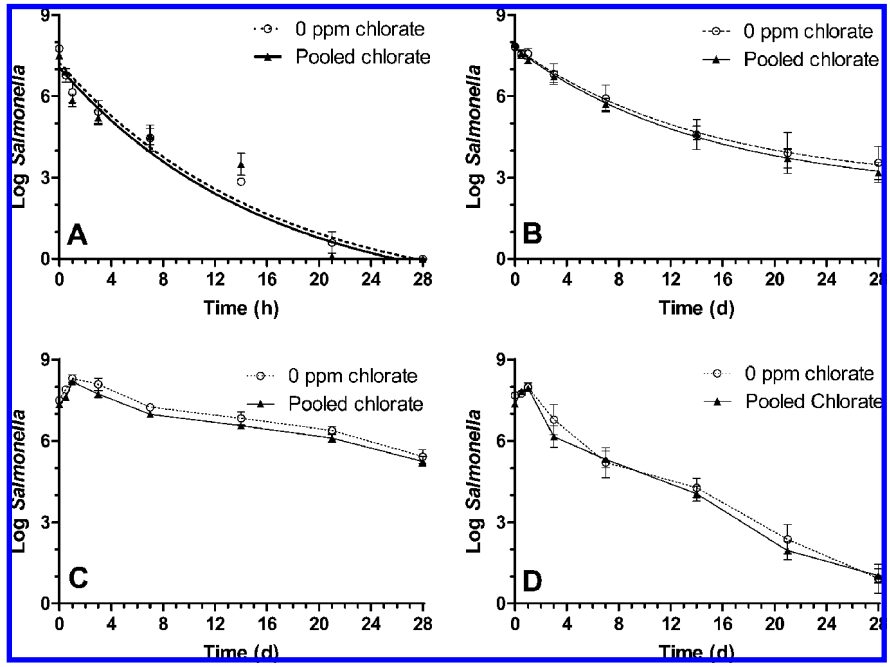

Figure 2. Effect of chlorate (solid lines) on the survival of *Salmonella enterica* relative to controls (hatched lines) in batch cultures containing live (**A**, **B**) or killed (**C**, **D**) fecal bacteria under aerobic (**A**, **C**) or anaerobic (**B**, **D**) conditions.

Table 3. Chlorate Half-Lives (Days; Estimated Using either Linear or First-Order Nonlinear Least Square Regression) in Batch Incubations Containing Live Fecal Bacteria, Live Fecal Bacteria Fortified with *E. coli* O157:H7 and *Salmonella* Typhimurium, or Autoclaved Fecal Bacteria Fortified with *E. coli* O157:H7 and *Salmonella* Typhimurium

			temperature		
atmosphere	fecal bacteria	pathogens present[c]	5 °C	20 °C	30 °C
aerobic	live[a]	no	28.0	1.4	1.0
anaerobic	live[a]	no	28.7[d]	4.3	1.3
aerobic	live[a]	yes	2.9	0.3	0.2
anaerobic	live[a]	yes	5.9	0.4	0.1
aerobic	killed[b]	yes	17.6[d]	1.0	0.2
anaerobic	killed[b]	yes	19.4[d]	1.1	0.4

[a] Fresh cattle feces were extracted 1:1 with phosphate buffered saline, and the extract was used to inoculate batch incubations [b] Fresh cattle feces were extracted 1:1 with phosphate buffered saline, and the extract was autoclaved and used to inoculate batch incubations [c] *E. coli* O157:H7 and *Salmonella* Typhimurium were fortified into either fresh or autoclaved fecal extracts and were used to inoculate batch incubations. [d] Estimated using linear regression, where half-life = 0.5(100%)/slope; data could not be fit to first order.

Anderson et al. (*27*), who found that *E. coli* present in liquid cattle manure and swine wastes, respectively, were susceptible to 5 to 10 mM chlorate (532 to 1064 mg/L).

Half-lives of chlorate at 20−30 °C were less than 5 days regardless of whether bacteria were incubated under aerobic or anaerobic conditions. Thus, chlorate was rapidly degraded to chloride by 1) pathogens only, 2) native fecal bacteria only, and 3) the mixture of pathogens and native fecal bacteria. Half-lives of chlorate were considerably longer at 5 °C, ranging from 3 to 30 days. Although chlorate has been used as a herbicide for over a 100 years (*28*), as a defoliant (*29*) and as a tool to induce flowering of tropical fruit trees (*30*), few complete studies have been published on its environmental fate and stability. Stability of chlorate in soils after herbicidal application has been reported to be highly variable, with chlorate disappearing from soil in 6 months to 5 years, depending upon soil moisture and temperature (*31*). The disparate longevity of chlorate may have much to do with the variance in chlorate application rate that occurs with agricultural and non agricultural applications. For example, the application rate of chlorate for industrial (noncrop) herbicidal use is up to 120 g/m^2, whereas herbicidal applications relating to food crops are limited to a maximum of 1.2 g/m^2 (*32*). The fact that chlorate is transformed to chloride by soil bacteria was recognized as early as 1928 (*33*) and it was subsequently realized that chlorate degradation was more rapid in manure amended soils than in soils amended with nitrogen fertilizers (*34*). Studies published in more recent years have shown that soil type, application rate, and soil moisture are major determinants in the degradation of chlorate after application to induce flowering of longan plants. Typically, chlorate applied to soils at 34 mg/kg was totally degraded by about 2 weeks in flooded soils and by 8 weeks in soils at 100% of their maximum water holding capacity (MWHC; 35); soils amended with 330 mg/kg chlorate did not degrade appreciable quantities of chlorate over 8 weeks when MWHCs were 100% or less. Addition of reducing equivalents in the form of glucose, sucrose, fructose, or succinate, greatly accelerated the degradation of chlorate, regardless of soil moisture (*35, 36*). Collectively, our results are consistent with previous research showing that chlorate degradation is temperature dependent, is limited by available reducing equivalents, and is rapid when moisture is not limiting.

When incubations contained only *E. coli* O157:H7 and *Salmonella* (at 20 and 30 °C) the periods associated with the most rapid rates of chlorate depletion (0−24 h) corresponded to periods of *E. coli* O157:H7 and *Salmonella* growth (∼1 log unit for each). Such growth in the presence of an antimicrobial agent suggests that chlorate reduction by *E. coli* O157:H7 or *Salmonella* may occur through multiple routes. Current theory states that bacterial susceptibility to chlorate arises in those organisms that express respiratory nitrate reductase under anaerobic conditions (*27, 37*). According to this hypothesis, a membrane-bound but cytoplasmically oriented nitrate reductase (Nar) cometabolizes chlorate to chlorite, which is highly toxic and causes bacterial cell death. The expression of Nar is induced by nitrate and it represents the major respiratory nitrate reductase present in anaerobically grown coliforms (*38*). Native chlorite is a highly effective disinfectant when added externally to a broad spectrum of bacterial cells (*39–41*); thus the specificity

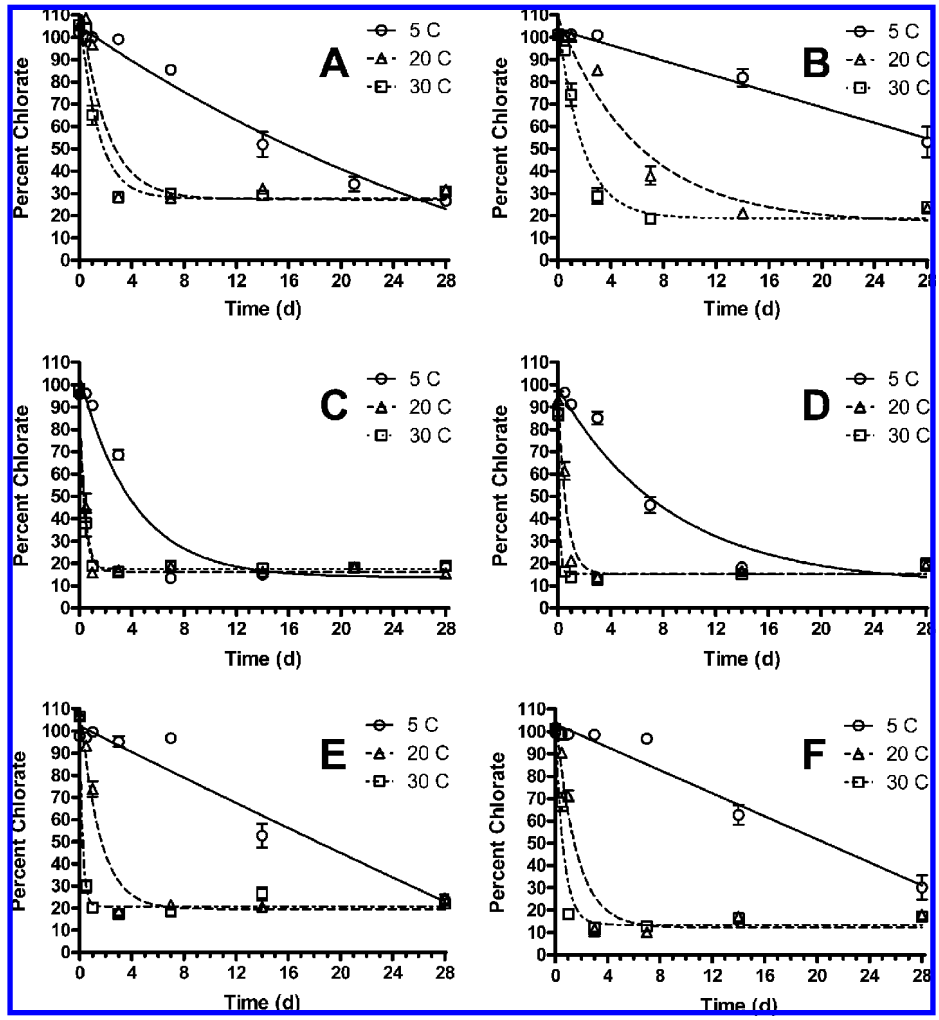

Figure 3. Chlorate stability in incubations containing fecal bacteria extracts (**A**, **B**), fecal bacterial extracts plus *E. coli* 0157:H7 and *Salmonella enterica* (**C**, **D**), and autoclaved (killed) fecal bacteria plus *E. coli* O157:H7 and *Salmonella enterica* (**E**, **F**). For each set of incubations, [^{36}Cl]chlorate, dissolved in bovine urine, was added to a mixture of soil, dried manure, and a fresh manure extract to achieve final chlorate concentrations of 0, 17, 33, and 67 ppm. In addition, incubations were conducted at three temperatures (5, 20, 30 °C) under both aerobic (**A**, **C**, **E**) and anaerobic (**B**, **D**, **F**) conditions.

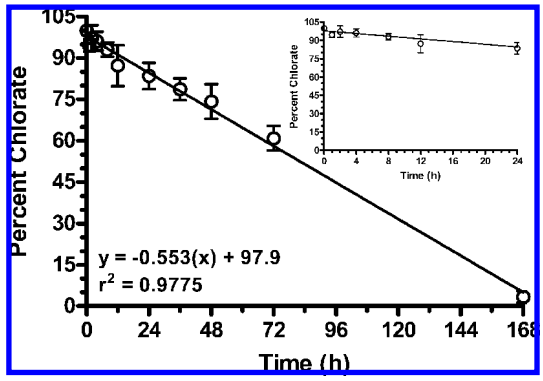

Figure 4. Decomposition of chlorate over a 168-h period (100 ppm starting concentration) in 10-L lagoons ($n = 3$) fortified with sediment and slurry collected from a beef cattle feedlot. Soluble [^{36}Cl]chlorate was measured by liquid scintillation counting of lagoon liquids after precipitation of [^{36}Cl]chloride with silver nitrate. (Inset) Chlorate degradation during the initial 24 h of the incubation period.

of chlorate is presumably due to the intracellular production of chlorite by only those species expressing Nar.

Although the hypothesis that chlorate is activated to chlorite by nitrate reductase has recently garnered attention because of the potential food safety applications of chlorate salts, bacterial susceptibility to chlorate via the enzymatic conversion of chlorate to chlorite was first proposed by Quastel et al. in 1925 (*42*) and this proposal was again supported in 1952 by Gosksøyr (*43*). The hypothesis was based, and sustained, on the putative measurement of chlorite in bacterial incubation media.

Quastel et al. (*42*) indirectly measured chlorite production from resting "*Bacillus coli*" after incubation of the bacteria in media containing a final chlorate content of 1% (i.e., 10000 ppm). Chlorite was measured by observing the liberation of iodine after the addition of starch and acetic acid to the bacterial cells. Because chlorine dioxide could be produced after the acidification of chlorate (*44*), the measurements of Quastel et al. (*42*) cannot be considered definitive because chlorine dioxide could also be titrated in such a manner. Goksøyr (*43*) measured chlorite produced in bacterial incubations using both paper chromatography and titration. However, the detection limit of chlorite with paper chromatography analysis was "abnormally high" (Goksøyr's words; the stated detection limit was 5 mM or 250 ppm (*43*)) so that "the method could not be used generally in this work". As an alternative to the insensitive paper chromatography method, Goksøyr determined chlorite by titration with the undefined reagent "KJ" and potassium sulfate under acidic conditions. Similar to Quastel's (*42*) analysis, the use of acid in an analysis with large amounts of chlorate and available reductant could lead to artifacts. In addition, Goksøyr (*43*)

Table 4. Summary of Studies Investigating the Efficacy of Chlorate Salts in Livestock-Based Test Systems

test system	target bacteria	host species	matrix, route	chlorate level[a] mM or %	chlorate level[a] ppm	dose administration	duration	ref
in vitro	*Salmonella* Typhimurium	bovine	ruminal fluid	1.25; 5 mM	133; 532	solution	24 h	*3*
	E. coli O157:H7	bovine	ruminal fluid	1.25; 5 mM	133; 532	solution	24 h	*3*
in vivo	*E. coli* O157:H7	bovine	oral	100 mM	10640	drinking water		*5*
in vivo	*E. coli* O157:H7	bovine	oral	0.01; 0.05%	4800; 20000	diet	1 day, 5 days	*4*
in vivo	*E. coli* O157:H7	bovine	oral	35.7 mM	3800	drinking water	1 day, 5 days	*4*
in vivo	*E. coli* O157:H7	ovine[b]	oral	0.6; 1.2; 2.4%	30000; 60000; 120000	diet	point	*7*
in vivo	*E. coli* O157:H7	ovine	oral	100 mM	10640	drinking water	24 h	*6*
in vivo	generic *E. coli*	bovine	oral	0.12; 0.23%	1200; 2300	feed	24 h	*8*
in vivo	*Salmonella*	meleagris	oral	7.5; 15, 30; 60 mM	798; 1596; 3192; 6384	water	14, 26, 38 h	*61*
in vivo	*Salmonella*	gallus	oral	15 mM	1596	water	48 h	*62*
in vivo	*Salmonella*	gallus	oral	7.5; 15; 30 mM	798; 1596; 3192	water	24 h	*63*
		gallus	oral	15 mM	1596	water	24, 48 h	*63*
in vivo	*Salmonella enteritidis*	gallus	oral	15 mM	1596	water	unknown	*64*
in vitro	*Salmonella*	gallus	CE broth	15 mM	1596	solution	48 h	*65*
in vitro	*Campylobacter jejuni*	gallus	chicken wings	50; 100 mM	5320; 10640	solution	1, 3, 5, 10, 20 min	*66*
in vivo	*E. coli* O157:H7	porcine	oral	100; 200 mM	10640; 21280	water (bolus)	24 h (3 doses)	*67*
in vivo	*Salmonella*	porcine	oral	100 mM	10640	water (bolus)	16 h	*68*
in vivo	*Salmonella*	porcine	oral	100; 200 mM	10640; 21280	water	24, 36 h	*69*
in vivo	*Salmonella*	porcine	oral		800	feed	14 days	*70*
in vivo	*Salmonella*	porcine	oral		~400	water	5 days	*71*
in vitro	*Salmonella*	porcine	feces	5; 10 mM	532; 1064	water	24 h	*27*

[a] Chlorate concentrations (ppm) are reported as sodium chlorate equivalents [b] 55 kg sheep consuming 1.1, 2.2, or 4.4 g sodium chlorate per kg body weight; assume feed consumption is 3.5% of body wt or ~ 2 kg/day

questioned the specificity of the titration used in the study described by their manuscript. Perhaps the most convincing evidence that chlorite is produced as an intermediate during chlorate metabolism was presented by Sanchés-Crispín et al. (*45*) who published paper chromatography autoradiograms of [^{36}Cl]chlorate metabolites produced by *E. coli* K12. In this study, chlorite was apparently produced in membrane vesicles of wild type *E. coli* K12 and *E. coli* K12 strain 356 (PA601), a mutant resistant to chlorate during aerobic growth (*46*). In live cells, chlorite was detected in anaerobic cultures of only *E. coli* K12 strain 356 (PA601) incubated with 1.3 mM (108 ppm) of chlorate. Replication of the paper chromatographic techniques used by Sanchés-Crispín et al. (*46*) in our laboratory afforded good resolution between chlorate and chlorite, and chlorate and chloride, but very poor resolution between chloride and chlorite (data not shown).

Thus, the hypothesis that chlorite produced by nitrate-respiring bacteria is responsible for the effects of chlorate on pathogens such as *E. coli* O157:H7 and *Salmonella* Typhimurium has not been verified using modern analytical techniques. Nevertheless, this hypothesis—which is reasonable (*47*) considering that chlorite is the two electron reduction product of chlorate— is widely held to be fact (*9, 19, 27, 48*).

Regardless of the veracity of the chlorate to chlorite hypothesis as a lethal mechanism for pathogens, an additional pathway, or pathways, for chlorate reduction is necessary to explain the extensive chlorate reduction observed in the present study. One known pathway is through (per)chlorate reductase which catalyzes the reduction of perchlorate and(or) chlorate in bacteria isolated from a variety of diverse aerobic and anaerobic environments (*11, 49–53*). Although these microorganisms typically respire perchlorate or chlorate under anaerobic conditions, mixed cultures have been demonstrated to be capable of reducing perchlorate at redox potentials as high as +180 mV (*54*). (Per)chlorate reductases, in concert with chlorite dismutases (*11, 55, 56*) rapidly and efficiently catalyze the reduction of perchlorate and chlorate to chloride ion. Although chlorate reductase converts chlorate to chlorite, chlorite has not been measured as a metabolic intermediate in (per)chlorate-reducing bacteria, presumably because of chlorite dismutase's low k_m, high V_{max}, and high chlorite turnover rate (*10, 55*). Any chlorite produced within bacteria expressing chlorite dismutase is rapidly and completely reduced to chloride ion. Chlorate-reductase expressing bacteria have been isolated from human and animal waste lagoon slurries and sludges, but attempts to isolate such bacteria from ruminal sources have not been successful (*26*).

Although enzyme-based chlorate reduction mechanisms likely exist and are responsible for a good portion the chlorate reduction in our study, the nonenzymatic chemical reduction of chlorate cannot be discounted. For example, Oliver et al. (*26*) showed that 6 and 17% of the 300 and 100 ppm [^{36}Cl]chlorate, respectively, present in incubations of autoclaved ruminal fluid was converted to chloride ion during 24-h anaerobic incubations (37 °C). In addition, chlorate present in beef cattle tissues stored at 3 °C was converted to chloride ion during a 2-wk storage period (*14*), presumably through nonenzymatic means. The linear relationship between chlorate reduction and time in the artificial lagoon (**Figure 4**) also suggests that chlorate was being reduced as reductant became available, presumably through bacterial action. The chemical reductants have not yet been identified, but in ruminal-based systems hydrogen sulfide would be a good candidate.

Regardless of the mechanism of chlorate conversion to chloride, the initial rates of its reduction were high in our urine-feces-soil incubation systems, especially as temperature increased. In almost all incubations at 20 and 30 °C, chlorate was not completely reduced to chloride, with typically 20 to 30% of the chlorate remaining. The slowing of chlorate reduction after its initial rapid metabolism is likely a result of the depletion of available reducing agents or cosubstrates. Microbial chlorate reduction is dependent upon the availability of usable reducing equivalents (*57, 58*).

Chlorate degradation in an artificial beef cattle lagoon was slower than in the soil-manure-urine mixtures, but was nevertheless complete within approximately 7 days. The near linear rate of chlorate degradation suggests that the lagoon microcosm differed greatly from the microcosm present in the fecal-urine extracts. The lagoon systems remained extremely anaerobic (i.e., reduction potential of <-400 mV) throughout the 7-day test period. Whether chemical or bacterial reduction of chlorate was

occurring was not determined, but the linear rate of reduction suggests that chemical reduction was occurring as reductants were being generated.

Collectively, results from our laboratory suggest that chlorate is subject to reduction to chloride at multiple steps within a ruminant production system. For example, dietary chlorate is converted to chloride in the rumen (*26*), and absorbed chlorate is likely converted to chloride after its absorption into the blood stream, with the concomitant oxidation of hemoglobin and other substrates (*59, 60*). Low concentrations of chlorate were also converted to chloride in beef tissues stored at 3 °C (*14*). When chlorate enters the waste stream, either through urination or defecation, it is subject to rapid destruction by fecal bacteria or lagoon processes.

Chlorate had no measurable effects on the loads of pathogens tested in the present study; as such, there is no evidence that excreted chlorate residues would have a secondary disinfection effect on feedlot floors. From an environmental perspective, however, this study demonstrates that chlorate was rapidly degraded to chloride ion under both aerobic and anaerobic conditions in a moist fecal mixture. Rapid chlorate reduction to a natural product in manure, and its complete reduction to chloride after 7 days in an artificial lagoon suggests that commercial uses of chlorate in feedlots with properly maintained waste handling facilities would represent minimal risk to the environment.

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