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Multiplex Fluorogenic Real-Time PCR for Detection and Quantification of *Escherichia coli* O157:H7 in Dairy Wastewater Wetlands

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Surface water and groundwater are continuously used as sources of drinking water in many metropolitan areas of the United States. The quality of water from these sources may be reduced due to increases in contaminants such as *Escherichia coli* from urban and agricultural runoffs. In this study, a multiplex fluorogenic PCR assay was used to quantify *E. coli* O157:H7 in soil, manure, cow and calf feces, and dairy wastewater in an artificial wetland. Primers and probes were designed to amplify and quantify the Shiga-like toxin 1 (*stx1*) and 2 (*stx2*) genes and the intimin (*eae*) gene of *E. coli* O157:H7 in a single reaction. Primer specificity was confirmed with DNA from 33 *E. coli* O157:H7 and related strains with and without the three genes. A direct correlation was determined between the fluorescence threshold cycle (C_T) and the starting quantity of *E. coli* O157:H7 DNA. A similar correlation was observed between the C_T and number of CFU per milliliter used in the PCR assay. A detection limit of 7.9×10^{-5} pg of *E. coli* O157:H7 DNA ml^{-1} equivalent to approximately 6.4×10^3 CFU of *E. coli* O157:H7 ml^{-1} based on plate counts was determined. Quantification of *E. coli* O157:H7 in soil, manure, feces, and wastewater was possible when cell numbers were $\geq 3.5 \times 10^4$ CFU g^{-1}. *E. coli* O157:H7 levels detected in wetland samples decreased by about 2 logs between wetland influents and effluents. The detection limit of the assay in soil was improved to less than 10 CFU g^{-1} with a 16-h enrichment. These results indicate that the developed PCR assay is suitable for quantitative determination of *E. coli* O157:H7 in environmental samples and represents a considerable advancement in pathogen quantification in different ecosystems.

Constructed wetlands are commonly used for biotreatment of urban runoff and agricultural waste, such as dairy waste wash water from milk cows and on-farm waste from manure piles. These wastes harbor different bacterial species including human pathogens. Present management practices for dairy wash water on most commercial farms involve long-term storage in ponds where evaporation, percolation into groundwater, or spraying onto crops and/or disposal on land occurs. A constructed wetland treatment system maximizes the utility of existing storage ponds, reduces sediment loading in the wash water ponds through on-site treatment, increases pond capacity, and decreases the need to clean and scrape storage ponds. The goal is to have a final effluent water from the wetland that is suitable for on-site reuse and reduces the amount of contaminants entering groundwater supplies as a result of percolation of wash water stored in ponds and sprayed on disposal lands. This final product is expected to enhance water and air quality; reduce biological oxygen demand, nitrate-nitrogen, phosphorus, and ammonia emissions; and inactivate protozoan parasites and pathogens present in dairy wash water.

The wash waters from dairy and beef cattle operations harbor different bacterial species including human pathogens such as enterohemorrhagic *Escherichia coli* O157:H7. *E. coli* O157:H7 causes a wide spectrum of disease symptoms in humans, such as diarrhea ranging from mild to bloody, hemorrhagic colitis, and complications including hemolytic-uremic syndrome (HUS) and seizures that are particularly severe in children (14). *E. coli* O157:H7 strains are generally lysogenized with one or more phages carrying genes for Shiga-like toxins (7, 22). Shiga-like toxins play a major role in the pathogenesis of hemorrhagic colitis and HUS through cytotoxic effects on cells of the kidneys, intestines, central nervous system, and other organs (14, 23). *E. coli* O157:H7 also harbors a 43-kb pathogenicity island, termed the locus of enterocyte effacement, containing virulence attributes required for the formation of attaching and effacing lesions on the target host cells (17, 19, 26, 33). The formation of the attaching-effacing lesion phenotype requires interactions between intimin, a bacterial cell surface protein, and a translocated intimin receptor (Tir) (2, 17, 19, 20). Tir is secreted by bacterial cells into the host cells, where it is localized into the host cell membrane and serves as a docking point for bacterial intimin (15). Intimin and Tir are encoded by the genes *eae* and *tir*, respectively, which are present in the locus of enterocyte effacement (1). The intimin proteins of pathogenic *E. coli* strains are highly divergent in the amino acid sequence at the carboxy terminus, and based on this sequence variation, intimins have been classified into five

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TABLE 1. Nucleotide sequences of primers and fluorogenic probes

Primer or probe[a]	Sequence (5′→3′)	T_m (°C)	Location[b] within the target gene	Gene[c] detected
stx1 forward	GAC TGC AAA GAC GTA TGT AGA TTC G	60	90–114	*stx1* (150 bp)
stx1 reverse	ATC TAT CCC TCT GAC ATC AAC TGC	59	240–217	
stx1 probe	TGA ATG TCA TTC GCT CTG CAA TAG GTA CTC	70	116–145	
stx2 forward	ATT AAC CAC ACC CCA CCG	59	184–201	*stx2* (200 bp)
stx2 reverse	GTC ATG GAA ACC GTT GTC AC	58	392–373	
stx2 probe	CAG TTA TTT TGC TGT GGA TAT ACG AGG GCT TG	69	204–235	
eae forward	GTA AGT TAC ACT ATA AAA GCA CCG TCG	59	2494–2524	O157:H7 *eae* (106 bp)
eae reverse	TCT GTG TGG ATG GTA ATA AAT TTT TG	59	2599–2574	
eae probe	AAA TGG ACA TAG CAT CAG CAT AAT AGG CTT GCT	69	2572–2540	

[a] Primers and probes were designed by using the program Primer Express version 1.0 (PE Applied Biosystems). The melting temperature (T_m) of the primers ranged from 58 to 60°C, and that of the probes ranged from 68 to 70°C. The probes were conjugated with fluorescent reporter dyes FAM 490 (*stx1*), HEX 530 (*stx2*), and Texas red 575 ($eae_{O157:H7}$) at the 5′ ends and with the quencher dye BHQ 1 for FAM and HEX and BHQ 2 for Texas red at the 3′ ends.
[b] The positions of the oligonucleotides are listed relative to the initiation codon (+1 adenine) of the respective gene.
[c] The nucleotide sequences used in the design of these primers and probes were retrieved from GenBank under accession no. M16625 (*stx1*), X07865 (*stx2*), and AF081182 ($eae_{O157:H7}$).

TABLE 2. Specificity of conventional multiplex PCR assay for detection of the *stx1* and *stx2* genes of STEC and the *eae* gene of *E. coli* O157:H7

Bacterial strain type tested	Strain no.	Serotype	Genotype[a]			Origin/disease or status
			stx1	*stx2*	*eae*	
Nontoxigenic *E. coli*	63	O78:H16	−	−	−	Dog
	912	OX3:H11	−	−	−	Pig/normal
EPEC	1861	O119	−	−	−	Human/diarrhea
	1987	O111:H2	−	−	−	Human
	1988	O26:NM	−	−	−	Human
	5722	O55:NM	−	−	+	Human
ETEC	431	O101	−	−	−	Pig/diarrhea
	1477	O149:H10	−	−	−	Pig/diarrhea
STEC	5702	O103:H2	+	−	+	Human/HUS
	5354	O157:H7	+	−	+	Calf/normal
	3048	O157:NM	+	+	+	Calf
	5264	O157:NM	+	+	+	Calf/normal
	4178	O157:H7	+	+	+	Human/HUS
	5705	O121:H19	+	+	−	Human/HUS
	3883	O111:NH	+	−	+	Calf/normal
	5710	O26:H11	+	+	+	Human/HUS
	2871	O157:H7	−	+	+	Calf/normal
	2725	O157:H7	+	+	+	HUS
	5570	O157:H7	+	+	+	Human/diarrhea
	3128	O113	+	+	−	Human/diarrhea
	2799	O157:NH	−	+	+	Calf/normal
	5709	O111:NM	+	+	+	Human/HUS
	5981	O157:NM	−	−	+	Calf
	5701	O126:H27	+	−	−	Human/HUS
	3861	OX3:H2	+	+	+	Calf/normal
	2409	O157:H7	+	−	+	Food
	1043	O22	+	−	+	Calf/diarrhea
	2873	O157:NM	+	+	+	Calf/normal
	5708	O45:H2	+	−	−	Human/HUS
	4700	O157:H7	+	+	+	Calf
	3081	O157:H7	+	+	+	Calf
	1524	O132	−	−	+	Rabbit/diarrhea
	3244	O119:H16	−	+	−	Calf/normal

[a] The presence of these genes had been determined by Sharma et al. (28) by fluorogenic PCR with DNA probes specific for these genes and confirmed in this study. +, gene detected by multiplex PCR; −, gene not detected by multiplex PCR.

subgroups with *E. coli* O157:H7 strains occurring in type Y (25).

Since dairy and beef cattle are considered important reservoirs for *E. coli* O157:H7 (6, 34), this pathogen could potentially enter the drinking water supply from cattle waste wash water. As wastewater is being increasingly used in the irrigation of crops, there is a danger of inadequate treatment to eliminate bacterial pathogens. Also, recent alterations in methods of food production and delivery to consumers have exacerbated problems with pathogenic bacteria such as *E. coli* O157:H7. The increasing popularity of organic food production has led to widespread composting of animal manure for use in crop production. Problems have often occurred as a consequence of temperatures during composting being insufficient to kill enteric bacteria.

Methods for the effective treatment of wastewater and manure are essential to ensure the safety of drinking water and crops. To ascertain the effectiveness of wastewater treatment, it is imperative to develop methods that would allow rapid and sensitive detection of bacterial pathogens in agricultural and urban water runoffs, soils, and cattle manure before and after treatment of these important recyclable resources. The availability of methods for effective treatment and detection of pathogens in environmental samples would reduce the incidence of human illnesses by preventing the transmission of bacterial pathogens to humans. This in turn would save millions of dollars in health-related medical costs and food recalls.

PCR has become an important method for the rapid, sensitive, and specific detection of bacterial pathogens. Recent advances in the synthesis of fluorogenic probes and the development of instrumentation for continuous monitoring of fluorescence have facilitated the development of real-time PCR assays for specific, automated detection and quantitation of amplified gene products (10). Real-time multiplex PCR has been used for the detection and quantification of *E. coli* O157:H7 in food and clinical samples (4, 21, 27, 28), but absolute quantification of this pathogen by this technology has not been tested vigorously with wastewater and environmental samples.

In this study, a real-time multiplex PCR assay with specific

TABLE 3. Sensitivity of real-time multiplex PCR assay for detection and quantification of the *stx1* and the *stx2* genes of STEC, *eae* of *E. coli* O157:H7, and related strains

Bacterial strain type	Strain no.	Serotype	Genotype	C_T of gene:		
				stx1	*stx2*	*eae*
Nontoxigenic *E. coli*	63	O78:H16		42.3	–[a]	–
Salmonella enterica serovar Typhimurium	3244	O119:H16		46.9	–	–
EPEC	5722	O55:NM	*eae*	38.1	47.7	43.8
ETEC	431	O101	*stap*	38.8	–	–
STEC	5701	O116:H27	*stx1*	24.6	–	47.8
	4718	O157:H7	*stx1 stx2 eae*	24.8	29.2	26.0
	4700	O157:H7	*stx1 stx2 eae*	25.1	31.1	27.6
	3081	O157:H7	*stx1 stx2 eae*	23.9	28.4	26.1
	5699	O91:H21	*stx2*	39.2	26.3	–

[a] –, C_T not determined by real-time PCR.

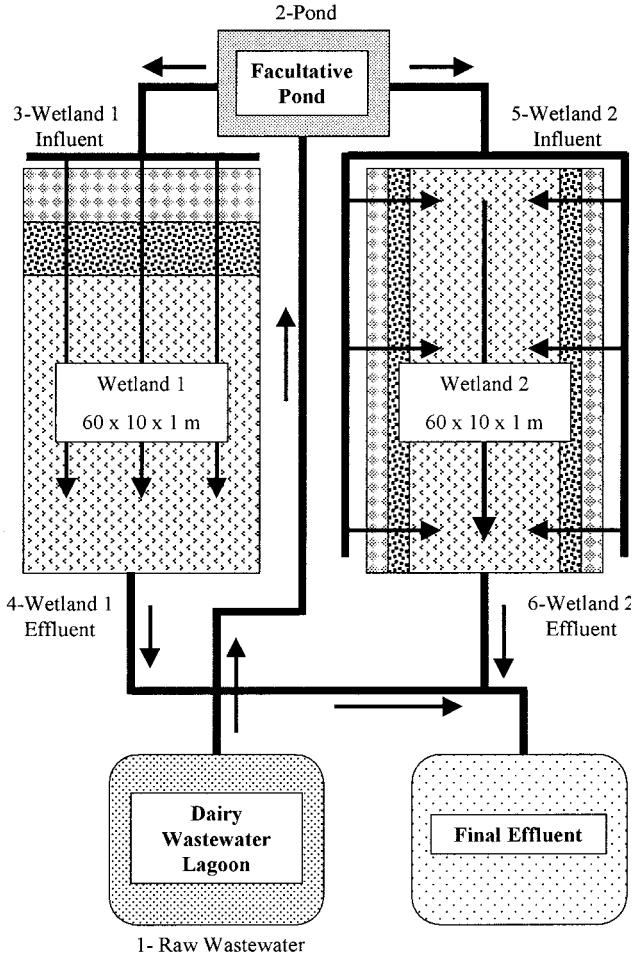

FIG. 1. Schematic of the dairy wash water wetlands used as a treatment facility for the removal of dairy waste and other compounds. All wastewater received in the wetlands was from the milking parlor. The wash water was drained into the raw water lagoon (1), moved through 3-in.-diameter polyvinyl chloride pipes by gravity to an aeration pond, and passed through two parallel wetlands. Water samples were collected from the raw dairy wastewater lagoon (1), facultative pond (2), wetland 1 influent (3), wetland 2 influent (5), wetland 1 effluent (4), and wetland 2 effluent (6).

probes (Table 1) was used to detect and quantify *E. coli* O157:H7 in dairy wastewater prior to and following treatment by a subsurface constructed wetland. The main benefit of simultaneous multiplexing with quantification is cost efficiency and a higher throughput. The objective of the present study was to develop a real-time multiplex PCR assay to detect and quantify *E. coli* O157:H7 and to determine its suitability for monitoring the effectiveness of a subsurface constructed wetland system in reducing *E. coli* O157:H7 levels in dairy waste wash water. The real-time PCR method was also evaluated for the detection and quantification of *E. coli* O157:H7 in soil samples seeded with known numbers of the organism. An iCycle iQ detection system (Bio-Rad, Hercules, Calif.) consisting of a thermal cycler, an optical module, and detection software was used for the PCR assay validation. This instrument monitored incremental increases in fluorescence at successive PCR cycles in real time and used this data to establish a threshold cycle (C_T) for the assay. During the early rounds of amplification, the changes in fluorescence are negligible and beyond the sensitivity of the system detector. At some point during cycling, a change will register with the detection system. This is called the threshold cycle (C_T) and is proportional to the amount of the starting amount of nucleic acid (10). Since the threshold cycle is inversely proportional to the copy number of a target gene, this allowed for the construction of standard curves by using samples containing 10-fold dilutions of target genes.

MATERIALS AND METHODS

Bacterial strains and culture media. The specificity of the multiplex PCR was determined with 33 different strains of *E. coli* O157:H7 and other related strains (Table 2), most of which have been previously characterized (28). From the 33 strains, 9 *E. coli* O157:H7 and non-O157:H7 strains were further evaluated to determine the sensitivity of the multiplex real-time PCR assay (Table 3). All strains were obtained from the National Animal Disease Center (Ames, Iowa) and were cultured on Luria-Bertani broth agar and sorbitol-MacConkey (SMAC) agar plates at 37°C.

Primer and probe design. Primers and probes used for the detection and quantification of *E. coli* O157:H7 are shown in Table 1. The reporter dyes FAM (6-carboxyfluorescein), HEX (6-carboxyfluoroscein), and Texas red (sulforhodamine 101) were conjugated at the 5′ ends of the probes, and quencher dyes, Black Hole quencher (BHQ) dyes I and II (Biosearch Technologies, Novato, Calif.), were conjugated at the 3′ ends. The FAM-, HEX-, and Texas red-labeled probes were used for the detection of the *stx1*, *stx2*, and *eae* genes, respectively, and were synthesized by Biosearch Technologies. The BHQ dye was used as the quencher dye because of its broad quenching spectrum and a lower signal-to-noise ratio than that of other quenching dyes.

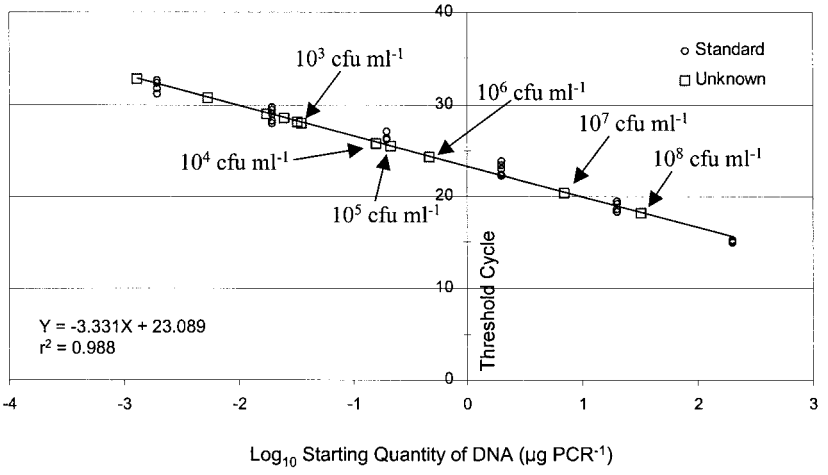

FIG. 2. Standard curve for the real-time PCR analysis of *E. coli* O157:H7 strain 3081. The C_T values are plotted against the corresponding *E. coli* O157:H7 DNA concentrations. Template DNA was extracted from *E. coli* O157:H7 DNA, diluted 10-fold, and used as a known DNA concentration in every experiment. DNA was extracted from *E. coli* O157:H7 strain 3081 in amounts between 6.4×10^{-2} and 6.4×10^{8} CFU ml^{-1}. The reaction efficiencies of the *stx1* and *eae* genes were above 99%, while that of the *stx2* gene was about 93%.

DNA isolation from pure culture and environmental samples. Genomic DNA was isolated from a pure culture of *E. coli* O157:H7 strain 3081, grown for 12 h at 37°C, and extracted with the Qiagen tissue kit (QIAamp DNA Mini kit; Qiagen, Valencia, Calif.) according to the manufacturer's protocol. Total bacterial community DNA was extracted from environmental samples to determine the detection limits of *E. coli* O157:H7 strain 3081 by real-time PCR. The DNA was extracted from 500 mg of soil or fecal samples or 100 ml of water with UltraClean soil, fecal, and water DNA kits (MO BIO, Inc., Solana Beach, Calif.) according to the manufacturer's protocol. The 100 ml of water was filtered and concentrated into 0.25 ml for DNA extraction with the MO BIO water DNA kit.

Laboratory seeding experiments. Arlington sandy loam soil was obtained from the University of California, Riverside, Agricultural Experiment Station. Soil was removed from the top 15 cm with a stainless steel shovel, passed through a 4-mm-pore-size stainless steel sieve, and treated with 500 mg of methyl bromide kg^{-1} to fumigate the soil. The soil was stored at 4°C and tested by bacteriological and PCR techniques to ensure that it was *E. coli* O157:H7 negative prior to use. An overnight culture of *E. coli* strain 3081 was serially diluted in tryptic soy broth, and the number of CFU of bacteria in each dilution was determined by plating on cefixime-tellurite (CT)–SMAC agar with BCIG (5-bromo-4-chloro-3-indoxyl-β-D-glucuronide) containing 0.05 mg of cefixime liter^{-1} and 2.5 mg of tellurite liter^{-1} (LAB M; IDG). The average titer (CFU milliliter^{-1}) of three replicates was determined. For the spiked experiment, 10 g of soil was seeded with a 0- to 10-fold dilution series of *E. coli* O157:H7 strain 3081 in flasks containing 90 ml of modified Luria-Bertani broth consisting of 8 mg of vancomycin liter^{-1}, 0.05 mg of cefixime liter^{-1}, and 10 mg of cefsulodin liter^{-1}. The inoculated flasks were incubated at 37°C with agitation at 160 rpm. Samples were vortexed for 30 s, and 2-ml aliquots were taken after 0, 2, 8, 16, and 24 h for DNA extraction. The DNA extracted from the enrichment broth was used as a template for real-time PCR. In experiments to compare real-time PCR with the conventional culture method, the enrichment broths of seeded soil samples were also plated onto CT-SMAC agar plates. Sorbitol-negative colonies were enumerated.

Layout of wetlands, sampling scheme, and microbiological analysis. The project design included two wetlands operating in parallel, in addition to a raw and a facultative pond for central collection of wash water prior to treatment (Fig. 1). The constructed wetlands utilized a horizontal subsurface flow system where wash water treatment occurred beneath the surface of the gravel, which was planted with shallow but densely rooted herbaceous plants. Wetland 1 was an end-loading design, where the wash water entered through multiple inlets into coarse gravel, reeds (*Phragmites communus*), and bulrushes (*Scirpus validus*) and then drained into the collection box at the end of the basin. Wetland 2 was a side-loading basin, where wash water entered through multiple inlets along both sides of the wetland and passed through a narrow gravel bed, containing reeds and bulrushes, before being collected through a perforated pipe along the center of the wetlands which drained to the collection box.

Bacterial biofilms on the gravel supported the microbial degradation of nutrients in the wash water. The deep-rooted vegetation from bulrushes transported oxygen to the anaerobic zone, allowing for the nitrification of ammonium and the subsequent denitrification of nitrate in the wash water. Six sample locations were used as indicated in Fig. 1. Samples were collected and plated within 6 h. Fresh cow and calf fecal samples, as well as manure samples, were also collected for bacterial enumeration. Fresh fecal samples were collected within minutes of defecation with a stainless steel shovel, and the top portion was taken for laboratory analysis. Manure samples were taken from piles of fecal materials that had been deposited for about 2 weeks. DNA extraction of environmental samples was completed within 48 h of collection. All samples were collected from a commercial dairy farm in Chino, Calif., adjacent to the constructed wetland between December 2000 and September 2001.

One gram or 1 ml of environmental samples was added to 9 ml of tryptic soy broth, vortexed briefly, serially diluted, and plated for the enumeration of total heterotrophic bacteria, total *E. coli*, and *E. coli* O157 on tryptic soy agar, SMAC, and CT-SMAC agar, respectively. The plates were incubated at 25°C for heterotrophic bacterial counts and 37°C for total *E. coli* and *E. coli* O157 for 24 h. Six sorbitol-negative, translucent colonies per sample were tested by multiplex PCR to determine the presence of the three genes. Additionally, isolates that were sorbitol positive or β-glucuronidase positive (red-pink colonies with a purple center or green colonies) were enumerated as other *E. coli* strains.

Real-time multiplex PCR assay for quantification of *E. coli* O157:H7 strain 3081. A single primer-probe reaction was compared with a multiplex primer-probe reaction to determine any significant differences in the C_T values. *E. coli* O157:H7 strain 3081 genomic DNA (7.9 pg ml^{-1}) was amplified in both single and multiplex reactions. To demonstrate the detection range of multiplex PCR, 10-fold serial dilutions containing 7.9×10^{0} to 7.9×10^{-9} pg of genomic DNA ml^{-1} were assayed for the *stx1*, *stx2*, and *eae* genes. PCR was performed in a 50-μl volume containing 200 μM deoxynucleoside triphosphates (dNTPs), 2 μl of genomic DNA from each concentration, 2.5 U of AmpliTaq Gold polymerase, 5 μl of 10× TaqMan buffer (PE Applied Biosystems, Foster City, Calif.), 0.3 μM (each) primer, 0.1 μM (each) probe, and 3 mM $MgCl_2$. Genomic DNA purified from *E. coli* O157:H7 strain 3081 was used as a template for the positive control. Reaction mixtures were dispensed into a 96-well, thin-wall PCR plate (Bio-Rad), covered with optically clear sealing film, and centrifuged briefly. PCR was performed with the iCycle iQ thermal cycler with the following cycle conditions: denaturation at 95°C for 10 min and 50 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 40 s, followed by a 5-min extension at 72°C and holding at 4°C.

The iCycle iQ real-time PCR detection system and software were used for data analyses. This system used a thermal cycler, an optical module, and detection software to quantify PCR products in real time, as revealed by the increase of fluorescence signal by 5′-nuclease activity during the amplification process. The threshold cycle (C_T) for each standard was plotted against the log of starting quantity to construct the standard curve used to quantify genes in the unknown samples.

Multiplex PCR quantification of environmental samples. Template DNA (2 μl) was added to 48 μl of the reaction mixture as described above, except that the

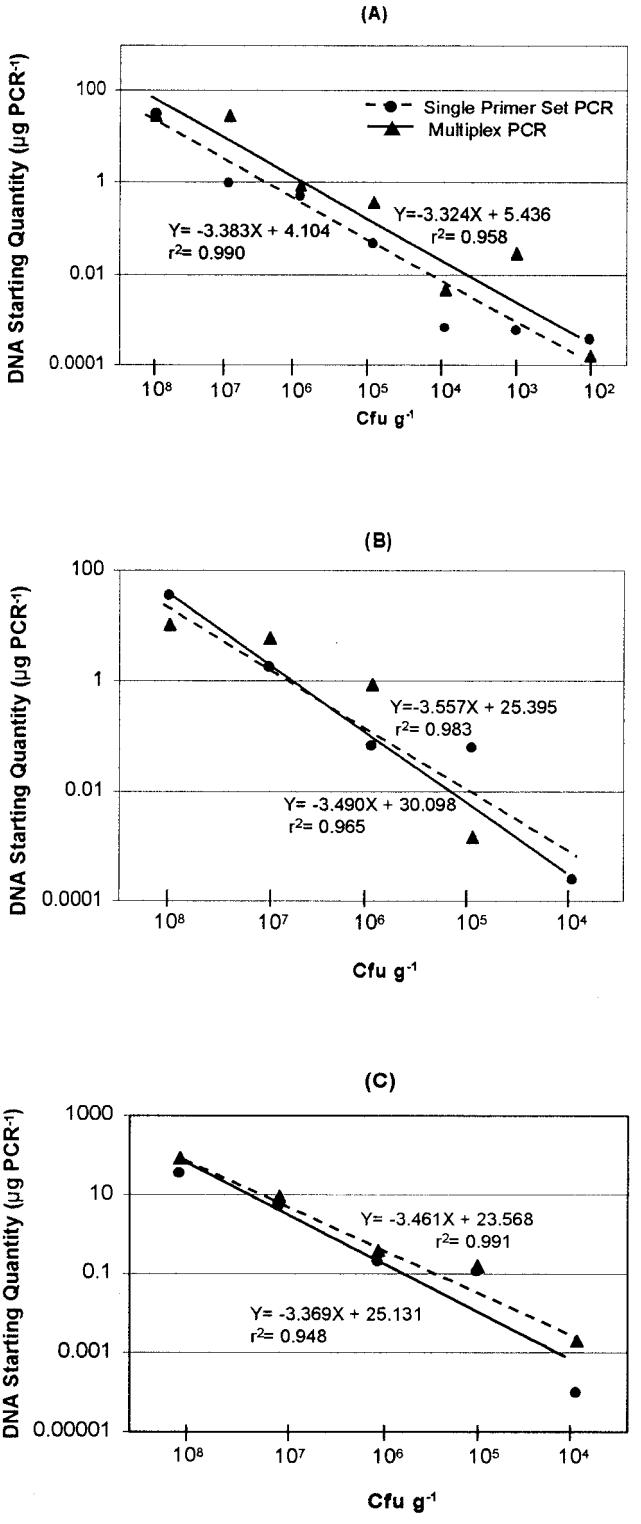

FIG. 3. Determination of detection limits between the single reactions and the multiplex reactions in spiked soil. Standard curves for multiplex versus single-gene real-time PCR analysis of *E. coli* O157:H7 are shown for the three genes. A linear relationship was maintained between 6.4×10^3 and 6.4×10^8 CFU g^{-1} for the *stx1* gene (A) and 6.4×10^4 and 6.4×10^8 CFU g^{-1} for the *stx2* gene and the *eae* gene, respectively (B and C, respectively).

concentrations of $MgCl_2$ and dNTPs were optimized to 5 mM and 450 μM, respectively. Amplification conditions were as described above for all environmental samples. *E. coli* O157:H7 concentrations in environmental samples were confirmed quantitatively by assaying serial dilutions (7.9×10^0 to 7.9×10^{-9} pg ml^{-1}) of strain 3081 DNA in the same plate. Dilution series and environmental samples were tested in triplicate.

Standardization and amplification efficiency. Standard curves generated from plotting C_T versus $\log_{10}$ of starting DNA quantities (picograms) were used for determining the detection limit of the assay. The standard curve was constructed by using known quantities of genomic DNA (7.9×10^0 to 7.9×10^{-9} pg ml^{-1}) extracted from samples containing 6.4×10^{-2} to 6.4×10^8 CFU of *E. coli* O157:H7 ml^{-1}. The concentration of the extracted DNA was measured by an Ultrospec 4000 spectrophotometer with Swift II application software (Pharmacia Biotech, Cambridge, England), and numbers of CFU $milliliter^{-1}$ were determined by plating culture dilutions on CT-SMAC. The titers (CFU $milliliter^{-1}$) of *E. coli* O157:H7 present in unknown samples were determined from the standard curve. For a comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of the standard curves were calculated by performing a linear regression analysis with the iCycle iQ software. A mixture of all PCR reagents without any DNA was used as a negative control. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency will generate a slope of −3.32.

Standardization of DNA quantities between known and unknown samples was accomplished by dividing total numbers of CFU of *E. coli* O157:H7 strain 3081 $milliliter^{-1}$ by the mean starting DNA concentration of that CFU $milliliter^{-1}$ from the instrument analysis. This resulted in a CFU $milliliter^{-1}$ index, which was used as a multiplier to calculate the CFU $milliliter^{-1}$ for all unknown samples. The CFU $milliliter^{-1}$ index was obtained from the highest DNA quantity to estimate CFU $milliliter^{-1}$ from lower DNA quantities. This approach was used because the instrument can give reports in either concentrations or copy numbers, and for environmental samples, it is easier to understand results in actual numbers than in concentrations or copy numbers. For example, in the spiked soil calculated numbers of CFU $milliliter^{-1}$ from plates for *E. coli* O157:H7 strain 3081 were 6.4×10^8 after 16 h of growth at 37°C. The mean starting quantity from the instrument was 2.8×10^1 CFU ml^{-1}, resulting in a CFU $milliliter^{-1}$ index of 2.29×10^7. This number was used as a factor for the determination of CFU $milliliter^{-1}$ of unknown samples with starting mean DNA concentrations determined by the instrument analysis. This process allowed for the establishment of a consistent method for calculating DNA quantities and the corresponding CFU $milliliter^{-1}$ in unknown samples. At the same time, DNA from serial dilutions with known CFU $milliliter^{-1}$ was included in every reaction to cross check the accuracy of the calculations.

Statistical analysis. Data analyses of environmental and laboratory samples were performed with SAS software (*SAS/STAT User's Guide*, *Release 6.03*; SAS Institute Inc., Cary, N.C.). Analyses of variance, means, and standard deviations were conducted to determine whether there were significant differences among mean quantities of pathogens for each sampling point.

RESULTS AND DISCUSSION

Specificity and sensitivity of multiplex real-time PCR assay. The ability of the multiplex PCR assay to distinguish *E. coli* O157:H7 from other serotypes of *E. coli* was determined by analyzing 33 Shiga-toxigenic *E. coli* (STEC) and non-STEC *E. coli* strains (Table 2). These strains were selected from a variety of serotypes with various combinations of *stx* and *eae* genes. PCR analysis of the 33 strains yielded amplification results consistent with previously published features of these strains (28). The sensitivities of FAM-, HEX-, and Texas red-labeled probes in specifically detecting and quantifying *stx1*, *stx2*, and *eae* genes were determined by plotting the log DNA starting quantities of nine strains used for the test. Three different DNA concentrations with C_T values of 23.9, 28.1, and 30.8 for *stx1*, *stx2*, and *eae* genes, respectively, from *E. coli* O157:H7 strain 3081 pure culture were tested in the real-time PCR assay to determine the sensitivities of the primers and probes in a multiplex reaction from different *E. coli* strains. Only C_T values for the highest concentrations are shown (Table 3). Of the nine

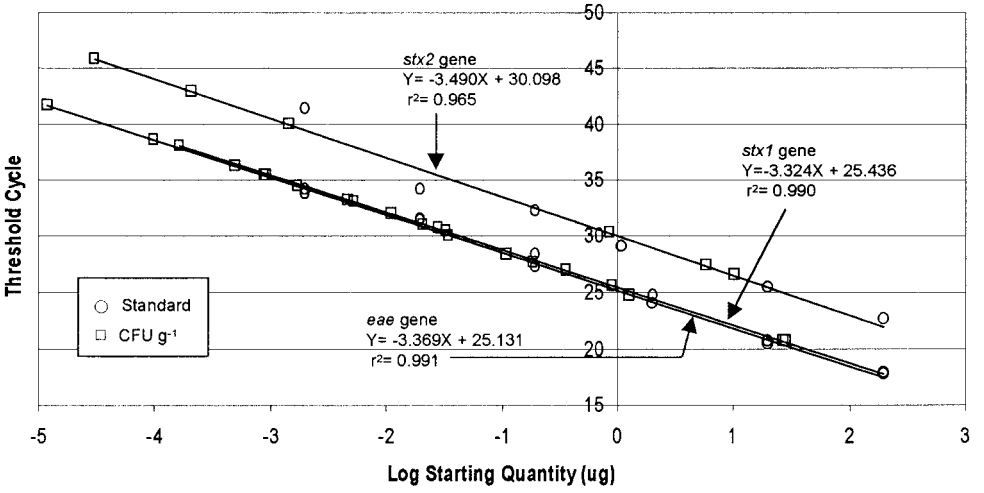

FIG. 4. Standard curves for multiplex real-time PCR analysis of *E. coli* O157:H7 in spiked soil. The reaction efficiencies for the *stx1* and *eae* genes were higher than that for the *stx2* gene as demonstrated by the closeness of the standard curves. These standard curves were used for the detection and quantification of *E. coli* O157:H7 from the enrichment experiment.

bacterial strains, *E. coli* O157:H7 strains 3081, 4700, and 4718 had C_T mean values of 25 or less for the *stx1* gene (150-bp amplicon size), 31 or less for the *stx2* gene (200-bp amplicon size), and 27.6 or less for the *eae* gene (106-bp amplicon size) when 5 to 10 pg of DNA ml^{-1} was used as template. When *E. coli* O157:H7 strains that carry the *stx1* gene, the *stx2* gene, or the *eae* gene were tested, their C_T mean values were also comparable to those in the multiple reaction. The non-STEC strains (nontoxigenic *E. coli*, enteropathogenic *E. coli* [EPEC], and enterotoxigenic *E. coli* [ETEC]) had mean C_T values (38 to 47) outside the sensitivity limit of the assay for all three genes.

Standard curves, detection limits, and amplification efficiencies. The detection sensitivity of the quantitative real-time PCR assay was determined by testing triplicate sets of genomic DNA prepared from serial dilutions (6.4×10^{-2} to 6.4×10^{8} CFU ml^{-1}) of *E. coli* strain 3081 (Fig. 2). The results were reported as threshold cycle numbers versus log starting quantities of DNA. Positive signals were found in all dilutions except those where the DNA concentrations were below 7.9×10^{-5} pg ml^{-1}. The C_T values were also plotted against CFU $milliliter^{-1}$ (6.4×10^{-2} to 6.4×10^{8} CFU ml^{-1}) in 10-fold serial dilutions of the *E. coli* O157:H7 culture used for extracting genomic DNA. Based on this approach, a correlation was observed between the C_T and the CFU $milliliter^{-1}$ of the

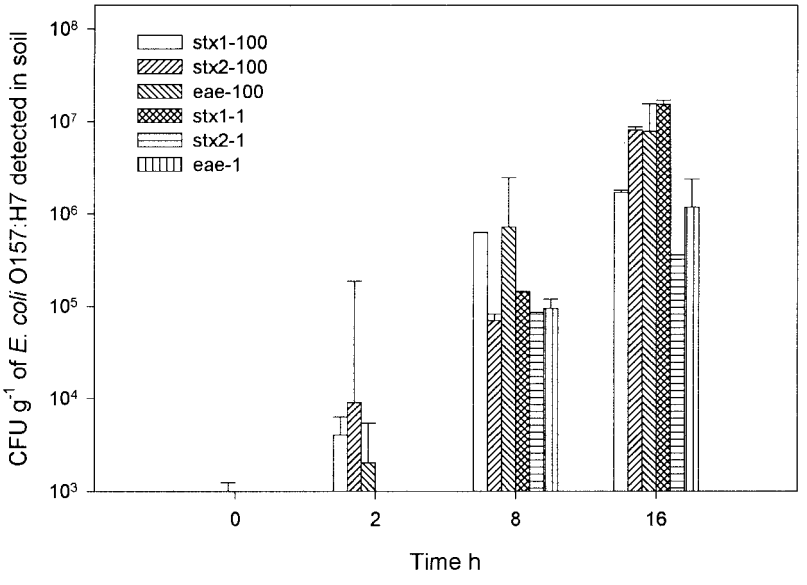

FIG. 5. Detection and quantification of *E. coli* O157:H7 in soil spiked with different concentrations of pathogens and after 0 to 16 h of enrichment. The sensitivity of the multiplex PCR assay from the standard curve in Fig. 4 was used for the detection and quantification of the *stx1* and *stx2* genes of STEC and the *eae* gene of *E. coli* O157:H7 in spiked soil. Concentrations shown are for samples collected after incubation times of 0, 2, 8, and 16 h. Standard errors were derived from the means of triplicate data points.

starting quantity of *E. coli* O157:H7 DNA. A detection limit of 7.9×10^{-5} pg of starting DNA ml^{-1}, equivalent to 6.4×10^3 CFU ml^{-1}, was determined. The efficiency of each assay was calculated from the slope of the standard curve. The standard curves generated from the PCR data resulted in reaction efficiencies of 93.6, 99.9, and 99.6% for the *stx2*, *stx1*, and *eae* genes, respectively, with correlation coefficients of 0.96, 0.99, and 0.98, respectively (Fig. 2 has data for the *eae* gene).

Recovery of *E. coli* O157:H7 from spiked soil. The ability to quantify *E. coli* O157:H7 in environmental samples was tested by spiking different dilutions of *E. coli* O157:H7 into methyl bromide-fumigated soil. Bacterial DNA was extracted from inoculated soil samples and used as the template in the multiplex PCR. Since the assay with spiked soil samples involved a multiplex real-time PCR assay, an effort was made to maximize and equalize the efficiency of amplification among each of the three primer-probe sets. Cross-reactivity between fluorophores was determined by comparing the mean C_T values of wells containing one primer-probe set to those of wells containing primers and probes for all three genes in the spiked samples. By adjustment of the concentrations of dNTPs and $MgCl_2$ to 450 μM and 5 mM, respectively, C_T values of single primer-probe PCRs differed by ≤5% from the C_T values generated in the multiplex PCR containing all three primer-probe sets (Fig. 3). Under these optimized conditions, the linearity between the C_T values and the target concentrations was observed between 3.5×10^3 (*stx1*), 3.5×10^4 (*stx2* and *eae*), and 3.5×10^8 CFU ml^{-1} dilutions of *E. coli* O157:H7. This demonstrates that quantification of target DNA was possible within this range. The detection sensitivity in soils spiked with 10-fold serial dilutions of *E. coli* O157:H7, therefore, ranged between 3.5×10^3 CFU ml^{-1} and 3.5×10^8 CFU ml^{-1} for the *stx1* gene (Fig. 3A) and 3.5×10^4 CFU ml^{-1} and 3.5×10^8 CFU ml^{-1} for the *stx2* and *eae* genes (Fig. 3B and C).

Effect of enrichment on detection sensitivity of multiplex PCR. Most bacterial pathogens, including *E. coli* O157:H7, are present in very low numbers in soil, wastewater, and feces. Soil and feces also contain substances that are inhibitory to PCR and amplification efficiencies (31, 32). The amplification efficiency and the efficiency of the probe cleavages have direct effects on the C_T measurements of the DNA starting quantities (11, 16, 24). Precise C_T determination depends upon the efficient performance of the PCR amplification and detection of the reporter fluorophores. This problem is very critical when dealing with environmental samples (30). According to these authors, this problem was overcome by using probes designed to take advantage of quenching by fluorescence resonance energy transfer. Detection was improved in this study by the use of dark quenchers (BHQ dyes) with a very broad fluorescence spectrum within the context of fluorescence resonance energy transfer. This aided in the successful detection of equivalent numbers of *E. coli* O157:H7 in spiked soil.

The detection of very low levels of bacterial contamination in soils and feces requires that the samples be cultured for a few hours in an appropriate enrichment broth. Enrichment dilutes out the inhibitory substances and provides conditions conducive to the growth and multiplication of bacterial pathogens to a detectable number. Plotting the mean C_T values against the starting quantities of DNA generated the standard curve that was used to estimate *E. coli* O157:H7 concentrations

TABLE 4. Concentrations of total *E. coli* and *E. coli* O157 detected in environmental samples by culture methods

Site[a]	Value (CFU ml^{-1}) for the month of:								
	February[b]	April[b]	July[b]	August[b]	September[b]	April[c]	July[c]	August[c]	September[c]
Raw	$2.0 \times 10^4 \pm 0.4 \times 10^3$	$3.9 \times 10^7 \pm 4.2 \times 10^5$	$1.6 \times 10^6 \pm 1.7 \times 10^5$	$1.1 \times 10^7 \pm 4.2 \times 10^4$	$5.0 \times 10^3 \pm 1.7 \times 10^3$	$3.8 \times 10^6 \pm 3.4 \times 10^4$	$1.6 \times 10^6 \pm 1.5 \times 10^4$	$1.1 \times 10^6 \pm 5.2 \times 10^4$	$1.0 \times 10^2 \pm 1.8 \times 10^1$
Pond	$2.2 \times 10^4 \pm 1.0 \times 10^3$	$5.6 \times 10^4 \pm 7.4 \times 10^3$	$1.2 \times 10^3 \pm 2.8 \times 10^2$	$1.5 \times 10^3 \pm 2.5 \times 10^2$	$1.2 \times 10^3 \pm 2.8 \times 10^2$	$8.0 \times 10^2 \pm 1.2 \times 10^2$	$8.2 \times 10^2 \pm 2.7 \times 10^2$	$4.2 \times 10^2 \pm 1.0 \times 10^2$	$8.9 \times 10^2 \pm 1.2 \times 10^2$
W1 Inf	$1.1 \times 10^4 \pm 1.3 \times 10^3$	$4.7 \times 10^4 \pm 3.9 \times 10^3$	$2.4 \times 10^3 \pm 6.9 \times 10^2$	$2.4 \times 10^3 \pm 6.9 \times 10^2$	$1.1 \times 10^4 \pm 6.5 \times 10^2$	$5.0 \times 10^3 \pm 2.3 \times 10^3$	$2.1 \times 10^3 \pm 1.3 \times 10^2$	$6.3 \times 10^3 \pm 1.5 \times 10^2$	$5.1 \times 10^2 \pm 8.6 \times 10^1$
W1 Eff	$2.4 \times 10^2 \pm 0.2 \times 10^2$	$7.5 \times 10^2 \pm 1.4 \times 10^2$	$1.3 \times 10^2 \pm 5.9 \times 10^1$	$1.3 \times 10^2 \pm 5.9 \times 10^1$	$1.0 \times 10^3 \pm 5.9 \times 10^1$	$8.3 \times 10^1 \pm 1.3 \times 10^0$	$4.8 \times 10^1 \pm 2.8 \times 10^0$	$4.8 \times 10^1 \pm 3.2 \times 10^0$	$7.0 \times 10^1 \pm 5.8 \times 10^0$
W2 Inf	$1.7 \times 10^6 \pm 1.6 \times 10^3$	$5.2 \times 10^4 \pm 3.8 \times 10^3$	$2.5 \times 10^3 \pm 1.2 \times 10^3$	$2.5 \times 10^3 \pm 1.3 \times 10^3$	$1.2 \times 10^4 \pm 1.3 \times 10^3$	$1.5 \times 10^3 \pm 1.1 \times 10^3$	$1.8 \times 10^3 \pm 1.4 \times 10^2$	$1.2 \times 10^3 \pm 2.5 \times 10^2$	ND[d]
W2 Eff	$1.0 \times 10^4 \pm 0.9 \times 10^2$	$6.6 \times 10^2 \pm 5.7 \times 10^1$	$2.2 \times 10^2 \pm 8.0 \times 10^0$	$2.2 \times 10^2 \pm 8.0 \times 10^0$	$3.1 \times 10^1 \pm 1.0 \times 10^0$	$3.2 \times 10^2 \pm 1.5 \times 10^1$	$3.2 \times 10^2 \pm 1.8 \times 10^2$	$1.2 \times 10^2 \pm 5.9 \times 10^0$	$8.9 \times 10^2 \pm 2.8 \times 10^1$
Calf F	NA[e]	$6.1 \times 10^7 \pm 6.2 \times 10^4$	$3.7 \times 10^7 \pm 1.2 \times 10^5$	$3.7 \times 10^6 \pm 1.1 \times 10^5$	$1.9 \times 10^7 \pm 1.2 \times 10^6$	$3.7 \times 10^6 \pm 2.4 \times 10^3$	$3.2 \times 10^6 \pm 2.4 \times 10^4$	$2.5 \times 10^6 \pm 3.8 \times 10^4$	NA
Cow F	NA	$7.8 \times 10^7 \pm 4.1 \times 10^4$	$9.6 \times 10^5 \pm 5.1 \times 10^5$	$2.5 \times 10^6 \pm 5.2 \times 10^5$	$3.6 \times 10^6 \pm 1.9 \times 10^5$	$3.1 \times 10^4 \pm 1.9 \times 10^3$	$6.7 \times 10^4 \pm 6.9 \times 10^2$	$6.7 \times 10^4 \pm 8.2 \times 10^2$	NA
Manure	NA	$6.5 \times 10^7 \pm 1.3 \times 10^3$	$1.5 \times 10^6 \pm 7.8 \times 10^5$	$9.5 \times 10^5 \pm 7.3 \times 10^5$	$1.4 \times 10^6 \pm 4.9 \times 10^4$	$2.4 \times 10^5 \pm 3.7 \times 10^3$	$3.1 \times 10^7 \pm 5.8 \times 10^5$	$1.7 \times 10^7 \pm 4.8 \times 10^5$	NA

[a] All solid samples were from calf feces (Calf F), cow feces (Cow F), or manure, and the liquid waste samples were from the facultative pond (Pond), the raw pond (Raw), wetland 1 effluent (W1 Eff), wetland 1 influent (W1 Inf), wetland 2 effluent (W2 Eff), and wetland 2 influent (W2 Inf).

[b] Total *E. coli* numbers from different samples determined on SMAC medium.

[c] Total *E. coli* O157 numbers determined on CT-SMAC medium.

[d] ND, not determined.

[e] NA, samples not collected for analysis.

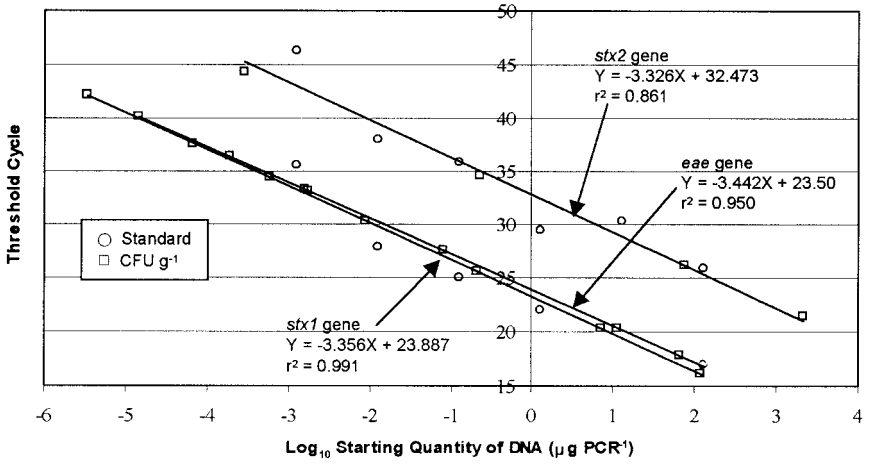

FIG. 6. Standard curves for the multiplex real-time PCR analysis of *E. coli* O157:H7 from calf and cow feces, manure, and wetlands wastewater. *E. coli* O157:H7 concentrations were determined for the samples collected in August and September. Similar curves were done for December, February, April, and July.

in spiked soil samples (Fig. 4). The multiplex PCR assay allowed detection of 1.58×10^7, 7.52×10^6, and 4.96×10^6 CFU of *E. coli* O157:H7 g^{-1} in soil with the *stx1*-, *stx2*-, and *eae*-specific primer-probe sets, respectively, from soil spiked with 3.5×10^8 CFU of *E. coli* O157:H7 strain 3081 g^{-1} (data not shown). The detection limit of this PCR assay in soil samples without enrichment (0 h) was 3.5×10^4 CFU g^{-1}. At this concentration, the assay efficiencies were within 5% of each other and were within the linear range of the standard curve. The concentrations were equivalent to 4.10×10^4, 4.88×10^4, and 4.85×10^4 CFU of *E. coli* O157:H7 g^{-1} based on detection of the *stx1*, *stx2*, and *eae* genes, respectively. After 2 h of enrichment, the assay detected 6.7×10^3 CFU g^{-1} on the average for the three genes when the soil was spiked with 100 CFU of *E. coli* O157:H7 ml^{-1} (Fig. 5). After 8 h, the soil samples spiked with 1 CFU ml^{-1} resulted in the detection of 1.60×10^5 CFU g^{-1} on the average for the three genes and 1.15×10^6 CFU g^{-1} when 100 CFU ml^{-1} was used as the inoculum density. By using a single enrichment process, the real-time PCR assay detected between 1 and 10 CFU of *E. coli* O157:H7 g^{-1} after 16 h of enrichment in artificially inoculated soils. The presence of heterotrophic bacterial flora at a level of 10^7 CFU g of $soil^{-1}$ before enrichment had no effect on the detection sensitivity of this assay (data not shown). The detection sensitivity obtained in this assay was comparable to that of conventional multiplex PCR methods (13). The use of enrichment along with the real-time PCR approach described here offers the possibility for sensitive detection of *E. coli* O157:H7 in soil within 1 day. This approach has successfully been used for relative quantification of *E. coli* O157:H7 in other matrices (3, 21, 28).

Concentrations of total *E. coli* and *E. coli* O157 in feces, manure, and wetlands. Total *E. coli* and *E. coli* O157 concentrations in different matrices from wetland sites 1 to 6 (Table 4) were determined between February 2001 and September 2001 by culture methods on SMAC and CT-SMAC agar. The numbers of total *E. coli* bacteria in the samples ranged from 1.1×10^2 (wetland 2 effluent) to 1.8×10^7 (cow and calf feces, manure, and raw wash water) CFU ml^{-1}, and the numbers of *E. coli* O157 bacteria ranged from 48 (wetland 1 effluent) to 3.1×10^7 in manure samples. There was an overall reduction of 3 to 4 logs of total *E. coli* and *E. coli* O157 in this wetland

TABLE 5. Concentrations of *E. coli* O157:H7 detected in environmental samples by real-time PCR

Site[a]	Result (CFU ml^{-1}) for gene and month						
	stx1					*stx2*	
	February	April	July	August	September	February	April
Raw	$1.3 \times 10^5 \pm 3.2 \times 10^4$	$4.9 \times 10^4 \pm 1.1 \times 10^3$	$1.5 \times 10^4 \pm 5.1 \times 10^3$	$1.1 \times 10^4 \pm 4.4 \times 10^3$	$8.1 \times 10^3 \pm 2.2 \times 10^3$	ND[b]	$2.2 \times 10^4 \pm 3.2 \times 10^3$
Pond	$2.3 \times 10^3 \pm 1.5 \times 10^2$	$7.4 \times 10^4 \pm 2.1 \times 10^3$	$1.1 \times 10^3 \pm 5.6 \times 10^2$	$1.7 \times 10^4 \pm 7.1 \times 10^3$	$1.9 \times 10^4 \pm 6.2 \times 10^3$	ND	$2.2 \times 10^4 \pm 2.2 \times 10^4$
W1 Inf	$4.1 \times 10^3 \pm 3.2 \times 10^3$	$1.1 \times 10^5 \pm 3.4 \times 10^4$	$3.1 \times 10^3 \pm 4.1 \times 10^3$	$3.3 \times 10^4 \pm 5.2 \times 10^3$	$3.4 \times 10^4 \pm 2.2 \times 10^3$	$1.7 \times 10^4 \pm 1.1 \times 10^2$	$4.4 \times 10^3 \pm 3.1 \times 10^3$
W1 Eff	$3.2 \times 10^3 \pm 3.0 \times 10^3$	$5.3 \times 10^3 \pm 4.2 \times 10^3$	$3.4 \times 10^2 \pm 3.0 \times 10^3$	$8.3 \times 10^3 \pm 2.2 \times 10^3$	$1.0 \times 10^4 \pm 1.2 \times 10^3$	ND	ND
W2 Inf	ND	$8.0 \times 10^4 \pm 1.5 \times 10^4$	$2.3 \times 10^3 \pm 8.2 \times 10^2$	$4.1 \times 10^4 \pm 6.2 \times 10^3$	$5.0 \times 10^3 \pm 6.2 \times 10^3$	ND	$3.2 \times 10^4 \pm 3.3 \times 10^4$
W2 Eff	ND	$7.6 \times 10^3 \pm 2.3 \times 10^3$	$1.8 \times 10^3 \pm 4.2 \times 10^2$	$1.2 \times 10^4 \pm 9.1 \times 10^4$	$4.3 \times 10^3 \pm 6.1 \times 10^2$	ND	ND
Calf feces	$1.2 \times 10^4 \pm 2.2 \times 10^3$	$5.9 \times 10^3 \pm 5.2 \times 10^3$	$4.8 \times 10^3 \pm 3.8 \times 10^3$	$2.9 \times 10^4 \pm 2.2 \times 10^3$	$6.0 \times 10^3 \pm 8.3 \times 10^3$	ND	$4.9 \times 10^3 \pm 3.5 \times 10^3$
Cow feces	ND	$2.7 \times 10^5 \pm 1.1 \times 10^5$	$3.2 \times 10^3 \pm 2.2 \times 10^3$	$1.6 \times 10^4 \pm 1.1 \times 10^3$	$4.2 \times 10^4 \pm 6.3 \times 10^3$	ND	$6.5 \times 10^4 \pm 3.6 \times 10^4$
Manure	$4.5 \times 10^3 \pm 4.2 \times 10^2$	NA[c]	ND	$2.7 \times 10^4 \pm 9.1 \times 10^3$	$9.5 \times 10^4 \pm 5.2 \times 10^3$	$1.2 \times 10^2 \pm 2.2 \times 10^1$	NA

[a] All solid samples were from calf feces (Calf F), cow feces (Cow F), or manure, and the liquid waste samples were from the facultative pond (Pond), the raw pond (Raw), wetland 1 effluent (W1 Eff), wetland 1 influent (W1 Inf), wetland 2 effluent (W2 Eff), and wetland 2 influent (W2 Inf).
[b] ND, not determined.
[c] NA, samples not collected for analysis.

demonstration project. The most efficient unit of the project in removing total *E. coli* and *E. coli* O157 was the facultative pond. To evaluate the effects of seasonal variations on the concentration of total *E. coli* and *E. coli* O157, samples were grouped and mean separation was carried out by the least significant difference test. The concentrations of total *E. coli* and *E. coli* O157 were not significantly different among the six sampling points in February, April, and September but were highly significant in July and August. On the average, the highest concentration of total *E. coli* and *E. coli* O157 was recorded in April and the lowest was recorded in September at all the sampling points in the wetlands.

Quantification of *E. coli* O157:H7 from fecal and wetland samples by real-time PCR. Successful quantification of *E. coli* O157:H7 in spiked soils led to the evaluation of wetland and fecal samples in this assay. The standard curves from August and September from a single experimental plate are shown as an example (Fig. 6). Quantitative real-time PCR analysis of the manure, fecal, and wetland samples for the three genes with the standard curves revealed linearity between the C_T values and the starting quantities of DNA representing 10^4 to 10^8 CFU g^{-1}. Amplification efficiencies for *stx1* and *eae* genes were slightly higher than that for the *stx2* gene in all the standard curves. These standard curves were used for estimating the numbers of *E. coli* O157:H7 in cattle or calf feces, manure, the facultative pond, the raw pond, and wetland influents and effluents over 1 year. Table 5 shows the number of *E. coli* O157:H7 bacteria detected over several months in feces, manure, wastewater, and wetland influents and effluents. Temporal fluctuation was noticed in the levels of *E. coli* O157:H7 in these samples. Most samples collected during February and September did not contain detectable levels of the three genes compared to samples collected in April. Samples from April showed significantly higher levels ($P < 0.05$) of *E. coli* O157:H7 than did those from the other months since the presence of the three genes was used for confirmation of the pathogen. The numbers quantified in these samples were in agreement with the work of Zhao et al. (34) and Shere et al. (29), who reported that concentrations of *E. coli* O157:H7 in cattle feces ranged from 10^2 to 10^5 CFU g of feces^{-1}.

The concentrations of *E. coli* O157:H7 obtained by real-time PCR (Table 5) were very close to the numbers obtained by the traditional culture methods on CT-SMAC (Table 4). The developed PCR assay, however, has the advantage of higher throughput, higher reproducibility, and less time required to screen many samples. While PCR may sometimes detect dead cells and degraded DNA, the data from this study showed only a small proportion of this artifact to be of concern for absolute quantification of pathogens in the environment with ribosomal DNA as a template for amplification. Therefore, the ability to quantify *E. coli* O157:H7 in the environment without using culture methods will be very helpful for developing models of pathogen transport in the environment and, subsequently, for risk assessment. Currently, most models and transport studies demonstrating the risk of *E. coli* O157:H7 in the environment depend on culture techniques (9). Other studies have looked at the detection of *E. coli* O157:H7 in soil, water, and feces (5, 8, 13). In this study, a fluorescent signal was converted into target cell densities and related directly to cell densities in soil, manure, feces, and wash water. This approach was made possible by relating the target DNA to the CFU milliliter^{-1} of a cultured *E. coli* strain. This procedure is in contrast to other studies of *E. coli* O157:H7, where presumptive detection by TaqMan PCR was used to estimate the population size of *E. coli* O157:H7 (4, 21). The quantification strategy used here was successful due to prior knowledge of *E. coli* O157:H7 DNA copy numbers and genome size. This strategy has also recently been applied to the detection and quantification of methyl *tert*-butyl ether-degrading strain PMI by real-time PCR (12) and to total *E. coli* and *Pseudomonas fluorescens* (18).

The automated PCR amplification and detection of target gene amplicons described in this study are conducive for screening large numbers of environmental samples in a single assay. This method is a significant tool for monitoring large numbers of environmental samples contaminated with cattle feces or manure that are subsequently transported either by horizontal flow to larger bodies of water or by vertical movement to groundwater. This problem is critical in the Chino-Santa Ana River Basin, Calif., where the surface water and groundwater are major sources of drinking water and where the water quality is progressively deteriorating due to intensive dairy operation and disposal of untreated wastewater into the Chino Basin. The use of constructed wetlands in treating wastewater from farm operations may improve the quality of

TABLE 5—*Continued*

Result (CFU ml^{-1}) for gene and month								
stx2			*eae*					
July	August	September	February	April	July	August	September
$5.2 \times 10^3 \pm 6.6 \times 10^3$	ND	$1.6 \times 10^3 \pm 2.2 \times 10^2$	$5.2 \times 10^3 \pm 9.3 \times 10^3$	$3.3 \times 10^4 \pm 5.2 \times 10^3$	$2.1 \times 10^4 \pm 2.1 \times 10^3$	$1.2 \times 10^4 \pm 7.2 \times 10^3$	$1.6 \times 10^3 \pm 8.3 \times 10^2$
$1.4 \times 10^4 \pm 4.2 \times 10^4$	$8.7 \times 10^3 \pm 7.1 \times 10^3$	ND	$1.4 \times 10^4 \pm 4.2 \times 10^4$	$6.6 \times 10^4 \pm 2.2 \times 10^4$	$4.1 \times 10^3 \pm 9.2 \times 10^3$	$4.1 \times 10^3 \pm 3.2 \times 10^3$	$6.3 \times 10^3 \pm 8.1 \times 10^3$
$1.9 \times 10^5 \pm 2.1 \times 10^3$	$2.1 \times 10^4 \pm 3.1 \times 10^3$	ND	$1.9 \times 10^5 \pm 3.3 \times 10^5$	$1.4 \times 10^4 \pm 7.2 \times 10^4$	$3.1 \times 10^4 \pm 6.2 \times 10^3$	$1.2 \times 10^5 \pm 3.2 \times 10^3$	$2.0 \times 10^3 \pm 2.2 \times 10^3$
$3.4 \times 10^3 \pm 3.1 \times 10^2$	ND	ND	$4.3 \times 10^3 \pm 3.6 \times 10^3$	ND	$3.5 \times 10^3 \pm 3.4 \times 10^3$	$1.8 \times 10^3 \pm 3.2 \times 10^4$	$1.0 \times 10^3 \pm 2.5 \times 10^2$
ND	ND	ND	$6.8 \times 10^4 \pm 6.2 \times 10^4$	$4.6 \times 10^4 \pm 4.2 \times 10^4$	$1.2 \times 10^4 \pm 3.8 \times 10^3$	$2.1 \times 10^4 \pm 5.1 \times 10^3$	$8.7 \times 10^4 \pm 9.1 \times 10^3$
ND	ND	ND	ND	$8.6 \times 10^3 \pm 4.4 \times 10^3$	$6.2 \times 10^3 \pm 1.1 \times 10^3$	$6.9 \times 10^4 \pm 1.1 \times 10^3$	$9.7 \times 10^4 \pm 8.2 \times 10^2$
ND	ND	ND	ND	$9.8 \times 10^4 \pm 2.0 \times 10^3$	$9.6 \times 10^4 \pm 8.2 \times 10^3$	$6.7 \times 10^4 \pm 6.2 \times 10^3$	ND
ND	$8.9 \times 10^3 \pm 2.4 \times 10^2$	ND	ND	$2.7 \times 10^4 \pm 3.0 \times 10^4$	$5.8 \times 10^4 \pm 4.1 \times 10^3$	$1.2 \times 10^4 \pm 7.2 \times 10^3$	
$1.1 \times 10^3 \pm 7.2 \times 10^2$	ND	ND	$4.0 \times 10^2 \pm 2.3 \times 10^2$	NA	ND	$4.2 \times 10^4 \pm 4.2 \times 10^3$	$7.6 \times 10^2 \pm 2.1 \times 10^2$

water that drains into the Santa Ana River. The multiplex PCR assay in combination with culture methods can be a useful method for water districts in the area to monitor contamination by this pathogen in the major rivers and groundwater.

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Mention of trademark or proprietary products in this work does not constitute a guarantee or warranty of the property by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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