Evaluation of PCR-based Quantification Techniques to Estimate the Abundance of Atrazine Chlorohydrolase Gene *atzA* in Rhizosphere Soils

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There are many challenges in the accurate quantification of bacterial genes, such as the atrazine-degrading enzyme *atzA* from *Pseudomonas* sp. strain ADP, from soil samples. We compared four quantitative methods for enumeration of atrazine-degrading bacteria in rhizosphere environments and utilized the optimal probe-based real-time polymerase chain reaction (PCR)–based method in an ongoing bioremediation experiment to monitor *atzA* copy number over time. We compared three quantitative PCR (*q*PCR) based methods—quantitative competitive PCR and two real-time *q*PCR methods—to traditional dilution-plate counting techniques. The optimal real-time *q*PCR assay was then used to monitor *atzA* copy number over time in the robust atrazine-degrading *Pseudomonas* sp. strain ADP–spiked rhizosphere environment. The use of sensitive and reliable probe-based real-time *q*PCRs for the enumeration of bacterial catabolic genes allows for their detection from soil samples and monitoring of potential degradative populations over time. The addition of atrazine-biodegrading bacteria into atrazine-contaminated sites to remove entrapped atrazine is a promising approach for mitigating atrazine pollution and its metabolites. The methodology contained herein will allow for optimal monitoring of *atzA* in rhizosphere soil with or without the addition of biodegradative *Pseudomonas* sp. strain ADP of bacteria.

Atrazine (ATR, 2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) is an inhibitor of photosynthesis in broad-leaf weeds. The common use of ATR for the control of weeds in major crops like corn (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench], and sugarcane (*Saccharum officinarum* L.) has led to its high prevalence in the environment and led to the contamination of soil, surface water, and drinking water aquifers (Erickson and Lee, 1989). Because of ATR's potential endocrine disruption activities, phytotoxicity, and long half-life, public awareness and interest in removing ATR and its metabolites have increased significantly (Thurman et al., 1992; Lerch et al., 1998; Lerch and Blanchard, 2003; Orton et al., 2009).

A plausible method of reducing ATR in the environment is to introduce ATR degraders such as *Pseudomonas* sp. strain ADP. *Pseudomonas* sp. strain ADP is an archetypical bacterial strain originally isolated from an area of high atrazine contamination at a former atrazine dealer site (Mandelbaum et al., 1993, 1995). *Pseudomonas* sp. strain ADP has the ability to use ATR and its metabolites as a carbon source and sole nitrogen source, and contains the first gene involved in the degradation of atrazine to hydroxyatrazine, *atzA* (Mandelbaum et al., 1995; de Souza et al., 1996). *atzA* has been found among diverse species of bacteria, is widespread, and is highly conserved (de Souza et al., 1998a; Seffernick et al., 2000).

Unfortunately, the atrazine catabolic genes *atzABC* are unstable. Transposon insertion sequences flanking each gene result in a large (>40 kbp) unstable region in the pADP-1 plasmid (de Souza et al., 1998a,b; Martinez et al., 2001). These transposase sequences allow for both the loss of the *atzABC* genes and the atrazine-degrading phenotype, as well as the passage of these genes into other neighboring soil bacteria species (de Souza et al., 1998a). Additionally, the availability of alternative nitrogen sources alleviates the selective pressure on *Pseudomonas* sp. strain ADP to use atrazine as its sole nitrogen source and maintain the *atzABC* genes. The spontaneous loss of these genes and/or the pADP-1 plasmid leads to the loss of the atrazine-degradative phenotype (de Souza et al., 1998a; Garcia-Gonzalez et al., 2003).

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Abbreviations: ATR, atrazine; BSA, bovine serum albumin; CFU, colony forming unit; cPCR, competitive polymerase chain reaction; GC, gas chromatograph; MS, mass spectrometer; PCR, polymerase chain reaction; *q*PCR, quantitative polymerase chain reaction.

To fully understand the role of *Pseudomonas* sp. strain ADP in the degradation of ATR in native soil environments, it is critical that we be able to quantify the copy number of the ATR degrading genes present. A number of techniques for bacterial DNA extraction from soil samples as well as quantitative PCR methods have been developed in recent years for quantifying genes of interest in environmental samples (Lakay et al., 2006; Arbeli and Fuentes, 2007).

Competitive polymerase chain reaction (*c*PCR) can be troublesome due to a limited dynamic range and the time-consuming need to perform multiple dilutions and quantitations for each sample. Despite this, *c*PCR has been successfully utilized for the quantification of *atzC* and rDNA genes from atrazine-degrading communities in rhizospheres (Martin-Laurent et al., 2003; Piutti et al., 2002.)

Real-time quantitative PCR (*q*PCR) is less labor intensive and more accurate than traditional colony counting. Recent real-time *q*PCR method is based on the SYBR Green or the TaqMan probe-based chemistries (Ponchel et al., 2003). When appropriate standards are used, quantification of target DNA copy number can be obtained. The use of TaqMan probe-based real-time *q*PCR can provide a higher specificity to the target DNA than that achieved by the SYBR Green-based real-time *q*PCR (Matsenko et al., 2008). SYBR Green dye will bind to non-specific amplicons in the reaction mixture and can result in background signal from these non-target gene amplifications, whereas the probe-based real-time *q*PCR relies on the specific combination of correct primer binding and cleavage of the internal probe by the nuclease activity of the polymerase before a signal is detected.

It is necessary to develop effective quantitative PCR methods for enumeration of *atzA* and other catabolic genes in rhizosphere soils. These methods will be useful for monitoring the behavior, activity, and survival of *Pseudomonas* sp. strain ADP in rhizosphere environments. Because the *atzABC* genes are inherently unstable, precise fluctuations in copy numbers of these genes need to be obtained to determine their stability and the overall population of potential ATR degraders in a dynamic soil sample. Additionally, *Pseudomonas* sp. strain ADP has the ability to horizontally transfer the *atzABC* genes to neighboring bacteria. This, combined with the natural presence of *atzA* homologs among diverse soil isolates, necessitates its accurate enumeration in soil samples.

The rhizosphere is the site of the majority of ATR degradation (55–81%) in vegetative buffer strips and is the ideal place for the monitoring of *atzA* copy number (Lin et al., 2008). The goal of this study is to quantify *atzA* copy number in rhizosphere soil samples using four distinct quantitative methods and to apply the most accurate and reliable quantitative method for quantification of *atzA* in comparison to degradation kinetics in a rhizosphere environment using *Pseudomonas* sp. strain ADP.

Materials and Methods

Soil Inoculation and Media Conditions for Optimization and Method Comparison

Pseudomonas sp. strain ADP was cultured as described in Mandelbaum et al. (1995). Ten milliliters of a 50-mL overnight culture was inoculated into 1 L of R medium supplemented with ATR and incubated in a 37°C rotary shaker until the culture reached stationary phase OD_{600} of ~0.8. Modified-R medium contains 67 mM KH_2PO_4, pH 6.8, 2.0 g L^{-1} Na citrate, 0.2 mM $CaCl_2$, 0.2% glucose, 5 mL L^{-1} R-salt solution, and 1 mL L^{-1} trace metal solution, with 1 mL L^{-1} ATR suspension for broth or 5 mL L^{-1} for agar plates. The R-salt solution contains per liter of 80 g $MgSO_4 7H_2O$; 2 g $FeSO_4 7H_2O$; and 4 mL concentrate HCl. The trace metal solution contains per liter of 0.003 g Na_2MoO_4–$2H_2O$; 0.02 g $CoCl_2 6H_2O$; 0.01 g $ZnSO_4 7H_2O$; 0.003 g $MnCl_2 4H_2O$; 0.001 g $CuCl_2$–$2H_2O$; 0.002 g $NiCl_2 6H_2O$; and 0.03 g H_3BO_4. The ATR suspension is 1 g ATR suspended in 10 mL methanol.

Colony forming units (CFUs) were determined by plating replicates of each suspension on R plates and incubation at 30°C for 48 h. Colonies of *Pseudomonas* sp. strain ADP were verified to be able to utilize ATR by the appearance of ATR -clearing zones around the colonies (Mandelbaum et al., 1995). Serial 10-fold dilutions of bacterial culture suspension were prepared to achieve concentrations of 3.8×10^7, 3.8×10^6, 3.8×10^5, and 3.8×10^4 cells mL^{-1}. Rhizosphere soil was collected from root masses of 12-mo-old mature eastern gamagrass [*Tripsacum dactyloides* (L.) L.] grown in 7-L containers.

Plants were grown for 3 mo in a mixture of 60% sand and 40% Mexico silt loam (fine, smectitic, mesic Vertic Epiaqualfs) collected from the A horizon of the soil profile. The soil particle size consisted of 108 g kg^{-1} clay, 338 g kg^{-1} silt, and 557 g kg^{-1} sand; cation exchange capacity was 8.1 cmol kg^{-1}, and initial organic carbon content was 6 g kg^{-1}. One milliliter of each *Pseudomonas* sp. strain ADP diluted suspension was added to 1 g of rhizosphere soil to achieve overall concentrations of 1.94×10^7, 1.94×10^6, 1.94×10^5, and 1.94×10^4 CFU g^{-1}. Colony forming units were determined as above.

DNA Extraction from Soil

Soil (500 mg dry wt. equivalent) from each of the four inoculum concentrations aforementioned, and native control was collected in quadruplicate and subjected to DNA extraction with a FastDNA SPIN for Soil kit (MP Biomedicals, Solon, OH). After 2 h of gentle agitation in 0.1 M sodium phosphate buffer (pH 8.0) to remove residual humic acid, soil suspensions were processed according to manufacturer's directions. Extracted DNA was eluted in 100 μL DNase/RNase free water, and separated by electrophoresis on a 0.5% (w/w) agarose gel to demonstrate the lack of shearing of extracted DNA products, as has been reported for extraction procedures utilizing similar bead-beating methodologies (Zhou et al., 1996). We achieved a range of between 0.9 and 551 μg of DNA per extraction depending on the inoculation. DNA concentrations were determined as per the PicoGreen dsDNA Quantitation Kit (Invitrogen, Carlsbad, CA) and manufacturer's specification and using a Bio-Tek Synergy HTTR-1 plate reader.

Isolation of the Pseudomonas sp. Strain ADP DNA

Overnight cultures (10 mL) of *Pseudomonas* sp. strain ADP were prepared as above. Total DNA was isolated by sarcosyl treatment followed by phenol/chloroform extraction as previously described (Thompson et al., 2007). Total DNA was used as a template for construction of competitor PCR products and *atzA* plasmid standards as described in the following.

PCR Conditions and Optimization

The optimization of conditions and reagents for PCR are described in detail in the Supplemental Materials and Methods section and Supplemental Tables S1–S2 and were applied throughout this study.

One microliter of extracted DNA from spiked soil samples was used as template for PCR amplification combined with Premix Ex Taq 2X PCR Solution (Takara Bio, Shiga, Japan) and ddH_20 for a final volume of 50 μL. Polymerase chain reaction primers P. ADP Forward and P. ADP Reverse (Table 1) were then added at a final concentration of 0.5 μmol L^{-1}. Primers used in this study were those designed by de Souza et al. (1998a) to amplify a 444-bp fragment of *atzA* between nucleotides 472 and 915 with minimal specificity issues. We also added 1 μg mL^{-1} bovine serum albumin (BSA) to the PCR mixture for enhancement. Amplification of DNA was performed under the following conditions: 35 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 1 min. Amplicons were separated on 1.5% (w/w) agarose gels by electrophoresis. Band intensities were obtained via Multi-Gauge analytical software (Fujifilm, Tokyo, Japan). All PCRs were performed in triplicate.

Competitor Construction and Conditions

The DNA competitor for competitive PCR was designed and constructed using primers (cForward and cReverse) in Table 1 and the specifications detailed in the Competitive DNA construction kit (Takara Bio) The competitor was created to contain the primer binding sites obtained from de Souza et al. (1998a) with the 3′ end of the primers amplifying a 538-bp piece of lambda DNA included in the kit (~100 bp larger than the P. ADP Forward and P. ADP Reverse amplicon from PCR Conditions and Optimization). Amplicon copy number was calculated from the OD_{260} after purification of the PCR product using a Qiagen (Hilden, Germany) PCR purification kit. Dilutions of the competitor DNA were made to 10^8 through 10^2 competitor copies μL^{-1}. One microliter of a 1:100 dilution of DNA from spiked soil extracts was added to each PCR reaction plus 1 μg mL^{-1} BSA and 1 μL of each competitor template DNA dilution in series. Polymerase chain reaction cycle conditions, electrophoresis, and band intensities were performed as described above. The copy number of the *atzA* gene was determined by the ratio of target gene *atzA* (444-bp) amplicon to competitor DNA amplicon (538-bp). The log of this T/C ratio was plotted against the copy number of the competitor. *atzA* copy number was determined when log $(T/C) = 0$, when the amount of target DNA equals to the amount of competitor DNA. All competitive PCR reactions were performed at least in triplicate with each spiked soil dilution.

Quantitative Real-Time qPCR

The optimization of primer concentration and reagents can be found in Supplemental Material and Methods section and Supplemental Table S3. The TaqMan probe and the primer pair are listed in Table 1. Probe was designed using the Primer Express software (Applied Biosystems, Carlsbad, CA) to find an optimal probe with a melting temperature (T_m) that is 10°C greater than the primers (60°C compared with 50°C). Primer and probe specificity was tested by BLAST analysis (NCBI) to prevent known nonspecific binding targets that could be obtained in soil extracts and among *Pseudomonas* sp. strain ADP DNA. Final PCR mixtures consisted of 25 μL Premix Ex Taq (Perfect Real Time) (Takara Bio), 900 nM of each primer, 12.5 pmol of the TaqMan probe (Applied Biosystems, Carlsbad, CA), 1.0 μL of soil DNA extract in a 1:100 dilution, 1 μg mL^{-1} BSA, and RNase/DNase-free water to reach a final volume of 50 μL. All real-time *q*PCRs were initiated with activation of DNA polymerase at 95°C for 10 min and 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

The *atzA* gene was amplified by PCR (Primers P. ADP Forward and P. ADP Reverse) from *Pseudomonas* total DNA, cloned, correct plasmids purified from *Escherichia coli,* quantified, and used as standards for real-time *q*PCR. A standard curve was constructed at concentrations of 10^8 through 10^2 gene copies μL^{-1} and utilized to determine the copy number of the target *atzA* gene in the spiked soil samples. All real-time *q*PCR assays were performed in triplicate using a 7500 Fast real-time *q*PCR machine (Applied Biosystems, Carlsbad, CA). SYBR Green-based real-time *q*PCR assays used identical PCR primers and conditions as the aforementioned *q*PCR Taqman probe-based chemistry with the addition of a dissociation step at the end of 40 cycles. The SYBR Premix Ex Taq (Takara Bio) plus ROX Reference Dye II were utilized for the PCR mixtures, and the primer concentration of 900 nM was applied as described in the Supplement Information.

Growth Chamber Study and Pseudomonas sp. Strain ADP Inoculation

To determine the effects of *Pseudomonas* sp. strain ADP on the rhizodegradation of ATR, an incubation experiment was conducted in a walk-in chamber using mature eastern gamagrass grown in 7-kg containers, run in triplicate. Plants were allowed to grow to maturity (12 mo) in a Mexico silt loam. After three additional months of growth beyond maturity, the rhizosphere soil was collected and the soil moisture content was adjusted to 15%. An ATR solution containing 37 KBq of ^{14}C-ATR (ring-U-labeled *^{14}C-atrazine*, radiopurity > 98%, specific activity = 7.77 × 108 Bq $mmol^{-1}$; Sigma, St. Louis, MO) mixed with nonradioactive ATR solution was added to 20 g (dry wt. equivalent) of each soil to achieve the final concentration 1.5 mg kg^{-1}. The *Pseudomonas* sp. strain ADP culture was inoculated to each experiment unit to achieve the final concentration of 2 × 10^6 CFU g^{-1}. The ATR-treated spiked or control soil (20 g)

Table 1. Designed primers for real-time quantitative polymerase chain reaction (*q*PCR) and competitive real-time polymerase chain reaction (PCR).

Method	Primer
Real-time *q*PCR	
P. ADP Forward	GCACGGGCGTCAATTCTA (de Souza et al., 1998a)
P. ADP Reverse	CGCATTCCTTCAACTGTC (de Souza et al., 1998a)
atzA TaqMan MGB Probe	6FAM -ATCGGATGGACGGGCGCA-MGBNFQ
Competitive *q*PCR	
cForward	GCACGGACGTCAATTCTAGTACGGTCATCATCTGACAC
cReverse	CGCATTCCTTCAACTGTCAATACATCAAACGCCGCGAC

was incubated in sealed 500-mL Mason jars for 100 d at 25°C in the dark. Atrazine mineralization was measured using alkali traps (10 mL of 2 mol L^{-1} NaOH) placed in the Mason jars, and the traps were periodically replaced throughout the incubation period. One milliliter of NaOH solution collected from CO_2 trap was mixed with 4 mL of Ultra Gold AB scintillation cocktail (American Bioanalytical, Natick, MA). The $^{14}CO_2$ activity was quantified by Beckman LC6000 SC liquid scintillation counter (Brea, CA).

An additional set of rhizosphere soils was treated with same concentration of non-radiolabeled ATR followed by the same procedure for inoculation of *Pseudomonas* sp. strain ADP culture as described above. These samples were used to determine the ATR degradation rates over the incubation period. Soils were sampled at times 0, 1, 3, 5, 7, 9, and 35 d. All treatments were run in triplicate. The ATR in the 20-g soil was extracted with 250 mL of 90% acetonitrile (v/v) followed by 1 h of sonication. The final extracts were concentrated to 500 μL under the N_2 stream of flow. Analysis of ATR was performed using a Varian 3400cx gas chromatograph (GC) with a Hewlett-Packard (Palo Alto, CA) cross-linked methylsiloxane capillary column (12.5 m × 0.20 mm i.d.) coupled with a Varian Saturn 2000 ion trap mass spectrometer (MS) (Varian Inc., Walnut Creek, CA) as in Lin et al. (2008). The GC temperature program was 70°C for 1 min, ramped to 120°C at 50°C min^{-1}, ramped to 155°C at 38°C min^{-1}, and finally ramped to 290°C at 50°C min^{-1}, and this temperature remained constant for 12 min. Injector temperature was held at 250°C for 5 min. Splitless injection was used. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The transfer line between the GC and MS was held at 250°C, and the ion trap manifold was set to 250°C. A Varian 8200 AutoSampler was used to perform sample injection and solvent flushing of the needle.

Results and Discussion

PCR Amplification and Optimization

Efficient quantification of bacteria numbers in soil relies on the ability of PCR methods to be able to amplify target genes from the DNA of bacteria of interest in the proper soil environment. Serial 10-fold dilutions of *Pseudomonas* sp. strain ADP were added to rhizosphere soil to create bacterial numbers of 1.94×10^7, 1.94×10^6, 1.94×10^5, and 1.94×10^4 CFU g^{-1}. Soil samples spiked with *Pseudomonas* sp. strain ADP were prewashed and total DNA extracted. Polymerase chain reaction amplification of *atzA* from the soil DNA extracts was undertaken to determine the quality of the DNA and interference of any remaining PCR inhibitors in the samples. Polymerase chain reaction conditions, reagents, primer design, and specificity, as well as soil DNA extraction kit selection and supermix selection, were optimized as described in the Supplemental Materials and Methods section and Supplemental Tables S1–S2.

At higher inoculation concentrations (1.94×10^7 and 1.94×10^6 CFU g^{-1}), the soil extracted DNA amplified *atzA* efficiently when compared to purified *atzA* plasmid control template (Fig. 1B, lanes 2–4; Fig. 1C, lanes 2–3). At lower soil inoculums (1.94×10^4 and 1.94×10^5 CFU g^{-1}), *atzA* was not efficiently amplified on initial PCR amplification (Fig. 1A, lanes 1–2, 6–7). A 1:100 dilution of these extracted DNA

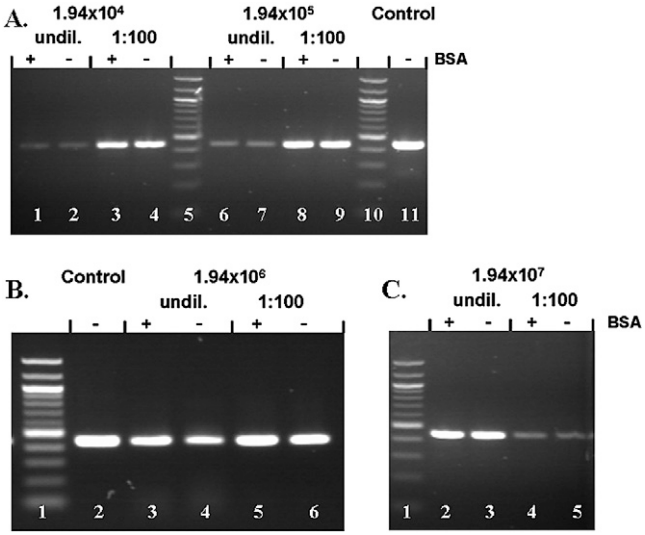

Fig. 1. Agarose gel electrophoresis of amplicons from real-time polymerase chain reaction (PCR) products obtained from soil extracts spiked *Pseudomonas* sp. strain ADP soil extracts. (A) PCR products (35 cycles) from soil extracts from inoculums of 1.94×10^4 colony forming units (CFU) g^{-1} (lanes 1–4) and 1.94×10^5 CFU g^{-1} (lanes 6–9). A 1:100 dilution of template DNA was performed on PCRs in lanes 3–4 and 8–9. Bovine serum albumin (BSA) was added to PCRs in lanes 1, 3, 6, and 8. A plasmid containing the cloned *atzA* gene was used as template for PCR in lane 11. Lanes 5 and 10 denote molecular weight standards (Promega [Madison, WI] 1-kb DNA ladder). (B and C) As in A, but PCRs utilizing the 1.94×10^6 and 1.94×10^7 CFU g^{-1} spiked soil extracts as templates, respectively. PCR conditions—Panel B: 30 cycles; Panel C: 28 cycles. Undil. = undiluted samples: A, lanes 1–2, 6–7; B, lanes 2–3; C, lanes 2–3.

templates resulted in greater amplification of *atzA*, surpassing even the identical undiluted templates (Fig. 1A, lanes 1–4, 6–9). Addition of BSA (Fig. 1) to enhance the PCR reaction appeared initially to lead to a minor increase in amplification, but this increase is not statistically significant (for 1.94×10^4 and 1.94×10^5 CFU g^{-1}, a mean increase of 11.75% ± 26% and 11% ± 22%, respectively, six reactions). Due to the presence of humic acid and other inhibitors in the DNA sample, a 1:100 dilution was necessary for accurate low copy number soil-extracted DNA. Additional inhibition can be overcome by use of high-activity DNA polymerases such as Ex Taq Polymerase (Takara Bio).

Real-time qPCR Analysis

Using the TaqMan probe-based real-time *q*PCR, we obtained consistent quantification of *atzA*. The TaqMan probe-based real-time *q*PCR system was highly sensitive, able to repeatedly and reliably detect both a 1:50 and 1:100 dilution of the 1.94×10^4 CFU g^{-1} spiked soil sample (Supplemental Fig. S1). A standard curve was generated from serial 10-fold dilutions of a plasmid-derived template containing a cloned copy of *atzA* (Supplemental Fig. S1). Other dilution concentrations of spiked soil sample did not give reliable results (Supplemental Table S2). The optimal dilution for real-time *q*PCR is 1:100, which suggests that the 1:50 dilution still has slight inhibition from PCR inhibitors in the DNA extracts. The TaqMan probe-based real-time *q*PCR assay was able to reliably detect less than 10 copies of template DNA per reaction (1.94×10^4 CFU g^{-1} spiked soil sample and 1:100 dilution) in the presence of PCR inhibitors with our optimized conditions.

Quantification of soil-derived samples was performed using the SYBR Green real-time *q*PCR chemistry and compared to a standard curve containing 10-fold dilutions of a plasmid containing a cloned copy of *atzA*. Comparison of results versus the standard curve demonstrated an inability of the SYBR Green real-time *q*PCR assay to accurately detect low quantities of target template (1.94×10^4 and 1.94×10^5 CFU g^{-1} with <10 and <100 copies of *atzA*, respectively) as shown in Supplemental Fig. S1. Amplification and quantification of real-time *q*PCR with the use of SYBR Green-based chemistry lead to equivalent results to the TaqMan probe-based chemistry for the higher spiked soil inoculums (1.94×10^6 and 1.94×10^7 CFU g^{-1}) (Fig. 2). But with the lower inoculums, the SYBR Green chemistry did not show the sensitivity needed to reliably detect the lower copy numbers found in the 1.94×10^4 and 1.94×10^5 CFU g^{-1} spiked soil samples (Fig. 2). The SYBR Green chemistry generated a background level within the soil samples at a Ct value > 29, which did not allow the accurate detection of soil DNA samples containing less than 50 to 100 copies of DNA template in our study (Supplemental Fig. S1).

Competitive PCR Analysis

To accurately quantify *atzA* gene copy via quantitative competitive PCR, serial dilutions of competitor template DNA were added to a static amount of soil template DNA. Use of the competitive PCR method was not sensitive enough to detect *atzA* in the 1.94×10^4 CFU g^{-1} spiked soil sample (Fig. 2). Enumeration of detectable spiked soil samples by competitive PCR gave greater counts than that achieved by traditional plate counting (Fig. 2). Polymerase chain reaction-based methods are generally considered more accurate than manual dilution and plate counting methods, but this difference could also be attributed to inherent flaws in the procedures or the presence of more than one copy of the pADP-1 plasmid (or *atzA*) in dividing cells. The inherent limitation of competitive PCR for rhizosphere soil quantification is its inability to enumerate the 1.94×10^4 CFU g^{-1} spiked soil sample.

A comparison of plate counting, *c*PCR, and real-time *q*PCR assay methods is shown in Fig. 2. All PCR-based techniques gave greater results than that achieved by dilution plate counting. The highest counts were obtained by competitive PCR, but the drawbacks to this technique included the inability to accurately quantify lower spiked soil samples (Fig. 2). Dilution of soil-derived templates 1:100 in a TaqMan probe-based real-time *q*PCR assay resulted in higher *atzA* counts than did a 1:50 dilution (Supplemental Fig. S1). The SYBR Green-based chemistry had comparative counts to TaqMan probe-based real-time *q*PCR at the higher inoculums but had a lower sensitivity in the low inoculums due to inherent background levels. Therefore, the real-time qPCR using TaqMan probe-based chemistry combined with a 1:100 dilution led to the best combination of sensitivity (<10 copies per reaction) and reproducibility in our assays.

Monitoring of atzA in an Ongoing Atrazine-Degradation Study

We then utilized the TaqMan probe-based real-time *q*PCR method to monitor *atzA* copy number in rhizosphere soil samples in a biodegradative environment. The ability to track *atzA*,

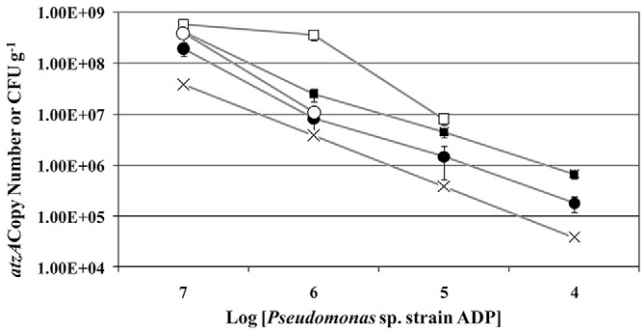

Fig. 2. Comparison of quantification techniques in this study. *atzA* copy number or colony forming units (CFU) per gram is plotted against the log number of *Pseudomonas* sp. strain ADP CFU g^{-1} in amended soil samples. (□) competitive PCR, (■) 1:100 dilution, TaqMan probe-based real-time quantitative polymerase chain reaction (*q*PCR), (●) 1:50 dilution, TaqMan probe-based real-time *q*PCR, (○) SYBR Green-based real-time *q*PCR, and (X) dilution plate counts on R media. Error bars denote standard deviation from at least three independent experiments.

both from *Pseudomonas* sp. strain ADP and from neighboring bacteria to which the *atzA* gene has been transferred, should provide an accurate picture of the state of these ATR-degrading bacteria over time. Eastern gamagrass was utilized as a surrogate plant rhizosphere for the addition of *Pseudomonas* sp. strain ADP (Lin et al., 2008). Samples from spiked and control rhizosphere environments (after removal of plants or roots) amended with ATR were periodically collected and analyzed for *atzA* copy number and ATR degradation. Additionally, *Pseudomonas* sp. strain ADP was added to bulk soil (no plant presence, past or present). In the bulk soil inoculation, bacterial *atzA* counts decreased steadily throughout the experiment (Fig. 3A). The eastern gamagrass rhizosphere environment temporarily stimulated *atzA* copy number (presumably by stimulating growth of the bacteria), but *atzA* copy number soon returned to levels similar to the control soil samples (Fig. 3A). This initial increase is consistent with reported increases in bacterial biomass and *atzC* copy number when exposed to the nutrients in a rhizosphere environment (Piutti et al., 2002; Martin-Laurent et al., 2003; Lin et al., 2008.) No *atzA* was detected in the unspiked control samples (Fig. 3A).

Over the first 10 d, the addition of the *Pseudomonas* sp. strain ADP to the eastern gamagrass rhizosphere soil greatly enhanced the degradation of ATR. After 24 h, there is a sharp increase in *atzA* copy number (60%) that corresponds with a dramatic decrease in the ^{14}C-atrazine concentrations of the soil (79.9 ± 2.3%) and its mineralization into $^{14}CO_2$ (20.9%, Fig. 3B and 3C). In the control unspiked rhizosphere soil (no detectable *atzA*), ^{14}C-atrazine degradation was minimal, with only an 8.6 ± 2.7% decrease in ATR observed after 9 d (Fig. 3B). This inherent degradation of ATR in the control soil (half-life of 4–57 wk) is similar to ATR degradation levels seen by natural processes (Best and Weber, 1974; Cohen et al., 1984).

After 7 d, in the eastern gamagrass rhizosphere environment supplemented with *Pseudomonas* sp. strain ADP, the soil was rid of residual ATR and mineralized 35.4% of it into $^{14}CO_2$. This level of degradation of ATR found on inoculation is considerably higher than that seen in other native rhizosphere degradation studies (Perkovich et al., 1996; Singh et al., 2004). These studies demonstrated the presence of a delay

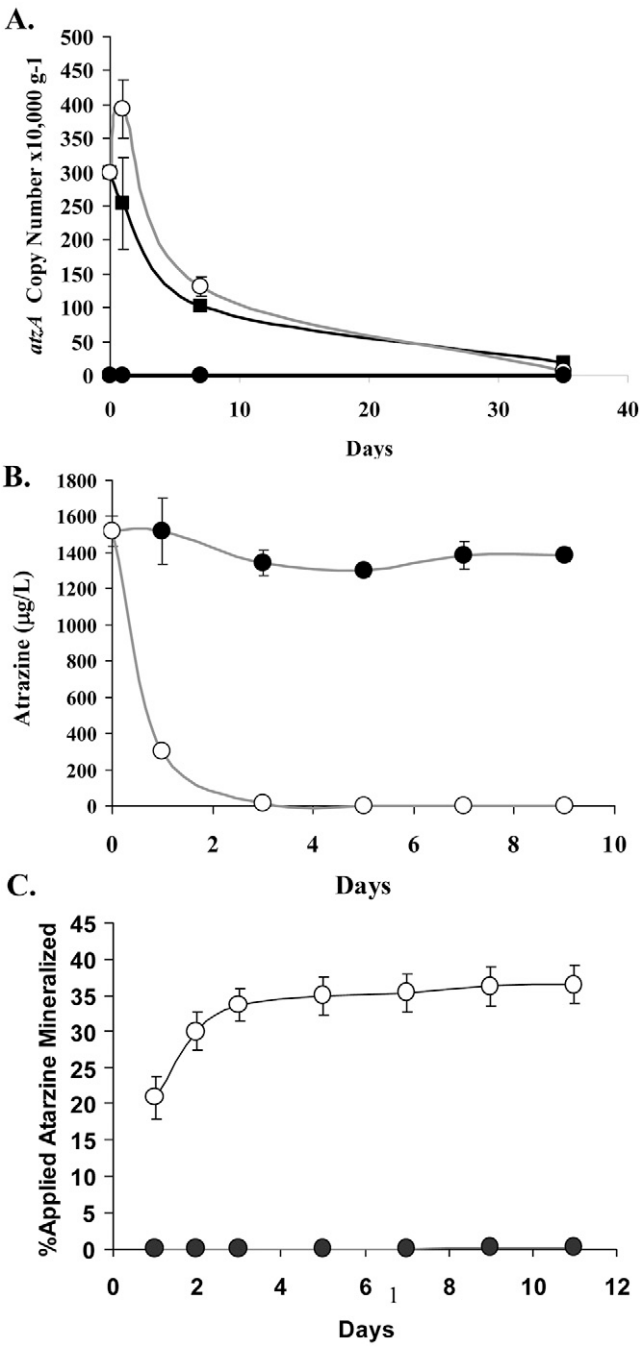

Fig. 3. Tracking of *atzA* copy number and atrazine-degradation kinetics during a time course. TaqMan probe-based real-time quantitative polymerase chain reaction (*q*PCR) estimated *atzA* copy number over time in atrazine-augmented rhizosphere environments and atrazine-degradation kinetics in eastern gamagrass rhizosphere environments uninoculated or inoculated with *Pseudomonas* sp. strain ADP. (A) Copy number of *atzA*: (○) *Pseudomonas* sp. strain ADP amended eastern gamagrass rhizosphere environment, (■) *Pseudomonas* sp. strain ADP amended bulk soil, (●) eastern gamagrass rhizosphere environment uninoculated (no detectable *atzA*). (B) Atrazine concentration: eastern gamagrass (EGG) rhizosphere environments uninoculated (●) or inoculated (○) with *Pseudomonas* sp. strain ADP. Total atrazine concentration of rhizosphere environments over time as determined by gas chromatography–mass spectrometry. (C) Total ^{14}C-atrazine atrazine mineralized into $^{14}CO_2$ in uninoculated (closed circle) or inoculated (open circle) soil.

in ATR degradation as biodegradative microorganisms in the rhizosphere increase in numbers in response to ATR, which is not apparent in our study when concentrated ATR-degraders such as *Pseudomonas* sp. strain ADP are added directly to rhizosphere environments. The remaining 64.6% of the ^{14}C was found among the metabolites of ATR and was not fully mineralized to CO_2 as of the end of the study (data not shown). The lack of complete mineralization may also be attributed to the lack of persistence of the *atzA* copy number and loss of the resultant degradative bacteria population in the soil. The eastern gamagrass rhizosphere soil alone was only able to mineralize 0.19% of ^{14}C-atrazine into $^{14}CO_2$ over the same time period (Fig. 3C). This increase in ATR-degradating bacteria in response to ATR was described previously (Piutti et al., 2002; Martin-Laurent et al., 2003). Those studies also demonstrated an increase in *atzC* copy number in rhizosphere soil that corresponded to the increase in mineralization of ATR. The astonishing rate we found (100% of ATR removed versus 8.6% in uninoculated eastern gamagrass soil) suggested that this eastern gamagrass rhizosphere environment supplemented with *Pseudomonas* sp. strain ADP fosters rapid ATR degradation. The addition of *Pseudomonas* sp. strain ADP to the rhizosphere of eastern gamagrass may be able to greatly enhance the grass's natural low level of ATR-degrading abilities (Lin et al., 2008).

The loss of *atzA* copy number over time suggests there was either no prolonged survival of the *Pseudomonas* sp. strain ADP bacteria in these rhizosphere environments or perhaps a loss of the unstable plasmid. This presumed loss of viability might be due to the loss of the ATR substrate as a carbon and nitrogen source for the bacteria, due to the fact that *atzA* copy number does decrease as soil concentrations of ATR drop (Fig. 3A and 3B). As this environment was artificially sealed to monitor ^{14}C-atrazine mineralization into $^{14}CO_2$, it is possible the aerobic bacteria would behave differently in a more relevant open system.

Conclusions

Quantification of soil microorganisms has its own unique set of problems. The presence of PCR inhibitors, nonculturable bacteria, and unknown species of soil bacteria combined with the difficulty in obtaining purified DNA all pose difficulties for accurate enumeration. We have compared competitive PCR and two real-time *q*PCR-based chemistries (SYBR Green and TaqMan probe-based) and have demonstrated that the TaqMan probe-based real-time *q*PCR has the lowest detection limit and highest degree of consistency in the enumeration of rhizosphere soil DNA samples.

The addition of *Pseudomonas* sp. strain ADP into rhizosphere environments leads to the quick and efficient degradation of atrazine into CO_2. The degradative potential (number of bacteria that retain *atzA* gene) of a rhizosphere may be monitored in real time with the use of the TaqMan probe-based real-time PCR methodology. We have demonstrated this methodology to be the most reliable and sensitive method for the detection of *atzA* in rhizosphere soil environments, and it may be utilized for the monitoring of *Pseudomonas* sp. strain ADP survivability and persistence. Additionally, the use of *Pseudomonas* sp. strain ADP in an environment with its natural ability to transfer *atzA* and other catabolic genes to the surrounding areas is a cause for concern, so the ability to track these genes in soil would be beneficial for transmission studies in the future.

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