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Discrepancies in qPCR-based gene quantification and their dependencies on soil properties, inhibitor presence, and DNA extraction kit types†

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The complexity and heterogeneity of soil samples necessitate the inclusion of extensive purification steps prior to genomic assays, such as quantitative PCR (qPCR). Although conventional DNA extraction kits have notably enhanced the convenience of the process, those designed for soil vary considerably in terms of reagents, time, and equipment. Therefore, purified gDNA quality varies depending on the DNA extraction kits used, which leads to discrepancies in gene quantification using qPCR. This issue can be amplified considerably when more complicated (or contaminated) soils are analyzed, even if extensive DNA extraction is employed. Here, we evaluated the influences of the DNA extraction method to the gene quantification using qPCR across soil types. Further Mg^{2+} ion spiking experiments were performed to observe multiple inhibitory effects on qPCR analysis performance. The results suggest that discrepancies in gene quantification are evident in the presence of qPCR inhibitors in soil samples. Furthermore, discrepancies in quantification results are exacerbated by gDNA template quality, which is attributed to DNA extraction. The observed multiple inhibitory effects underscore the importance of careful consideration of both DNA template quality and soil type to ensure more accurate gene quantification in soils.

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1. Introduction

Quantitative PCR (qPCR) is a leading technology for molecular biology in various fields, including soil microbiology. Since it was first reported in 1993,[1] it has been the preferred gene quantification method. Throughout the recent coronavirus disease 2019 (COVID-19) pandemic, qPCR has become well-known for pathogen detection. Prior to qPCR, DNA was extracted *via* cell lysis and purification to release genomic content from bacterial cells. Preparation of genomic DNA templates using conventional DNA extraction kits is considered the gold standard for qPCR assays.

The adaptation of qPCR to soil microbiology was initially challenging owing to soil sample complexity and heterogeneity, and physical and chemical property variations. Soils often contain substances that inhibit qPCR, including humic acids, polysaccharides, urea, phenolic compounds, cations, and heavy metals. These substances can remain in genomic DNA *via* co-precipitation during DNA extraction and inhibit qPCR enzymatic reactions.[2–5] For instance, qPCR is inhibited by low levels of humic acid through interactions with template DNA and *Taq* polymerase[6,7] as well as in the presence of Mg^{2+} ions, even after extensive purification.[8] The Mg^{2+} ion is a part of the qPCR reagent and functions as a cofactor of *Taq* polymerase during PCR. However, excess Mg^{2+} can interact with DNA and inhibit coagulation-based inhibition.[9,10] Therefore, DNA purification is necessary to remove these substances from soil samples and circumvent this potential inhibition.[11–15] Current DNA extraction kits for soil comprise multiple process steps as well as the use of multiple reagents, and are suitable even for the most delicate samples.

Although conventional DNA extraction has notably enhanced convenience for decades, methods designed for soil vary considerably in terms of reagents, time, and equipment. Therefore, the quality of purified gDNA varies depending on the DNA extraction kit used, leading to discrepancies in gene quantification using qPCR. This issue can be amplified when complicated or contaminated soils are analyzed, even if extensive DNA extraction is employed. Such discrepancies compromise the performance of qPCR analysis and are not easily circumvented, as each case varies. If the PCR assay discrepancy appears to be a false negative due to inhibition, the issue can become more serious (*e.g.*, detection of pathogens). Therefore, we revisited and evaluated this issue to identify any potential assay discrepancies and developed remedial actions for qPCR assays in soils.

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This study aimed to evaluate the impact of soil type and DNA extraction kit on the bacterial gene quantification using qPCR. The soils were sampled to assess various physicochemical properties, including soil texture, organic content, and Mg^{2+} ion content. We then investigated the quantification of the target gene from DNA templates extracted from *Pseudomonas putida*–seeded soils—a model bacterium commonly found in contaminated environments—using different DNA extraction kits. Further Mg^{2+} ion-spiking experiments were performed to observe multiple inhibitory effects on the performance of the qPCR assay.

2. Materials and methods

2.1. Soil sampling and properties

Soil samples were collected from four locations (Table S1†) at a depth of 0–15 cm using a stainless-steel trowel and stored in a polyethylene bag at −20 °C in a freezer before use. Before collection, large pieces of debris—including plant roots, fallen leaves, dead plants and animals, and stones—were removed. Samples for measuring the pH and Mg^{2+} ion concentrations were air-dried in the dark and sieved using 2 mm standard sieves. The clumped soil was broken into small pieces prior to sieving. Samples for moisture and organic matter content were sieved using 2 mm standard sieves without air drying. Conversely, samples for measuring exchangeable cations and soil materials were stored frozen (−20 °C) without sieving.

Soil properties—including moisture content, total organic content, pH, texture, and cation-exchange capacity—were determined. The soil moisture content (5 g) was measured using a protocol suggested by the soil pollution process test standards.[16] The total organic matter content of the soil was measured using the loss of ignition method in a furnace (SH Scientific, Sejong, Korea) for combustion at 550 °C for 4 h. The total organic content (%) was calculated by dividing the weight loss upon ignition by initial dry weight. All sieved soils (5 g) were subjected to pH measurements after adding 25 mL of deionized water and stirring at ambient temperature for 1 h. The pH was measured using a PB-10 Basic Benchtop pH meter (Sartorius, Göttingen, Germany). The Korean Society of Forest Environment Research (Namyangju, Gyeonggi-do, Korea) measured cation exchange capacity and soil texture.

The Mg^{2+} ions were extracted from the soil samples, and their concentration was measured using inductively coupled plasma mass spectrometry (ICP-MS, 7850, Agilent Technologies, Santa Clara, CA, USA). The soil sample (5 g) and 10 mL of 0.2 M hydrochloric acid (ACS reagent, 37%, Sigma-Aldrich, St. Louis, MO, USA) were placed in 40 mL amber glass bottles with a Teflon™ lining. The samples were then incubated overnight at ambient temperature (25 ± 1 °C) in an orbital shaker (SHO-2D; Daihan Scientific, Wonju, Kangwon-do, Korea).[17] Impurities were removed from the extract using a 0.2 μm syringe filter (PTFE, ADVENTEC Co., Tokyo, Japan). The Mg^{2+} ion standard (1000 mg L^{-1}, Merck, Darmstadt, Germany) solution was prepared at 0, 0.5, 1, 1.5, 2, 2.5, 5, and 10 mg L^{-1} by serial dilution with 0.2 M hydrochloric acid. The detailed parameters for the ICP-MS analysis are listed in Table S2.†

2.2. Bacteria pure culture

Pure bacterial cells of *Pseudomonas putida* (strain DSM 8368, DSMZ, Braunschweig, Germany) were cultured using autoclaved tryptic soy agar (soybean-casein digest agar medium, Difco™, Franklin Lakes, NJ, USA) and autoclaved tryptic soy broth (soybean-casein digest medium, Difco™). The culture was incubated in an orbital shaker (SHO-2D, Daihan) at an ambient temperature of 25 °C for 24 h. The *P. putida* culture was incubated until the optical density (OD) reached 1.0–1.2 to ready it for DNA extraction. The OD was measured at $\lambda = 600$ μm using a SpectraMax M2 spectrophotometer (Molecular Devices, San Jose, CA, USA).

2.3. DNA extraction using various kits

To investigate the influence of soil type and DNA extraction methods on qPCR results, various DNA extraction kits were applied to several soil samples. Prior to DNA extraction, pure bacterial cells of *P. putida* (OD ∼0.1, 9.6 mL) were seeded into all soil samples (500 mg) (Table S1†). The suitable concentration (OD 0.1) of bacterial cells was determined beforehand and is summarized in Fig. S1.†

DNA extraction from cell-seeded soil samples was conducted using three commercial kits—Kit A: Power soil pro kit (QIAGEN, Venlo, Netherlands), Kit B: FastDNA™ SPIN Kit (MP Biomedicals, Santa Ana, CA, USA), and Kit C: Nusoil kit (MACHEREY-NAGEL, Duren, Germany). Subsequent DNA extraction was performed according to the protocols provided by the three DNA extraction kits which included cell lysis, DNA binding, washing, and elution steps. Kit A is a DNA extraction kit that follows the most basic

Table 1 Comparison of DNA extraction kit protocols

DNA extraction process	Kit A	Kit B	Kit C
Cell lysis step	Chemical and physical lysis using beads	Chemical and physical lysis using beads	Chemical and physical lysis using beads
Inhibitor removal step	Remove inhibition factors using chemical precipitation	—	Remove inhibition factors using an inhibitor column filter
DNA binding step	Chemical DNA binding	Chemical DNA binding	Chemical DNA binding
Washing step	Two-step washing with ethanol-based washing reagent	One-step washing with ethanol-based washing reagent	Four-step washing with ethanol-based washing reagent
DNA elution step	Elution at room temperature (18–25 °C)	Elution at high temperature (55 °C)	Elution at room temperature (18–25 °C)

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protocol, which includes two washing steps using one spin filter. Kit B allows for relatively fast DNA extraction compared to that of the other kits and requires only one washing step using one spin filter. In addition, during the elution step, an incubation step is performed at a temperature higher than ambient temperature (25 ± 1 °C). Conversely, Kit C includes a filter to remove additional inhibitors following cell and before DNA binding. A singular spin filter involves four washing steps. The detailed features of each kit are listed in Table 1.

2.4. qPCR analysis

The following qPCR analysis was implemented for the genomic DNA extracted from all soils. First, a genomic standard was prepared for the qPCR analysis. Molecular cloning was performed to produce a plasmid that was used as a qPCR standard using the pCR2.2-TOPO vector and TOPO TA cloning kit (Invitrogen Thermo Fisher, Waltham, MA, USA). The gene inserted into the plasmid was the PCR amplicon (306 bp) of the PAH-RHD$_\alpha$ gene. Details of the experimental procedure are described in Fig. S3 and S4.† After measuring the plasmid concentration using a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA), the gene copy number was determined using eqn (1). qPCR standard solutions were serially diluted to 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8.

$$\text{Gene copy number} = \frac{6.02 \times 10^{23} \times \text{template DNA conc.} \times \text{volume of template DNA used in qPCR}}{\text{molecular weight of plasmid} + \text{molecular weight of interest DNA}} \quad (1)$$

The qPCR was performed using the Applied Biosystems QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific). Primers targeting a 306-bp region of the PAH-RHDα gene were synthesized by Bioneer (Daejeon, Korea; Table S3†). The qPCR was performed *via* a denaturation process at 95 °C for 15 min, followed by annealing at 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s for 40 cycles. The qPCR reaction (total 25 μL) composition was as follows: SYBR Green Master mix (2X; Thermo Fisher Scientific) 12.5 μL, forward primer (10 μM) 1 μL, reverse primer (10 μM) 1 μL, UltraPure™ DNase/RNase-free distilled water (Invitrogen) 5.5 μL, and template gDNA 5 μL. Afterwards, the elongation process was carried out at 80 °C for 10 s and 72 °C for 7 min.

2.5. Mg^{2+} ion-spiking experiment

A series of Mg^{2+} ion-spiking experiments were implemented to elucidate the effect of multiple inhibitors on the qPCR analysis of the soil samples. The Mg^{2+} ion stock solution was prepared by dissolving $MgCl_2 \cdot 6H_2O$ (DAEJUNG, Gyeonggi-do, Korea) in deionized water resulting in a final concentration of 1000 mg L^{-1} (equivalent to 120 mg L^{-1} of Mg^{2+} ion content). The Mg^{2+} ion stock solution was readied for use by autoclaving at 121 °C for 15 min.

To determine the inhibitory effect of Mg^{2+} ion content on the qPCR calibration curve, Mg^{2+} ions were added to the qPCR standard solution. Five points five microliter of diluted Mg^{2+} ion solutions (22 and 87 mg L^{-1} Mg^{2+} ion content) were added to the qPCR reaction, instead of deionized water, to achieve 5 and 20 mg L^{-1} Mg^{2+} ion content, respectively, resulting in a total volume of 25 μL reaction (equivalent to 0.1 and 0.5 μg Mg^{2+} in a reaction, respectively). The same volume (5.5 μL) of deionized water was subjected to the qPCR reaction as a negative control (0 mg L^{-1} Mg^{2+} ion content). The qPCR temperature program was as described above. Amplification and calibration curves were obtained and replotted using SigmaPlot software (12.5 version, Systat Software, Inc., San Jose, CA, US).

To examine the effect of a high Mg^{2+} ion concentration solely on the qPCR analysis, 2.4 mL of Mg^{2+} ion stock solution (120 mg L^{-1}) was added to the pure cell culture (9.6 mL, OD ~0.1) to achieve a final concentration of 24 mg L^{-1} (equivalent to 0.288 mg Mg^{2+} in a sample). Furthermore, the effect of a high Mg^{2+} concentration in the soil matrix was assessed by choosing sandy soil as the sample matrix among the four soil samples. A high Mg^{2+} ion concentration (325 μL of 120 mg L^{-1} stock solution, 78 mg kg^{-1} as the final concentration, equivalent to 0.039 mg Mg^{2+} in a sample) was spiked to 500 mg of the sandy soil to increase the Mg^{2+} ion content, while ensuring that the final concentration remained below 500 mg kg^{-1}, consistent with relevant environmental conditions.[18–20] The calculated final Mg^{2+} ion concentrations in the sandy soil are plotted in Fig. S6.† Notably, the same number of bacterial cells (9.6 mL, OD ~0.1) were seeded in the sandy soil. For the negative control, deionized water (2.4 mL for the pure cell sample and 325 μL for the soil sample) was added instead of Mg^{2+} ions.

The mixture was subjected to three different DNA extraction kits (Section 2.3). Subsequently, a qPCR analysis was performed as described in Section 2.4. The qPCR results of the Mg^{2+} ion-spiked pure cell cultures and Mg^{2+} ion-spiked sandy soils were compared.

2.6. Multiple inhibition experiment

Finally, target gene quantification was conducted using environmental soils to assess multiple inhibition effects. To examine the multiple inhibition in the qPCR assay, a high Mg^{2+} ion concentration (325 μL of 120 mg L^{-1} stock solution, 78 mg kg^{-1} as the final concentration, equivalent to 0.039 mg Mg^{2+} in each sample) was spiked to the three soils (Mountain, Paddy, and River), which were all seeded by pure cell culture (9.6 mL, OD ~0.1). The calculated final Mg^{2+} ion contents in the soils are presented in Fig. S6,† considering the original Mg^{2+} soil concentrations. For the negative control, 325 μL deionized water was added instead of the Mg^{2+} ion solution. Among the

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three DNA extraction kits, Kit C was chosen for the multiple inhibition experiment because it includes the most thorough purification steps (Table 1), representing the most reliable scenario to circumvent the inhibition in soils containing high concentrations of multiple inhibitors. Therefore, the mixture was subjected to DNA extraction using Kit C to obtain the DNA template for subsequent qPCR analysis following the procedure described in Section 2.4. Finally, the qPCR results of the additional Mg^{2+} ion-spiked soils were compared with those of the original soils (*i.e.*, without Mg^{2+} spiking).

3. Results and discussion

3.1. Soil properties

The physicochemical properties of the soil samples were investigated (Fig. 1 and Table S1†). Soil texture assessments determined the sand, silt, and clay contents of the soil samples (Fig. 1a). The mountain soil (denoted as "Mountain") is a loam soil with the highest clay content (17.3%) and lowest sand content (46.9%). The rice paddy soil (denoted as "Paddy") is a sandy loam soil with the second highest clay content (8.4%). The river sediment (denoted as "River") and sandy soil have a sand texture with 99.1 and 98.7% sand content, respectively.

The total organic content of the soil samples was measured to determine the amount of organic matter, which is a representative inhibitor of qPCR analyses (Fig. 1b).[4,6,7,21] The results showed that Mountain contained the highest total organic content (9.46 ± 0.16%) followed by 4.57 ± 0.15% in paddy, 1.18 ± 0.05% in river, and 2.01 ± 0.81% in sandy soil.

Mg^{2+} ion concentrations in the soil samples were measured using ICP-MS and quantified using a calibration curve (Fig. S5†). The results are presented in Fig. 1c and Table S1.† River contained the highest Mg^{2+} ion concentration of 287.52 ± 2.98 mg kg^{-1}, followed by Paddy (272.47 ± 2.62 mg kg^{-1}), with mountain and sandy soils showing a relatively lower Mg^{2+} ion content at 138.12 ± 1.79 and 95.75 ± 0.77 mg kg^{-1}, respectively. Additionally, we assessed other physicochemical parameters to understand soil properties (Fig. 1d). All four soil samples had slightly acidic pHs ranging from 4.50 to 5.59, with moisture contents ranging from 2.24 to 16.30%. They had varying soil cation exchange capacity values of ~<13 cmolc kg^{-1} (12.54 cmolc kg^{-1} in mountain; 3.85 cmolc kg^{-1} in paddy; 2.86 cmolc kg^{-1} in river; and 0.77 cmolc kg^{-1} in sandy soil).

3.2. Gene quantification discrepancies across soil types and DNA extraction kits

As organic matter content is generally considered a representative inhibitor of qPCR, we assessed qPCR performance using DNA extracted from soils containing different amounts of organic matter. Therefore, Mountain, Paddy, and River were selected because they possess high, medium, and low organic matter contents, respectively.

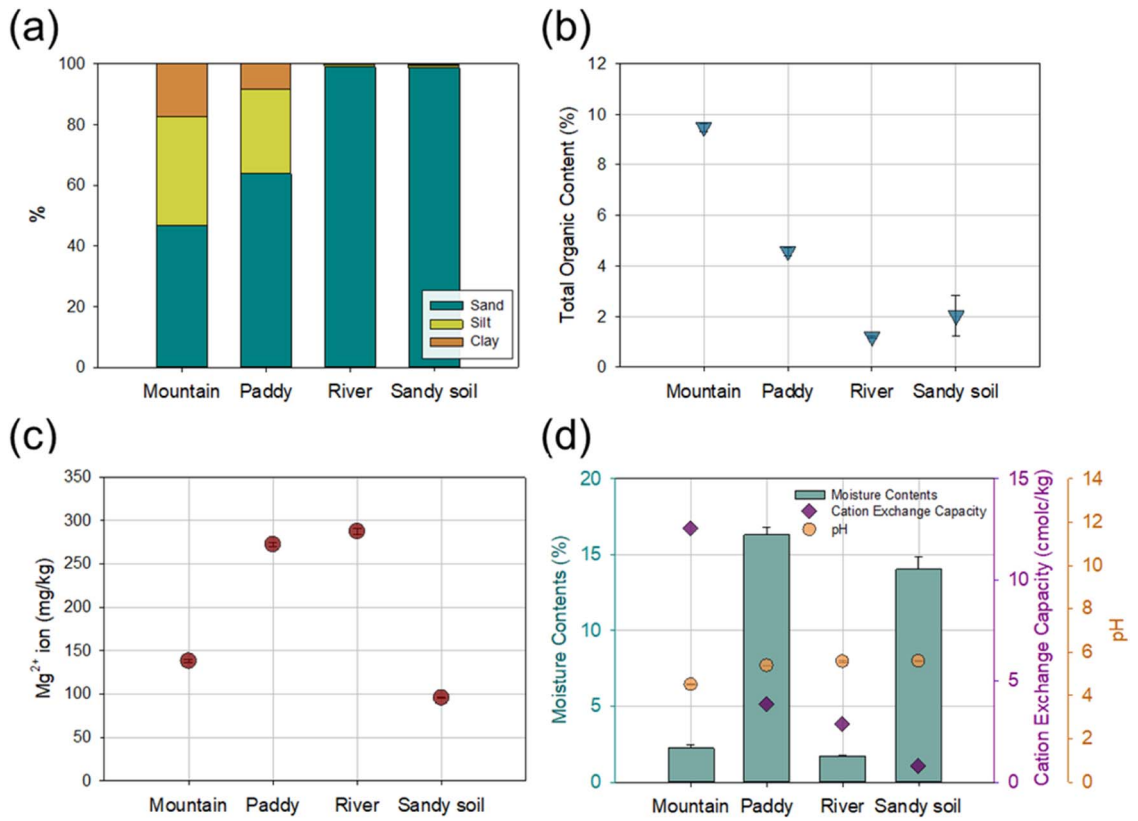


Fig. 1 (a) Soil texture analysis, (b) total organic content (%), (c) Mg^{2+} ion concentration, and (d) moisture content, cation exchange capacity, and pH of the four soil samples. The plots (or bars) and error bars represent the average of triplicated soil samples and standard deviation, respectively, except for soil texture analysis and cation exchange capacity. The same definition applies to the other figures unless otherwise stated.

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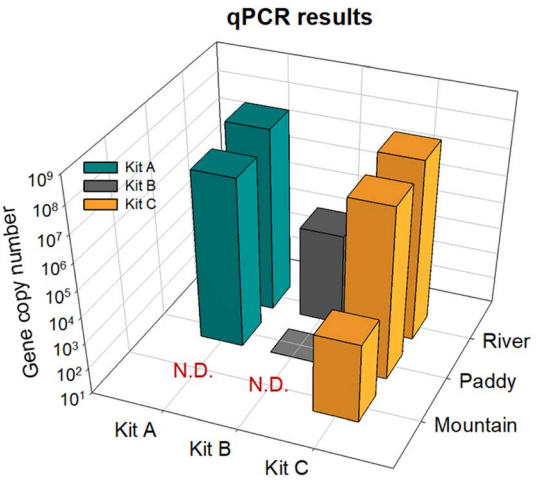


Fig. 2 Three-dimensional qPCR result plot of the extracted DNA template from each soil sample using different DNA extraction kits. Note that the results represent the average of triplicates and error bars are not shown for visual clarity.

The three-dimensional plot of Fig. 2 presents the qPCR result of the target gene (PAH-RHD$_\alpha$) of *P. putida*-seeded (all with the same amount of bacterial cells: 9.6 mL of OD 0.1) soil samples (*i.e.*, Mountain, Paddy, and River), which were extracted using three different DNA extraction kits. We observed a substantial discrepancy in the qPCR results depending on soil type with different organic matter content. Moreover, the discrepancy in gene quantification was pronounced depending on the type of template DNA extracted. The target gene in the DNA extracted by Kit A from Paddy and River was successfully quantified (2.5×10^7 and 7.9×10^7 gene copies, respectively) *via* qPCR, whereas that in Mountain was not detected (depicted as N.D. in Fig. 2). The gene in the extracted DNA from Mountain was also not determined (depicted as N.D. in Fig. 2) when it was treated by Kit B. Moreover, the target gene in the extracted DNA using Kit B from Paddy was close to N.D. (in the order of 10^{-1} gene copy number). Those from River were underestimated by two orders of magnitude compared with those of Kits A and C. In contrast, the target gene in the extracted DNA from the three soils treated with Kit C could be quantified by qPCR, even though Mountain presented a slight underestimation (three orders of magnitude). This observation suggests that substantial discrepancies were found in qPCR results depending on soil type and DNA extraction kit used. Further investigation is required to understand the influence of template DNA quality on qPCR results.

3.3. Effect of template DNA quality on qPCR performance

The DNA extraction kits selected here showed major differences in the inhibitor removal and washing steps (Table 1). Briefly, Kits A and C had additional inhibitor removal steps with chemical precipitation and physical separation using column filtration, respectively, whereas Kit B did not have any inhibitor elimination procedures after cell lysis. Moreover, Kit C had the most thorough washing steps (four), whereas Kit B had only one rinsing step for impurities in the template DNA. Therefore, Kit B was the fastest in terms of the time required for the entire procedure.

To elucidate the relationship between the discrepancy in qPCR results and type of DNA extraction (Fig. 2), it is important to understand the differences in DNA template qualities resulting from the selected DNA extraction kits. Therefore, we first examined DNA recovery, purity, and Mg^{2+} ion removal in bacterial cell-seeded soils using these kits (Fig. 3). The overall DNA concentrations were observed within the range of 7–24, 7–125, and 10–24 ng μL^{-1} for Kits A, B, and C, respectively (Fig. 3a). Kits A and C exhibited similar DNA yields in the Mountain (*t*-test, *P*-value > 0.770) and River (*t*-test, *P*-value > 0.734) samples, whereas Kit B presented an erroneously large variation in DNA extraction for all soil types (*t*-test, *P*-values << 0.001 when comparing Kit B with Kits A and C, indicating statistically significant differences). This result was consistent with previous studies that reported significant variations in DNA yield depending on the extraction kit used.[22–25]

To further evaluate the variation in the three kits, the purity of the extracted DNA was examined based on 260/280 and 260/230 ratios, which generally indicate the level of contamination of extracted DNA by protein and organic matter, respectively. According to the NanoDrop™ 2000 instructions and,[26] it is recommended to obtain the 260/280 ratio in a range similar to 1.8 and 260/230 ratio in a range of 2.0–2.2 in well-purified DNA samples. As shown in Fig. 3b, DNA templates were successfully purified from Mountain, Paddy, and River by Kits A and C while avoiding protein contamination (overall ratio of 1.9–2.0), whereas the extracted DNA by Kit B presented a slightly lower 260/280 ratio of 1.4–1.7 in the three soils. In comparison, the range of 260/230 ratios differed remarkably for each kit (Fig. 3c, ANOVA test, *P*-value << 0.001 for all soil type), with the lowest values for Kit B (<0.3). Although the DNA template extracted using Kit C exhibited higher 260/230 ratios (>0.9), all template DNA samples seemed to contain residual organic matter, resulting in low 260/230 ratios. These results indicate that all three kits struggled to remove organic matter in the soil samples to a level sufficient for qPCR, despite the common belief that they should have shown some level of bias among the kits.

The Mg^{2+} ion removal efficiency also varied according to the kit type (Fig. 3d), but we did not observe any notable differences between soil samples using the Kit A and C (ANOVA test, *P*-value = 0.258 and 0.991, respectively). This indicates that Mg^{2+} ion contents in the soil samples were within the removable ranges determined through the purification steps suggested by each kit. Kit B exhibited relatively lower and varied Mg^{2+} ion removal efficiency (ANOVA test, *P*-value << 0.001) with residual Mg^{2+} ion concentrations of ~5.6–6.1 mg L^{-1} in the extracted DNA from the three soils, whereas Kit C presented marginal residual Mg^{2+} ion concentrations of <1.1 mg L^{-1} in all samples.

This can be explained by comparing the kit protocols (Table 1). The variation in the purity results of the 260/230 ratio and Mg^{2+} ion removal may have occurred because of the purification differences. The absence of the inhibitor removal and simplified washing steps (*i.e.*, one step) in Kit B resulted in a large deviation in the DNA recovery and purification of bacterial

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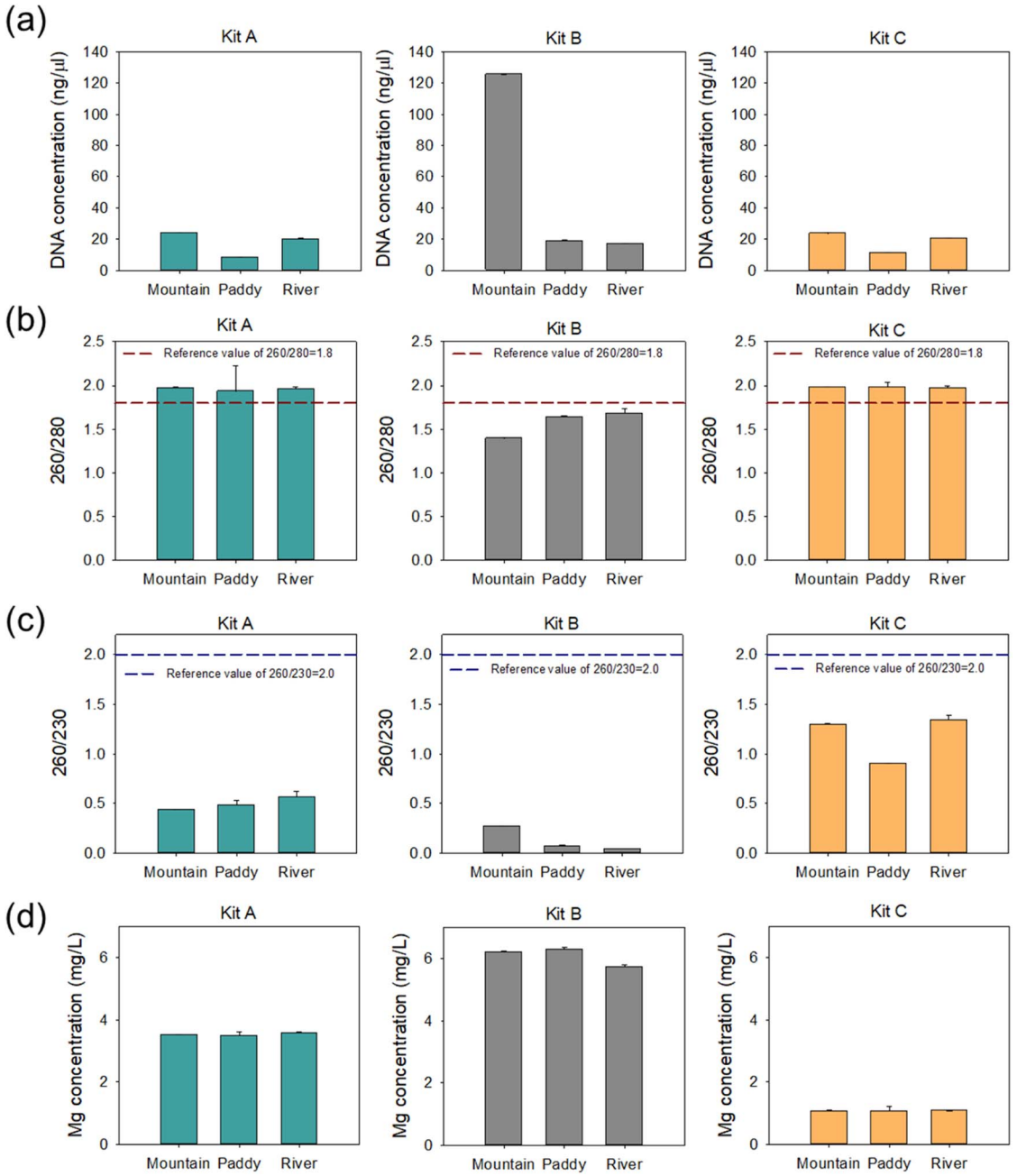


Fig. 3 (a) Concentration of extracted microbial DNA, (b) the 260/280, and (c) 260/230 ratios of the extracted DNA templates using different DNA extraction kits from each soil sample, and (d) residual Mg^{2+} ion concentrations in extracted DNA templates from soil samples using the three DNA extraction kits.

debris, organic matter, and Mg^{2+} ions in the soil matrix. In contrast, inhibitor column filtration and intensive purification steps (*i.e.*, four steps) in Kit C resulted in a more thoroughly purified DNA template from the soil samples. Referring to the qPCR results (Fig. 2), differences in the quality of the DNA template influenced by its extraction caused a considerable discrepancy in quantifying the target genes.

3.4. Investigation of Mg^{2+} ion inhibition in qPCR analysis

As mentioned earlier, residual organic matter in DNA templates acts as a predominant inhibitor during qPCR, and its interference behavior has often been investigated.[4,6,27] Here, the inhibition caused by organic matter in the soil was also dominant, resulting in a considerable discrepancy in gene quantification (Fig. 2). However, in more realistic scenarios, more than one inhibitor exists in soil samples. Therefore, we further investigated the discrepancy in coexisting inhibitors-effected qPCR results by employing Mg^{2+}, which is another well-known qPCR inhibitor easily found in soil samples.[8,21,28]

The Mg^{2+} ion causing qPCR inhibition was first determined by observing the amplification plots and Ct values constructed by using the PAH-RHD$_\alpha$ gene standards. Fig. 4a–c presents the

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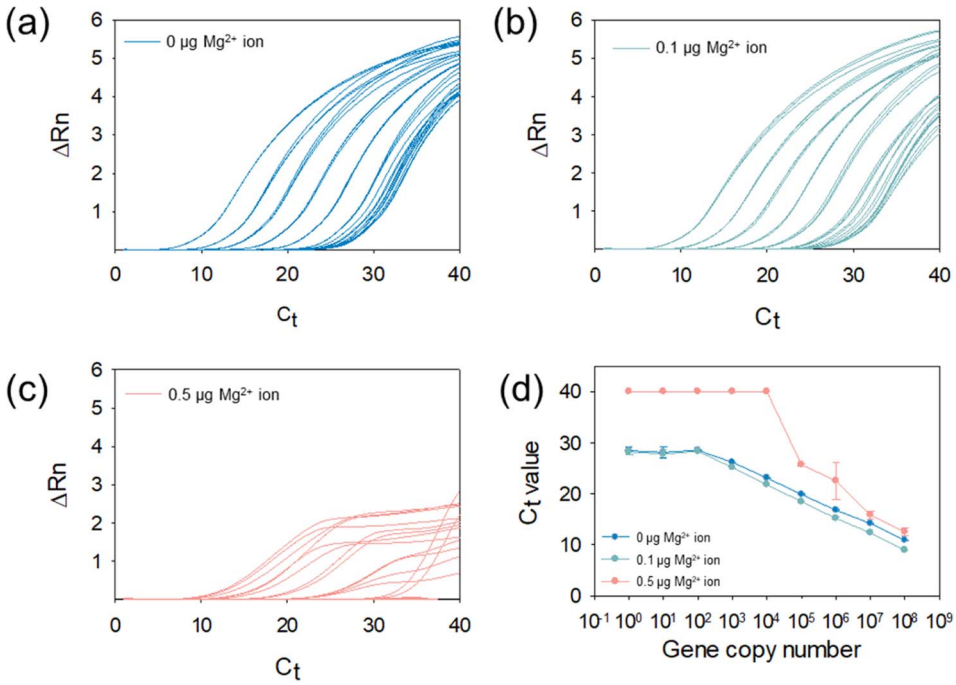


Fig. 4 Amplification curves of qPCR standard samples with (a) 0 μg Mg^{2+} ion (negative control), (b) 0.1 μg Mg^{2+} ion, and (c) 0.5 μg Mg^{2+} ion. (d) Ct values of qPCR with artificially added Mg^{2+} ions (0, 0.1, and 0.5 μg).

amplification plots of the PAH-RHD$_\alpha$ gene with varying amounts of Mg^{2+} (*i.e.*, 0, 0.1, and 0.5 μg Mg^{2+} per reaction, equivalent to 0, 5, and 10 mg L^{-1} Mg^{2+}, respectively) in the standard plasmid for qPCR. The signal-interfered amplification plot was observed in the presence of 0.5 μg of Mg^{2+} ions (Fig. 4c) in the plasmid template solution, exhibiting non-replicability and low ΔR_n. In comparison, the PAH-RHD$_\alpha$ gene was successfully amplified in the absence of Mg^{2+} ions (Fig. 4a) and presence of 0.1 μg of Mg^{2+} ions (Fig. 4b), and was well-replicated and had high ΔR_n values. Since the poor quality of amplification plots with low ΔR_n values refers to the occurrence of inhibition during the PCR reaction, this result infers that the gene quantification can be hindered by an excess amount of Mg^{2+} ions (*e.g.*, 0.5 μg Mg^{2+} ions per qPCR reaction solution).

We compared the Ct values from each amplification plot with the gene copy number (Fig. 4d). In the presence of 0.1 μg Mg^{2+} ions, the qPCR assay showed similar Ct values to that without Mg^{2+} ions across the entire gene copy number range assessed (from 10^0 to 10^8). This result also suggests the negligible inhibitory effects on the gene quantification caused by 0.1 μg of Mg^{2+} ions. However, in the presence of 0.5 μg of Mg^{2+} ions, the overall Ct values were higher than those with or without 0.1 μg of Mg^{2+} ions. Moreover, a higher Ct value was observed when the gene copy number was $<10^4$. Thus, the lower limit of detection became two orders of magnitude higher than that without Mg^{2+} ion inhibition when the qPCR reaction solution contained 0.5 μg of Mg^{2+} ions. Consequently, we confirmed that the inhibition was caused by an excessive amount of Mg^{2+} ions (0.5 μg of Mg^{2+} ions per reaction solution, equivalent to 20 mg L^{-1} Mg^{2+} ions in the solution) disrupting the calibration curve of the qPCR assay under the reaction conditions in this study, which was far less than the optimum Mg^{2+} ion concentration for PCR (*i.e.*, 2–5 mM in a reaction, equivalent to 49–122 mg L^{-1}).[29]

3.5. Multiple inhibition in qPCR analysis

The target gene quantification results using extracted DNA templates from the Mg^{2+} ion-spiked pure cell culture (0.288 mg Mg^{2+} in a sample, equivalent to 24 mg L^{-1} Mg^{2+}) and sandy soil (0.039 mg Mg^{2+} in a sample, equivalent to 78 mg kg^{-1} Mg^{2+}) are compared in Fig. 5. The target gene in the pure cell culture with high Mg^{2+} spiking was successfully quantified through DNA extraction using all three kits, even though the template extracted by Kit B presented a slight underestimation (~0.52 orders of magnitude compared to that of the other two kits; Fig. 5a).

The target gene in sandy soil with a high Mg^{2+} ion content was hardly quantified using the DNA extraction kits (Fig. 5b), even though the spiked amount of Mg^{2+} in sandy soil was 10 times lower than that in the pure cell sample. The DNA template prepared using Kit A exhibited a slightly lower gene copy number than the mean gene copy number obtained from the Mg^{2+} ion-spiked pure cell culture when extracted from the soil matrix. Moreover, the target gene in the DNA extracted from sandy soil using Kit B could not be detected by qPCR. However, the DNA extracted using Kit C only successfully demonstrated target gene quantification using the soil matrix, even with a high Mg^{2+} ion content, presenting a gene copy number similar to that of the Mg^{2+} ion-spiked pure cell culture. Although sandy soil has a simple composition without critical inhibitors, the matrix effect of the soil may notably affect the quality of the

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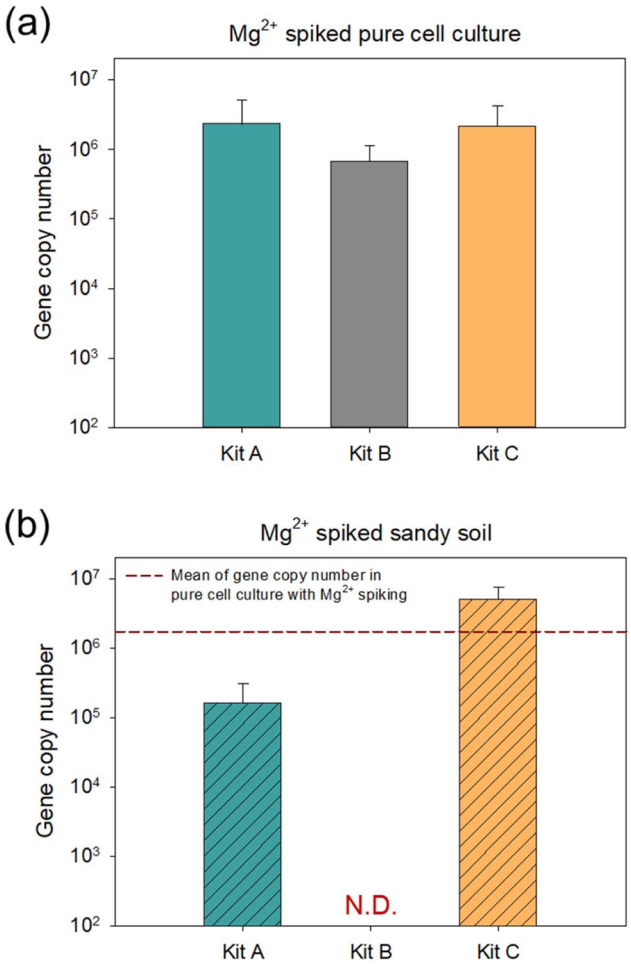


Fig. 5 Comparison of gene copy number of extracted DNA templates from Mg^{2+} ion-spiked (a) pure cells and (b) sandy soil sample using different DNA extraction kits.

extracted DNA templates. In sand containing less than 100 mg kg^{-1} of Mg^{2+} ion (equivalent to 0.1%) as a single inhibitor, a soil-specific DNA extraction kit was sufficient to obtain DNA acceptable quality for qPCR.[8] However, in actual sandy soil with a more complex composition, discrepancies in qPCR results appeared to be magnified depending on the type of DNA extraction kit used.

Overall, an apparent discrepancy in the qPCR results was observed when the sample matrix contained a high inhibitor content in soils compared to that of the pure cell culture. In addition, the different DNA template extraction procedures further exacerbated the inconsistency in the qPCR results of the soil samples, consistent with the results shown in Fig. 2 and 3.

An exacerbated discrepancy in target gene quantification was observed in environmental soil samples (*i.e.*, Mountain, Paddy, and River) with high Mg^{2+} ion-spiking. As shown in Fig. 6, the target gene in the DNA templates extracted from Paddy and River were successfully quantified despite the high Mg^{2+} ion concentration, which reached final levels of 350.47 and 365.52 mg kg^{-1}, respectively, due to Mg^{2+} addition (Fig. S6†). However, in Mountain with Mg^{2+} spiking, the target gene was

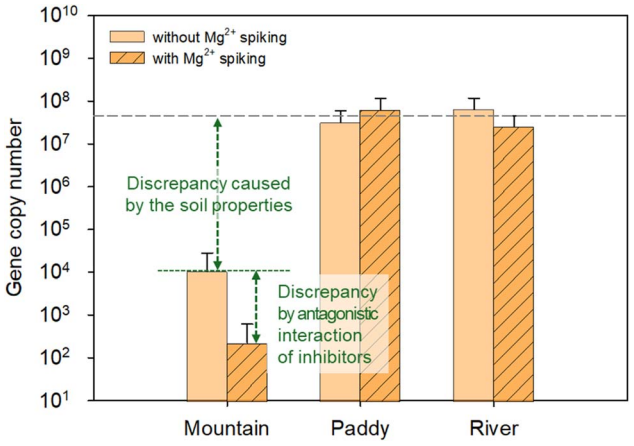


Fig. 6 Comparison of the gene copy number of the extracted DNA template by Kit C from soil samples with and without Mg^{2+} ion-spiking. Note that the qPCR results from the soils without Mg^{2+} ion-spiking are replicated in Fig. 2.

quantified at a 2.3×10^2 gene copy number, which is reduced by two orders of magnitude compared to that in Mountain without Mg^{2+} ion-spiking (Fig. 2). This reduction may have resulted from an antagonistic interaction between the high organic matter content of Mountain and elevated Mg^{2+} ion concentration, which reached a final level of 216.12 mg kg^{-1} due to spiking (Fig. S6†).

These results suggest that discrepancies in gene quantification are evident in the presence of qPCR inhibitors. Furthermore, discrepancies in the quantification results are exacerbated by the DNA template quality, which is attributed to DNA extraction. The possibility of multiple inhibitory effects caused by soil properties and DNA extraction kits was also observed.

This study highlights the importance of selecting appropriate DNA extraction kits based on soil characteristics. For fine-textured soils with high organic content, kits equipped with effective metal ion inhibitor removal steps such as chelation are recommended. In cases where soils have relatively low or unpredictable levels of inhibitors, kits that offer a balanced inhibitor removal and DNA yield, along with versatile extraction protocols, are preferable to ensure reliable qPCR results. If significant inhibition persists despite using a high-performance kit, further dilution or post-purification of the DNA template should be considered to improve qPCR performance.

4. Conclusion

Here, we observed a substantial discrepancy in qPCR results in varying soil types, as well as in the quality of DNA templates, which is mainly determined by DNA extraction kits. Gene quantification by qPCR was performed using *P. putida* cell-seeded soil samples with different properties such as high organic content (Mountain), medium organic with high Mg^{2+} ion content (Paddy), and low organic with high Mg^{2+} ion content (River). The qPCR results were inconsistent depending on soil type, indicating hindrance caused by matrix effects and

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inhibitors in the soils. Moreover, the discrepancy caused by the quality of the DNA template was more evident. We also found that target gene quantification was challenging, mainly due to the organic matter in all three soils, which may not have been completely removed by the extraction kits. Subsequently, interrelated inhibition during gene quantification was investigated by spiking Mg^{2+} ions at a high concentration (78 mg kg^{-1} Mg^{2+}) to hinder qPCR. The discrepancy in gene quantification was exacerbated when the organic-rich soil (Mountain) contained Mg^{2+} ions, even with the most thorough DNA extraction (Kit C). This finding highlights the need to carefully consider the relationship between inhibitors and DNA template quality to ensure accurate application of qPCR in environmental soil analyses.

Data availability

Data are available upon request from the authors.

Author contributions

A. S. and E. L. performed the experimental design. E. L. performed the experimental setup and measurements. A. S., H. L., and E. L. analyzed the data. E. L. and H. L. drafted the manuscript. A. S. and H. L. reviewed and revised the manuscript. All authors have approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced this study.

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