Synthetic oligonucleotides as quantitative PCR standards for quantifying microbial genes

Xingguo Han[1], Karin Beck[2], Helmut Burgmann[3], Beat Frey[4], Beat Stierli[1], and Aline Frossard[1]

[1]Swiss Federal Institute for Forest Snow and Landscape Research WSL
[2]Eawag Swiss Federal Institute of Aquatic Science and Technology
[3]Eawag
[4]Swiss Federal Institute for Forest Snow and Landscape Research

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Abstract

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Xingguo Han[1,*],

1. Forest Soils and Biogeochemistry, Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Birmensdorf, Switzerland

2. Eawag, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland

* For correspondence: xingguo.han@wsl.ch; aline.frossard@wsl.ch

Forest Soils and Biogeochemistry, Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Zürcherstrasse 111, 8903 Birmensdorf, Switzerland

Tel: +41 44 739 28 07

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Key words : Synthetic DNA, Plasmid, Real-time quantitative PCR (qPCR), qPCR standards; Microbial and functional gene abundances

1. Introduction

Soil microorganisms are a critical component of the Earth system by contributing significantly to global elemental cycles through a complex network of biogeochemical reactions (Schimel & Schaeffer, 2012). In many ecosystems, microorganisms gain energy for growth and survival through breaking down organic matter (OM), using carbon (C) and nitrogen (N) to build up biomass and releasing the greenhouse gases carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) to the atmosphere (Canfield et al., 2005; Hutchins & Capone, 2022; Oertel et al., 2016). Therefore, quantifying microbial abundance (as a proxy for biomass) is crucial to assess the importance of the microorganisms and understand their role or functions in ecosystems.

Over the past few decades, numerous techniques have been employed to quantify the population size of specific microorganisms or groups of microorganisms in environmental samples or synthetic communities in microbial ecology. These include, but are not limited to, direct epifluorescence microscopy (EFM) (Caron, 1983; Kepner & Pratt, 1994), flow cytometry (FCM) (Deng et al., 2019; Frossard et al., 2012; Frossard et al., 2016), fluorescence in situ hybridization (FISH) (Bouvier & del Giorgio, 2003), catalyzed reporter deposition-FISH (CARD-FISH, (Eickhorst & Tippkotter, 2008; Schippers et al., 2005), phospholipid quantification (Phospholipid-derived fatty acids, PLFAs) (Frostegard et al., 1991; White et al., 1979), and real-time quantitative polymerase chain reaction (qPCR) (Brankatschk et al., 2012; Han et al., 2020; Han et al., 2016; Hartmann et al., 2014; Smith & Osborn, 2009).

Among these approaches, qPCR has been widely used in molecular biology, as this method has proved to be relatively cheap, straightforward and efficient with a high sensitivity, covering a linear range over 7-8 orders of magnitude, and high throughput. qPCR relies on optical reporter systems, either using a double-stranded DNA-binding fluorescent dye such as SYBR® Green or DNA probes dual-labeled with reporter dyes and quenchers, such as TaqMan™probes (Arya et al., 2005; Orlando et al., 1998; VanGuilder et al., 2008). Alongside measuring the abundance of the bacterial, archaeal and fungal communities (using general bacterial, archaeal or universal primers for the 16S rRNA gene (Takai & Horikoshi, 2000) or of the ITS region for fungi (Fierer et al., 2005)), qPCR has been applied for detecting and quantifying copy numbers of microbial functional genes involved in C and N cycling. Among the functions frequently studied in diverse environments using qPCR are CH_4 production (methyl coenzyme M reductase A:*mcr* A) and oxidation (particulate methane monooxygenase:*pmo* A), nitrogen fixation (nitrogenase: *nif* H), ammonia oxidation (archaeal and bacterial ammonia monooxygenase: *amo* A), nitrite reduction (nitrite reductase: *nir* S and *nir* K), nitrite oxidation (beta subunit of nitrite oxidoreductase: *nxr* B), N_2O production (nitric oxide reductase: *nor* B) and reduction (nitrous oxide reductase: *nos* Z), and organic phosphorus hydrolysis (alkaline phosphatase D: *pho* D) (Church et al., 2005; Han et al., 2020; Han et al., 2016; Henry et al., 2006; Leininger et al., 2006; Luo et al., 2017; Perez-Mon et al., 2022).

In spite of the advantage of being a straightforward method not including too many steps, qPCR has a major drawback. To quantify a specific gene, qPCR assays require the corresponding standard for calibration under the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Classically, standards have been produced by cloning a target sequence into a plasmid, amplifying genes via PCR, using genomic DNA directly, or acquiring commercially approved

biological standards (Dhanasekaran et al., 2010; Goodwin et al., 2018). However, these approaches often incur significant costs, in terms of time and money, and potentially generate contaminations, particularly when preparing multiple plasmid standards targeting different microbial genes in parallel. For instance, both PCR amplicons and plasmids need be purified before being used, procedure which is often causing contaminations (Cimino et al., 1991). Moreover, the quantification of plasmid copies per cell was shown to be unreliable (Conte et al., 2018; May et al., 2015). In recent years, there has been a growing interest to use artificially synthesized DNA and RNA sequences as qPCR standards. Synthesizing such sequences to produce standards is considerably faster, cleaner (low contamination risk) and also less expensive (following considerable reduction of the cost of custom DNA synthesis in recent years) compared to traditional plasmid standards. The synthetic gene fragments can be purchased in a length of 125 to 3000 base pair (bp) with none degenerate nucleotides of A, T, C and G (Conte et al., 2018; May et al., 2015). Up to now, most of the artificially synthesized standards have been used for medical purpose, focusing on viral or infectious microorganisms (Bandeira et al., 2020; Bivins et al., 2021; Fesolovich & Tobe, 2017; Lima et al., 2017; Magee et al., 2017; Munoz-Calderon et al., 2021; Tourinho et al., 2015), very few in environmental samples. The few studies using synthesized gene fragments as qPCR standards assessed bacterial 16S rRNA genes in hydrocarbon-contaminated soils (Gunawardana et al., 2014), 16S rRNA genes and *mcr* A in a biogas digester (May et al., 2015), and antibiotic resistance genes in environmental water, soil and faeces samples (Xu et al., 2019), and none in microbial ecology. We propose that, given the advantages, synthetic qPCR standards can and should be widely adopted for qPCR analysis of functional genes in environmental microbiology and microbial ecology. However, this new methodological approach should be thoroughly evaluated and compared to previous practice before being adopted.

Here, we designed qPCR standards for a number of frequently studied functional genes of the C, N and P cycle, and the ITS region and the 16S rRNA gene by synthesizing double-stranded DNA fragments obtained by generation of consensus sequences from alignments of microbial gene sequences. To provide a thorough evaluation of the effectiveness and reliability of synthetic DNA fragments as qPCR standards, we compared these newly synthesized qPCR standards with standards produced via plasmids in different qPCR assays, targeting several different taxonomic and functional genes of soil microorganisms.

2. Materials and Methods

2.1. Production of qPCR standards

Synthetic standards were designed for qPCR assays targeting bacterial 16S rRNA genes, fungal ITS and a broad range of genes involved in C and N cycling, *mcr* A,*pmo* A, *nif* H, *nos* Z, *amo* A, *nir* S,*nir* K, *nxr* B, *nor* B and *pho* D genes (Table 1). Synthetic DNA fragments were designed by aligning between 10 and 20 gene sequences per targeted gene with the software Geneious (version 9.1.8). The gene sequences were downloaded from the National Center for Biotechnology Information (NCBI). For each targeted gene, a consensus sequence was obtained from at least 10 downloaded sequences. The consensus sequences were created with most frequent nucleotide for each base of the aligned sequences, therefore not including degenerate nucleotides of A, T, C and G. Forward and reverse primer sequences were included in the consensus sequence to target the interest gene. The consensus sequences of synthetic DNA fragments for each gene are available in Supplementary Table S1. Double-stranded synthetic DNA fragments (between 250 and 650 bp) were then ordered from gBlocks gene fragments (Integrated DNA Technologies: IDT, Inc.), with 500 ng dry DNA in a tube for each target. Upon reception, the DNA was resuspended in nuclease-free water (H_2O) and stored at -20 degC freezer for long-term use. The copy numbers of synthetic DNA per microliter were calculated using the formula according to Godornes et al. (2007):

$$\frac{\text{γενε ςοπιες}}{\mu\Lambda} = \frac{\Delta\text{NA ςονςεντρατιον } \left(\frac{\nu\gamma}{\mu\Lambda}\right) \times 10^{-9} \times 6.022 \ \times 10^{23}}{}$$

english(fragment size bp) $\times$ 660 g/mol

where the Avogadro number is 6.022×10^{23}(molecules/mole), the fragment size is the length of the synthesized DNA (bp), and 660 is the average weight of a single DNA base pair (g/mol).

For several targeted genes, qPCR standards were also produced via plasmids. PCR products of the particular gene (bacterial 16S rRNA gene, fungal ITS region, *mcr* A, *pmo* A, *nif* H and*nos* Z) were cloned into the vector and competent cells using the pGEM-T Easy Vector System II Systems Kit according to the manufacturer's instructions (Promega, Madison, WI, USA) (Frey et al., 2011; Henry et al., 2006). Briefly, PCR reactions were conducted to amplify the targeted gene from DNA extracted from soils. PCR products were then inserted to the Vector (Ligation), which was added to the *E. coli* JM109 competent cells (Transformation). Transformed *E. coli* were then spread on Luria-Bertani (LB) agar plates with appropriate antibiotics. After 16-24 hours of incubation at 37 °C, colonies were observed on the plates, and only white colonies were picked up and incubated in liquid LB medium to grow with shaking over 24 h. The plasmids were extracted by Plasmid Miniprep Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The plasmids were further verified by Sanger sequencing: Colony PCR products of the selected marker genes were sequenced on both strands (up to 960 bp), according to Frey et al. (2008). Cycle sequencing was carried out using the Big Dye-Terminator Cycle Sequencing Kit, version 1.3 (PE Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The copy numbers of plasmid DNA per microliter were calculated using the following formula:

$$\frac{\text{γενε ςοπιες}}{\mu\Lambda} = \frac{\Delta\text{NA ςονςεντρατιον } \left(\frac{\nu\gamma}{\mu\Lambda}\right) \times 10^{-9} \times 6.022 \times 10^{23}}{}$$

english(3015 $bp + amplicon\ size\ bp$) $\times$ 660 g/mol

where the length of the pGEM-T Easy vector is 3015 bp.

Standards produced by synthetic DNA and plasmids were directly compared by qPCR (top bold six genes in Table 1). Synthetic DNA fragments for microbial functional genes *amo* A, *nir* S, *nir* K,*nxr* B, *nor* B and *pho* D, were not compared with homologous standards produced via cloning, but were tested and verified by qPCR assays.

2.2. qPCR of standards and DNA extracted from soils

The effectiveness of the standard fragments produced via synthetic DNA and plasmids clones were tested and compared in qPCR assays for different genes with soil DNA on a QuantStudio5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) by SYBR Green. qPCR reactions (10 µL) were composed of 5 µL GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), 0.1 µL 30 mg mL^{-1} bovine serum albumin (BSA), 0.5 µL 10 µM of each primer, 1.9 µL molecular-grade water and 2 µL DNA template. Soil DNA was diluted at a concentration of ˜2 ng/µL to avoid potential PCR inhibition. The different primers used in the reactions are shown in Table 1 and details on qPCR thermocycling conditions are described in Table 2 and Supplementary Table S2. Three standard dilution series per target gene (for both synthetic DNA standard and plasmid DNA standard) were obtained from 10-fold serial dilutions of standards with molecular-grade H_2O. The standard series ranged ranging from 10^1 to 10^8 copies per µL.

DNA was extracted from soils collected along an altitudinal gradient in Switzerland in August 2021 (soil samples from 530, 1159, 1844, 2336 and 2715 m.a.s.l, Supplementary Table S3). The soils varied in pH, total organic carbon, C and N content (Supplementary Table S3). Total DNA was extracted with the DNeasy

Powersoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified with PicoGreen (ThermoFisher Scientific, Cleveland, OH, USA), and subsequently diluted to 2.0 ng per μL by molecular-grade H_2O and measured in triplicate.

A standard curve for each gene was generated by plotting cycle threshold (Ct) or quantification (Cq) of cycle values of each dilution step against the corresponding log10 transformed number of gene copies in the standard. The amplification efficiency (E) was estimated using the slope of the standard curve with the formula: $E = (10^{-1}/\text{slope}) - 1$. The detection limit was 10 copies per μL according to the lowest concentration standard (10^1 copies per μL).

3. Results and Discussion

3.1. Performance of synthetic and plasmid DNA standards

For the six targeted genes which were tested with synthetic DNA and plasmid DNA standards (Table 1), both standards for qPCR quantifications yielded significantly ($P < 0.001$) linear calibration curves featuring a coefficient value (R^2) of > 0.99 (Fig. 1), together with similar high R^2 derived from the genes tested with synthetic DNA standards (Supplementary Fig. S1). The dilution series of both synthetic DNA and plasmid DNA standards exhibited smooth and exponential amplification curves (Fig. 2). Coefficients of variation of Cq values among the replicates for the standards in the range from 10^1 to 10^8 copies per μl were between 0.03% and 4.15%, which indicated good repeatability and reproducibility. Additionally, the slopes of the synthetic standards were similar to those of the plasmid standards, with only minor differences (Fig. 1). These, all together, provided compelling evidence that, similar to traditional plasmid standards, synthetic DNA standards of serial dilutions can be amplified effectively and produce high-quality and consistent standard curves. Comparable standard curves between synthetic DNA standards and traditional standards (PCR amplicons and plasmids of cloning) have been reported in previous studies targeting human mitochondrial gene (Conte et al., 2018), antibiotic resistance gene (Xu et al., 2019), human T-cell leukaemia virus type 1 (HTLV-1) (Bandeira et al., 2020) and HBV virus (Portilho et al., 2018). Yet, to the best of our knowledge, this is the first study to use synthetically designed and produced DNA fragments as qPCR standards targeting a broad range of genes involved in C and N cycling employed in microbial ecology.

Standard curves from both synthetic and plasmid standards showed high and similar amplification efficiency (E) values, confirming the reliability of synthetic gene fragments as qPCR standards (Fig. 1 and 2). PCR efficiency of the standard curves for 16S rRNA gene reached 0.84 for synthetic DNA and 0.96 for plasmid standards. It attained 0.98 and 0.90 for fungal ITS region for the synthetic and plasmid standard respectively. Efficiency values of the remaining four genes tested (*mcr* A, *pmo* A, *nif* H and *nos* Z), were all lower than 0.90, irrespective of plasmid or synthetic standards (Fig. 1). Ideally, an efficiency value over 0.90 is considered a well amplified standard and a qualified standard curve (Svec et al., 2015). However, often times due to the potential PCR inhibition, such as self-inhibition, polymerase and protein inhibition, primer specificity and contamination, E values can be as low as 0.70 (Luby et al., 2016; Xu et al., 2019). The slightly lower E value for synthetic 16S rRNA gene standard during PCR might be caused by a small peak (PCR byproduct) right before the main PCR product peak of 16S rRNA gene, especially the least diluted ones, implied by the melting curves (Supplementary Fig. S2A), which caused the differences in the standard curves from synthetic and plasmid standards. Lower E values of the genes *mcr* A, *pmo* A, *nif* H and *nos* Z from both synthetic and plasmid standards were likely also related to PCR inhibitions, in particular to the less diluted standards. Usually, 3.3 (a slope of – 3.3) cycles apart of the 10-fold dilutions were considered as an indicator of 100% PCR efficiency (Svec et al., 2015)). However, much higher Cq value differences between the dilution series were found for these four genes from both standards (Fig. 1), indicating an inhibition effect. Additionally, different instruments and volume for standard dilution also made huge differences in PCR efficiency, which turned out a larger volume transferred during dilution (10 μL) could increase the efficiency (Svec et al., 2015), while 2 μL was used in this study.

In spite of the similarity of qPCR standard curves between synthetic and plasmid standards, there were slight differences in the slopes and E values of standard curves between these two standards (Fig. 1). For *mcr* A, *pmo* A, *nif* H and *nos* Z, standard curves from synthetic standards were always steeper (higher absolute slopes) than those from plasmid standards, with Cq values of the least diluted standards from synthetic standards lower than those of the least diluted plasmid standards, even when the copy numbers of the least diluted standards from synthetic standards were lower than those from plasmid standards for *mcr* A and *pmo* A (Supplementary Table S4). This indicated a self-inhibition of the least diluted plasmid standards, which took more cycles (higher Cq values) to get fully amplified. For ITS region, standard curve from plasmid standard was slightly steeper than that from synthetic standard, which also indicated an effect of inhibition. In addition to self-inhibition, there might be also a conformation effect of non-linear plasmid standards, which could also impact PCR efficiency as well (Hou et al., 2010).

3.2. Microbial gene abundances in soils based on synthetic and plasmid DNA standards

In order to validate the reliability of our synthetic DNA standards, the abundances of the tested genes were quantified in eDNA extracts from soils by qPCR assays and the gene copy abundance calculated using standards from synthetic and plasmid DNA (Fig. 3). No amplification was observed in negative control reactions, confirming the absence of contamination during the reaction preparation steps. Overall, gene copies calculated with either standard curves (i.e. from synthetic or plasmid DNA) were not significantly different for all the genes studied across all soil samples except for few assays (marked with asterisks in Supplementary Fig. S3). These significant differences reflect the described differences of the standards curves thus that gene copy numbers calculated from synthetic standards were on average lower than those from plasmid standards (difference of 5.1±4.4% for *mcr* A, 15.4±1.2% for *pmo* A, 23.8±4.1% for *nif* H and 6.9±6.2% for*nos* Z) or higher for 16S rRNA gene (17.7±3.2%) and ITS region (41.6±16.4% higher). Gene copies varying within one log (10 times) were widely reported for qPCR quantifications of viruses with synthetic and plasmid standards, and such results have been considered good agreement of the two methods (Bandeira et al., 2020; Lima et al., 2017; Portilho et al., 2018; Tourinho et al., 2015).

Despite the described deviation, correlation of gene copies in the soil samples using the synthetic standard and copies by using plasmid standard, was significant with a squared coefficient (R^2) of over 0.99 (Linear correlations, all significant $P < 0.001$) for all the six tested genes (Fig. 3), which showed highly identical results with both standards. Similarly, high R^2 (0.83) based on Linear correlations were also observed in a study on human virus by qPCR when comparing synthetic DNA and plasmid DNA standards (Bandeira et al., 2020). Furthermore, when comparing the relative differences in gene copy numbers to the highest observed soil value within each gene, there were no any significant differences in the relative differences across all sites of all the six tested genes between synthetic and plasmid standards (Supplementary Fig. S4).

Therefore, considering the sensitivity and efficiency of qPCR, inhibition and anthropogenic interference (i.e. pipetting errors), differences in copy numbers within 50% variation in this study are very much acceptable, especially with gene concentrations reaching up to more than 10^{10} copies per dry gram soil. All taken together, our results demonstrated that the synthetic DNA standards are reliable for qPCR quantification of various taxonomic and functional genes in soils.

4. Conclusion

In this study, we designed qPCR standards for quantifying various taxonomic and functional genes used in microbial ecology, such as those involved in C and N cycling, by synthesizing double-stranded DNA sequences as gBlocks gene fragments. We show that synthetic DNA standards performed equally well as traditional plasmid standards in producing linear qPCR calibration curves, yielding precise and efficient results for a broad range of soils. The application of synthetic DNA standards for qPCR assays is however not limited to

soils, but can be recommended for all kind of genes from a large variety of environments, such as water, air and sediments, whenever qPCR is needed for gene quantification.

Author contributions

A.F. and X.H. designed the study. B.S., B.F., K.B. and H.B. provided standards from plasmid DNA. X.H. designed the synthetic DNA standards, conducted the experimental work, analyzed the data and wrote the manuscript with the input from all coauthors. The final version has been approved by all co-authors.

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Conflict of interest statement

The authors declare no competing interests.

Data availability statement

Supporting figures and tables are provided in the Supplementary Material. Raw data used in this study are available in DRYAD: https://doi.org/10.5061/dryad.9w0vt4bms.

Benefit-sharing statement

This study provides a new approach of applying synthetic DNA standards as qPCR standards for quantifying microbial genes, which will be of high interest to people working in diverse fields, such as microbial ecology, molecular ecology, microbiology, diagnostics, immunology, food science, etc.

ORCID

Dr. Xingguo Han: https://orcid.org/0000-0002-5753-5255

Dr. Helmut Bürgmann: https://orcid.org/0000-0002-5651-5906

Dr. Beat Frey: https://orcid.org/0000-0002-6391-3574

Dr. Aline Frossard: https://orcid.org/0000-0003-1699-6220

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Tables and Figures

Table 1. Primers for the quantification of total abundance of bacterial 16S rRNA genes and fungal ITS2 region, *mcr* A (methyl coenzyme M reductase A: methanogens), *pmo* A (particulate methane monooxygenase: methanotrophs), *nif* H (nitrogenase: N fixers),*nos* Z (nitrous oxide reductase: N_2O reducers), archaeal *amo* A and bacterial *amo* A (archaeal and bacterial ammonia monooxygenase: ammonia oxidizers), *nir* S and *nir* K (nitrite reductase: nitrite reducers), *nxr* B (beta subunit of nitrite oxidoreductase: nitrite oxidizers), *nor* B (nitric oxide reductase: N_2O producers) and *pho* D (alkaline phosphatase D: organic phosphorus hydrolyzers) by qPCR. The top six genes (bold fonts) were compared with both synthetic DNA standard and plasmid DNA standard.

Target gene	Primers	Primer Sequence (5'-3')	Amplicon size (base pair, bp)	Primers Reference
Bacterial 16S rRNA gene	**349F**	**AGG CAG CAG TDR GGA AT**	**˜460**	(Takai & Horikoshi, 2000)
	806R	**GGA CTA CYV GGG TAT CTA AT**		
ITS	**ITS3**	**CAH CGA TGA AGA ACG YRG**	**˜430**	(Frey et al., 2016; Tedersoo et al., 2014)
	ITS4	**TCC TSC GCT TAT TGA TAT GC**		
*mcr***A**	**ML-F(mcrA)32**	**GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC**	**˜476**	(Luton et al., 2002)
	ML-R(mcrA)23	**TTC ATT GCR TAG TTW GGR TAG TT**		
*pmo***A**	**A189F**	**GGN GAC TGG GAC TTC TGG**	**˜532**	(Holmes et al., 1995)
	A682r	**GAA SGC NGA GAA GAA SGC**		
*nif***H**	**PolF_115**	**TGC GAY CCS AAR GCB GAC TC**	**˜362**	(Poly et al., 2001)
	PolR_457	**ATS GCC ATC ATY TCR CCG GA**		
*nos***Z**	**nosZ-1F**	**WCS YTG TTC MTC GAC AGC CAG**	**˜249**	(Henry et al., 2006)

Target gene	Primers	Primer Sequence (5'-3')	Amplicon size (base pair, bp)	Primers Reference
	nosZ_1R	**ATG TCG ATC ARC TGV KCR TTY TC**		
archaeal *amo*A	Arch-amoAF	CTG AYT GGG CYT GGA CAT C	~635	(Francis et al., 2005)
	Arch-amoAR	TTC TTC TTT GTT GCC CAG TA		
bacterial *amo*A	amoA-1F	GGG GHT TYT ACT GGT GGT	~491	(Rotthauwe et al., 1997)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC		
nirS	cd3AF	GTS AAC GTS AAG GAR ACS GG	~425	(Throback et al., 2004)
	R3cd	GAS TTC GGR TGS GTC TTG A		
nirK	nirK_F1aCu	ATC ATG GTS CTG CCG CG	~473	(Hallin & Lindgren, 1999)
	nirK_R3Cu	GCC TCG ATC AGR TTG TGG TT		
nxrB	nxrB169f	TAC ATG TGG TGG AAC A	~485	(Pester et al., 2014)
	nxrB638r	CGG TTC TGG TCR ATC A		
*nor*B	qnorB2F	GGN CAY CAR GGN TAY GA	~260	(Braker & Tiedje, 2003)
	qnorB5R	ACC CAN AGR TGN ACN ACC CAC CA		
*pho*D	phoD-F733	TGG GAY GAT CAY GAR GT	~363	(Ragot et al., 2015)
	phoD-R1083	CTG SGC SAK SAC RTT CCA		

Table 2. qPCR thermocycling conditions for the quantification of each target gene.

	16S rRNA gene	**ITS**	*mcr***A**	*pmo***A**	*nif***H**	*nos***Z**	***amo*A** archaeal	***amo*A** bacterial	*nir***S**	*nir***K**	*nxr***B**	*nor***B**	*pho***I**
Stage: denaturation	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 min at 95 ºC	2 mi at 95 ºC
Stage 2: annealing temperature	53 ºC	58 ºC	50 ºC	56 ºC	55 ºC	55 ºC	53 ºC	55 ºC	58 ºC	57 ºC	57 ºC	57 ºC	58 ºC
Stage 3: melting temperature	60 ºC	60 ºC	50 ºC	60 ºC	50 ºC	50 ºC	60 ºC	60 ºC	60 ºC	60 ºC	60 ºC	55 ºC	60 ºC
Stage 4: cooling	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC

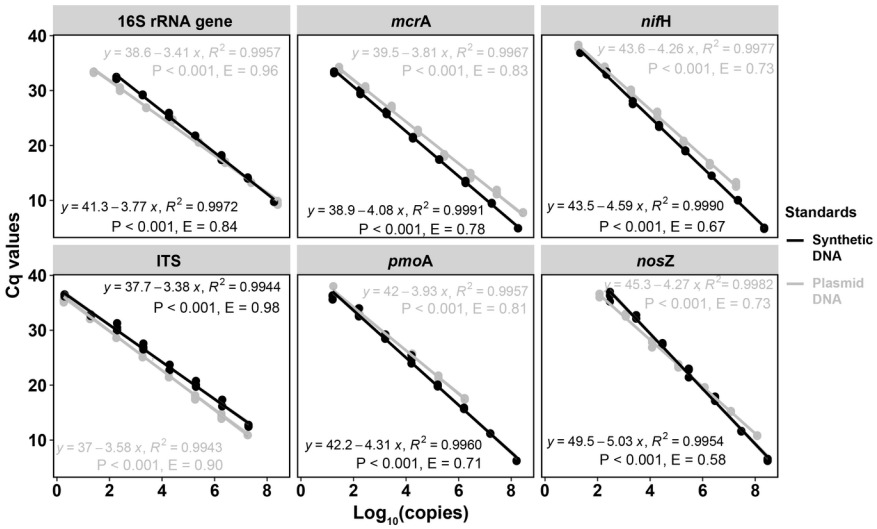

Fig. 1. Comparisons of qPCR standard curves between synthetic DNA and plasmid DNA standards. Standards were diluted by 10 times for each step from 10^8 to 10^1copies per µl. R^2 is the coefficient of determination. $P < 0.001$ indicates the significance of the linear regression. Gene copy numbers (copies per dry gram soil) were log10-transformed. E = (10-1/slope) – 1.

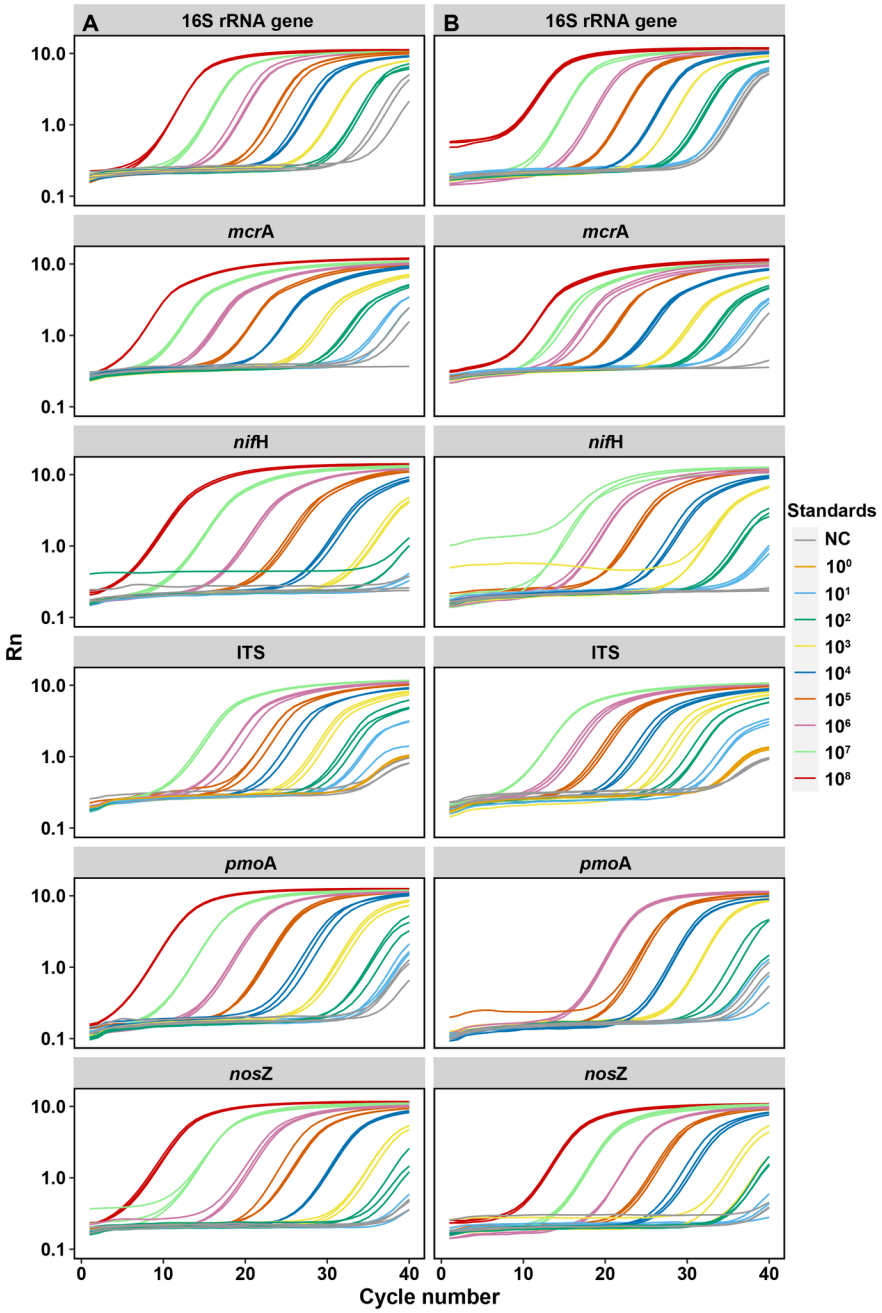

Fig. 2. Amplification curves of each target gene by serial dilutions between synthetic DNA (column A) and plasmid DNA (column B) standards. Standards were diluted by 10 times for each step from 10^8 to 10^1 copies per µl. NC: negative control. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye; i.e., Rn is the reporter signal normalized to the fluorescence signal of Applied Biosystems ROX Dye.

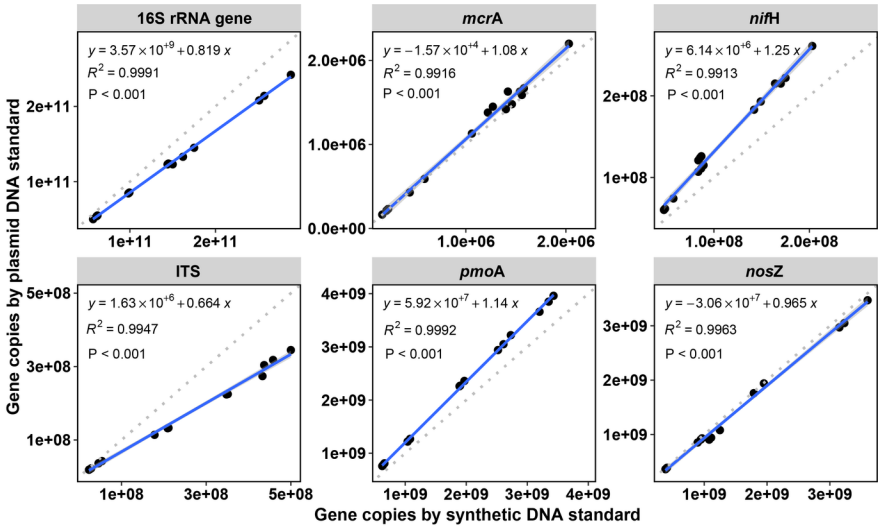

Fig. 3. Linear correlations between copy numbers in soil samples (copies per dry gram soil) of the tested genes by qPCR between synthetic DNA and plasmid DNA standards. R^2 is coefficient of determination. $P < 0.001$ indicates the significance of the linear regression. The grey dashed lines are 1:1 reference lines.