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# Microfluidic qPCR Enables High Throughput Quantification of Microbial Functional Genes but Requires Strict Curation of Primers

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Quantification of microbial functional genes enhances predictions of soil biogeochemical process rates, but reliance on low-throughput quantitative PCR (qPCR) limits the scope of ecological studies to a handful of targets. Here, we explore whether microfluidic qPCR (MFQPCR) is a viable high-throughput alternative for functional gene quantification, by evaluating the efficiency, specificity and sensitivity of 29 established and 12 newly designed primer pairs targeting taxonomic, nitrogen-cycling, and hydrocarbon degradation genes in genomic DNA soil extracts, under three different sets of MFQPCR assay conditions. Without curation, commonly-used qPCR primer pairs yielded an extreme range of reaction efficiencies (25.9–100.1%), but when conditions were optimized, MFQPCR produced copy-number estimates comparable to traditional qPCR. To guide microbial soil ecologists considering adoption of MFQPCR, we present suggestions for primer selection, including omission of inosines, degeneracy scores of $< 9$, amplicon sizes of $\leq 211$ bp, and GC content of 32–61%. We conclude that, while the nanoliter reaction volumes, rapid thermocycling and one-size-fits-all reaction conditions of MFQPCR necessitates more stringent primer selection criteria than is commonly applied in soil microbial ecology, the ability to quantify up to 96 targets in 96 samples makes MFQPCR a valuable tool for monitoring shifts in functional community abundances. MFQPCR will particularly suit studies targeting multiple clade-specific functional genes, or when primer design is informed by previous knowledge of the environment.

**Keywords: microfluidic qPCR, quantitative PCR, functional genes, nitrogen cycle, hydrocarbon degradation, microbial community, terrestrial ecology, biogeochemical cycles**

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## INTRODUCTION

Soil microbial communities perform a dazzling array of ecosystem services, from fixation of atmospheric nitrogen and the release of nutrients from rocks (Landeweert et al., 2001; Levy-Booth et al., 2014), to the degradation of organic pollutants and pesticides (Molina et al., 2009; Kumar et al., 2016). Advances in genomic technologies are rapidly expanding our knowledge of the genetic and taxonomic diversity of microbial communities, unearthing new biogeochemical pathways and

revealing whole clades of undescribed bacteria, at a pace that quantification technologies have struggled to keep up with (Jones et al., 2013; Hu et al., 2015; Widder et al., 2016; Kuypers et al., 2018). While high-throughput sequencing has become the mainstay of many microbial ecology and ecotoxicology laboratories, low-throughput quantitative PCR (qPCR) remains the method of choice for accurate quantification of total cell numbers, individual species or functional genes. In soil the quantification of microbial functional genes enhances the prediction of biogeochemical process rates (Hallin et al., 2009; Petersen et al., 2012; Bier et al., 2015; Powell et al., 2015; Graham et al., 2016; Breuillin-Sessoms et al., 2017), but traditional qPCR is laborious and costly, severely limiting the scope of microbial ecology investigations to quantification of just a handful of target genes.

Quantitative PCR uses DNA-complexing fluorophores and real-time detection to quantify the number of gene copies present in an individual reaction and is currently the gold-standard of gene quantification. By including a linear range of standards with known numbers of gene copies, qPCR is used to estimate starting concentrations of the target gene, often down to $10^1$ copies/ μL (Smith and Osborn, 2009). In recent years a number of alternatives to traditional qPCR have arisen which drastically improve throughput by either miniaturizing, automating, digitalizing or multiplexing reactions (Baker, 2010; Huggett et al., 2013). However, these methods do not simultaneously match both the accuracy and flexibility of qPCR. The most promising technologies that are addressing this gap include microarrays such as Geochip, which uses hybridization of an array of > 20,000 probes to semi-quantitate thousands of gene variants in a small number of samples (He et al., 2010); digital PCR, which uses sample partitioning into droplets or nanoliter chambers to detect individual copies of rare targets (Baker, 2012); and microfluidic qPCR (MFQPCR), which uses nanoliter reaction volumes and a system of pressurized valves and microfluidic channels to automate the mixing and thermocycling of up to 96 assays and 96 samples in a single chip (Spurgeon et al., 2008). While microarrays have proven a powerful tool for screening soils for the presence of functional genes (Yergeau et al., 2007), and digital PCR has its niche in detecting a few targets with high sensitivity, both are prohibitively expensive for ecological studies which require the analysis of large numbers of individual samples. MFQPCR has emerged as an attractive alternative, being cost-effective, easily customizable, and as it uses the same chemistry as traditional qPCR, theoretically produces directly comparable results (Miller et al., 2016).

First developed for reverse-transcriptase quantification of gene expression in humans (Spurgeon et al., 2008), Kleyer et al. (2017) recently reported the application of MFQPCR to quantify specific soil bacteria using species-specific primers in batch cultures and sterile sand. MFQPCR has also been successfully applied to environmental samples, detecting pathogenic bacteria and viruses in aquatic environments and in salmon (Ishii et al., 2013, 2014a,b; Byappanahalli et al., 2015; Miller et al., 2016; Bass et al., 2017; Sadik et al., 2017). These studies demonstrated that MFQPCR is sensitive to 2 copies/μL (Ishii et al., 2014a), is specific enough to distinguish between serotypes (Dhoubhadel et al., 2014), and per assay costs are less than half that of conventional qPCR (Ishii et al., 2014a; Miller et al., 2016). Until now, however, these studies have endeavored to use assays which conform to the parameters established in MFQPCR's development as a tool for quantification of gene expression in humans (Spurgeon et al., 2008). That is, using primer sets directed to highly specific target genes for quantification in relatively homogenous, high purity samples that have been purified of PCR-inhibiting contaminants.

Microfluidic qPCR achieves its high-throughput capacity by sacrificing much of the flexibility of qPCR. Recommended MQPCR conditions include primer pairs that are free from degeneracy, produce short amplicons of < 100 bp, and have melt temperatures close to 60°C, thereby enabling up to 96 assays to be run simultaneously, using identical reagent concentrations and thermocycling conditions (Spurgeon et al., 2008). Conversely, due to its low-throughput nature, traditional qPCR allows for assay-by-assay modifications of reaction conditions to suit specific primer pairs and sample types. This flexibility is particularly valuable in the quantification of microbial functional genes, where maximal coverage, rather than specificity, is desired (Gaby and Buckley, 2012). As microbial functional communities are taxonomically diverse, with several distinct clades performing a single substrate transformation, primers targeting a specific gene in one species have little predictive value for determining process rates in the whole community. Instead, degenerate primers are often used to target a single gene in one or multiple clades, with qPCR conditions optimized to ensure acceptable reaction efficiencies (Iwai et al., 2011; Wei et al., 2015; Gaby and Buckley, 2017). Other commonly used deviations from qPCR 'best-practice' include longer amplicons to straddle an active site combined with extended elongation times (Baldwin et al., 2003; Lueders and Von Netzer, 2017), the combination of mismatched bases and lower annealing temperatures (Ishii and Fukui, 2001; Frank et al., 2008; Edwards et al., 2011), and the use of additives to combat inhibitors such as humic acids present in soil DNA extracts (Dandie et al., 2007). Given the need for flexibility in primer design and the inability to customize individual assay conditions when using MFQPCR, it is necessary to establish what the boundaries of primer variability are for MFQPCR before it can be considered a high-throughput alternative for the quantification of microbial functional genes in environmental samples.

The aim of this study was to determine whether established qPCR primer pairs targeting microbial functional genes in soil could be applied to MFQPCR with comparable accuracy. As preliminary research showed a high assay failure rate (Crane, 2016), we aimed to determine which primer design parameters were crucial to assay success, by assessing performance of a wide variety of nitrogen cycle and hydrocarbon degradation primers under different combinations of primer concentrations and MFQPCR thermocycling conditions. We used soil DNA extracts from ongoing hydrocarbon ecotoxicology studies in subantarctic and Antarctic soils (Crane, 2016; Mcwatters et al., 2016) to evaluate the sensitivity, specificity and reaction efficiency of 29 established and 12 newly designed primer pairs and three sets of MFQPCR assay conditions. We then evaluated MFQPCR

accuracy by comparing MFQPCR and qPCR gene abundance estimates for a subset of genes and soil samples.

# MATERIALS AND METHODS

## Soil Samples and DNA Extraction

To evaluate MFQPCR reaction efficiencies, we used 217 soil DNA extracts from hydrocarbon ecotoxicology studies conducted on subantarctic Macquarie Island (Crane, 2016), and at Casey station, East Antarctica (Mcwatters et al., 2016). Samples from Macquarie Island were collected from a two-year *in situ* mesocosm study investigating the effect of a residual hydrocarbon mixture (spiked into clean soils) on native microbial soil communities. Samples from four separate biopiles at Casey Station were collected as part of a large-scale remediation project to evaluate hydrocarbon biodegradation rates and the effects of the active remediation treatments on the indigenous microbial population over 5 years. Soil samples (50g) were collected in sterile plastic 50 ml tubes, immediately sealed and stored at −20°C until analyzed. Total community genomic DNA (gDNA) was extracted in triplicate from 0.3 to 0.5 g soil using the FastDNA SPIN Kit for soil (MP Biomedicals) and quantified spectrophotometrically using the PicoGreen double-strand DNA kit (Life Technologies) on the ClarioSTAR® microplate reader (BMG Labtech). DNA lysates were diluted in nuclease- free water; Macquarie Island samples to an optimal range of 7–8 ng $\mu L^{-1}$, (min. 3.3 ng $\mu L^{-1}$, max 10.7 ng $\mu L^{-1}$) and Casey Station samples were all diluted 10-fold. An inter-plate calibrator (IPC) was generated by mixing 10 randomly selected Macquarie Island gDNA extracts to a final concentration of 8.7 ng $\mu L^{-1}$.

## Primers and Standard Generation

Primers targeting universal genes for Fungi, Bacteria, Archaea, Acidobacteria, and Betaproteobacteria were selected from the literature, in addition to a suite of primers for nitrogen cycling and hydrocarbon degrading genes in Bacteria and Archaea (**Table 1**). In accordance with qPCR best practice (Rodríguez et al., 2015), primer pairs with minimal degeneracy and small amplicon sizes were preferentially selected. Twelve additional nitrogen cycle primers with no degeneracy and amplicon sizes of < 200 bp were designed with PRISE2 (Huang et al., 2014) using sequences downloaded from FunGene (Fish et al., 2013) and GenBank. Standards for copy number quantification were generated either by PCR amplification of environmental gDNA (Shahsavari et al., 2016) or through artificially synthesized gBlock Gene Fragments (Integrated DNA Technologies) (**Table 2**). Representative sequences for gBlock standards were sourced from NCBI using Primer-BLAST (**Table S3**) (Ye et al., 2012) and each gBlock comprised of five different standard sequences. For PCR-derived standards, PCR reactions were conducted in 25 μL volumes containing 1 μL template, 1x GoTaq Flexi Buffer; pH 8.5 (Promega), 400 nM each primer (Integrated DNA Technologies), 250 μM each dNTP (Bioline), 160 μg ml-1 BSA, 0.625 U GoTaq polymerase (Promega) and optimized concentrations of $MgCl_2$ (Promega) (**Table S4**). Thermocycling conditions consisted of 94°C for 2 min, then 35 cycles of 94°C for 45 s, annealing for 45 s, 72°C for 45 s, with a final extension at 72°C for 10 min. Annealing temperatures were optimized for each primer pair (**Table S4**). PCR products were purified using QIAquick PCR purification columns (QIAGEN) and quantified spectrophotometrically. Copy numbers were calculated and standard curves generated using serial dilution from $10^2$ to $10^9$ copies/μL. Standards were pooled for use with MFQPCR, with final concentrations of $10^2$-$10^8$ copies/μL for EUK and Eub, and $10^1$-$10^7$ copies/μL for all other assays.

## MFQPCR

MFQPCR assays were run in three separate sets using Evagreen® chemistry and two different Fluidigm Dynamic Array™ Integrated Fluidic Circuits (IFCs): A single 48.48 IFC (Set A), three 48.48 IFCs (Set B), and a single 96.96 IFC (Set C). Primer selection (**Table 1**), primer concentration, extension times, sample source, and method of standard generation were different for each of the three Sets used (**Table 2**). An inter-plate calibrator sample (IPC) was run in triplicate across the three 48.48 IFCs in Set B (Bi, Bii, and Biii) to enable the evaluation of intra- and inter-run variation. Specific target amplification (STA) and MFQPCR were conducted at the Ramaciotti Center for Genomics (UNSW Australia, Sydney, Australia). Samples (gDNA) and 7-point standards were pre-amplified with a 50 nM primer pool using TaqMan PreAmp Master Mix (ThermoFischer Scientific). STA cycling conditions were 95°C for 2 min, then 14 cycles of 96°C for 15 s and 60°C for 4 min. Products were treated with 8 U Exonuclease I (New England Biolabs) at 37°C for 30 min and 80°C for 15 min, diluted 1 in 5 with DNA suspension buffer (TEKnova) and stored at −20°C overnight. For MFQPCR, gDNA and assays were loaded into the reaction chambers of a 48.48 or 96.96 IFC using an MX or HX IFC controller respectively, according to the manufacturer's Evagreen® protocol. The array was then placed in a BioMark HD™ for thermo-cycling; 95°C for 1 min, followed by 35 cycles of 96°C for 5 s and 60°C for 20 s or 25 s (**Table 2**), followed by melt curve analysis for 60–95°C at a ramp rate of 1°C/3 s.

## MFQPCR Data Analysis

Data were analyzed using the Real-Time PCR Analysis software, version 4.1.2 (Fluidigm), using default quality threshold of 0.65 and linear baseline correction. Peak sensitivity was set at 7, peak ratio threshold at 0.7, and melt temperature ($T_m$) ranges were set individually based on the peaks observed in standards, as per the manufacturers' recommendations. Both $T_m$ ranges and threshold cycle ($C_t$) values were manually normalized to the mean across intra-chip replicate assays. Individual reactions were excluded from analysis if they failed any of the melt curve quality parameters, were outside 0.5 $C_t$ of other replicates, or had a peak outside the set $T_m$ range. Calibration curves were created in the Calibration Curve View Module using the known copy numbers in standards, and the $R^2$ calculated from an OLS regression for each assay. Calibrated relative concentrations were then exported to MS Excel for conversion to copies/g of soil, analysis and modeling.

**TABLE 1 |** Primers pairs targeting taxonomic, nitrogen-cycling and hydrocarbon degradation genes selected for this study.

| Primer pair | Target | Primer | Sequence | Degeneracy score | Inosines | GC content (%) | Amplicon size (bp) | Set[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| 16s | Eubacterial 16s rRNA | 338F | ACTCCTACGGGAGGCAGCAG | 0 | 0 | 65 | 181 | A,C | Lane, 1991 |
| | | 519R | ACCGCGGCTGCTGGCAC | 0 | 0 | 76.5 | | | |
| 18s[b] | Fungal 18s rRNA | FR1 | AICCATTCAATCGGTAIT | 0 | 2 | 50.7 | 390 | A | Prevost-Boure et al., 2011 |
| | | FF390 | CGATAACGAACGAGACCT | 0 | 0 | 58.4 | | | |
| Acido | Acidobacteria 16s rRNA | Acido31f | GATCCTGGCTCAGAATC | 0 | 0 | 52.9 | 325 | B,C | Barns et al., 1999 & Muyzer et al., 1993 in Foesel et al., 2014 |
| | | 341r | CTGCTGCCTCCCGTAGG | 0 | 0 | 70.6 | | | |
| AlkB | alkane monooxygenase | AlkBF | AACTACATCGAGCACTACGG | 0 | 0 | 50 | 101 | A,B,C | Powell et al., 2006 |
| | | AlkBR | TGAAGATGTGGTTGCTGTTCC | 0 | 0 | 47.6 | | | |
| alkH[b] | alkane monooxygenase | alk-H1F | CIGIICACGAIITIGGICACAAGAAGG | 0 | 7 | 71.4 | 544 | A | Chénier et al., 2003 in Jurelevicius et al., 2013 |
| | | alk-H3R | GCITGITGATCIIIGTGICGCTGIAG | 0 | 7 | 69 | | | |
| almA | flavin-binding monooxygenase | almARTf | ATAGGTTAAATACGGTTCTCTGCAG | 0 | 0 | 40 | 262 | C | Wang and Shao, 2012 |
| | | almARTr | CAGCACTGGCCAGATAACTACG | 0 | 0 | 54.5 | | | |
| amoA1 | Bacterial ammonium oxidase | amoA1F | GGGGTTTCTACTGGTGGT | 0 | 0 | 55.6 | 491 | A | Rotthauwe et al., 1997 |
| | | amoA2R | CCCCTCKGSAAAGCCTTCTTC | 4 | 0 | 59.5 | | | |
| amoA2 | Bacterial ammonium oxidase | amoA-1Fmod | CTGGGGTTTCTACTGGTGGTC | 0 | 0 | 57.1 | 120 | A,B,C | Meinhardt et al., 2014 |
| | | GenAOBR | GCAGTGATCATCCAGTTGCG | 0 | 0 | 55 | | | |
| AOA2 | Archael ammonium oxidase | AOA2-F | GCAATCTATTACATGCTATTCA | 0 | 0 | 31.8 | 71 | C | This study |
| | | AOA2-R | TAGATAGTCATGATTGTTGCAT | 0 | 0 | 31.8 | | | |
| AOA8 | Archaeal ammonium oxidase | AOA8-F | CTATTCATAGTTGTAGTTGCTGTAA | 0 | 0 | 32 | 62 | C | This study |
| | | AOA8-R | ATGTAGTCTCCTGCGTTGAT | 0 | 0 | 45 | | | |
| AOB1 | Bacterial ammonium oxidase | AOB1-F | GTCTCCATGCTCATGTTC | 0 | 0 | 50 | 134 | C | This study |
| | | AOB1-R | GGAAAGCCTTCTTCGCC | 0 | 0 | 58.8 | | | |
| AOB26 | Bacterial ammonium oxidase | AOB26-F | TACTGGTGGTCGCACTA | 0 | 0 | 52.9 | 107 | C | This study |
| | | AOB26-R | GTGATCATCCAGTTGCG | 0 | 0 | 52.9 | | | |

*(Continued)*

**TABLE 1** | Continued

| Primer pair | Target | Primer | Sequence | Degeneracy score | Inosines | GC content (%) | Amplicon size (bp) | Set[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| BamA | 6-OCH-CoA hydrolase | Bam-sp9 | CAGTACAAYTCCTACACVACBG | 18 | 0 | 49.2 | 300 | B,C | Kuntze et al., 2008 |
| | | Bam-asp1 | CMATGCCGATYTCCTGRC | 8 | 0 | 58.3 | | | |
| BED[b] | Aromatic dioxygenases (toluene/benzene) | BEDemF | CAYGGVTGGGCBTAYGAYA | 72 | 0 | 66.5 | 307 | B | Iwai et al., 2008 & Iwai et al., 2010 in Iwai et al., 2011 |
| | | REVERSE BPHD-f3 | TCBGCIGCRAAYTTCCAGTT | 12 | 3 | 67.8 | | | |
| bProt | β-Proteobacteria 16s rRNA | S-C-bProt-0972-a-S-18 | CGAARAACCTTACCYACC | 4 | 0 | 50 | 231 | B,C | Pfeiffer et al., 2014 |
| | | S-C-bProt-1221-a-A-17 | GTATGACGTGTGWAGCC | 2 | 0 | 52.9 | | | |
| BssA[c] | Benzylsuccinate synthase (denitrifiers) | BssAf | ACGACGGYGGCATTTCTC | 2 | 0 | 58.3 | 130-132 | C | Beller et al., 2002 |
| | | BssAr | GCATGATSGGYACCGACA | 4 | 0 | 58.3 | | | |
| SRB-bssA[c] | Benzylsuccinate synthase (sulfate reducers) | SRBf | GTSCCCATGATGCGCAGC | 2 | 0 | 66.7 | 100 | C | Beller et al., 2008 |
| | | SRBr | CGACATTGAACTGCACGTGRTCG | 2 | 0 | 54.3 | | | |
| Cu1[b] | Fungal laccase | Cu1Fmod1 | ACGGTYCAYTGGCAYGG | 8 | 0 | 66.3 | ~200 | A | Edwards et al., 2011 |
| | | Cu2Rmod1 | GRCTGTGGTACCAGAAIGTNC | 8 | 1 | 60.1 | | | |
| Cu1bac | Bacterial laccase | Cu1AF | ACMWCBGTYCAYTGGCAYGG | 96 | 0 | 58.3 | 142 | B | Kellner et al., 2008 |
| | | Cu2R | GRCTGTGGTACCAGAANGTNCC | 32 | 0 | 56.8 | | | |
| Eub | Eubacterial 16s rRNA | Eub1048F | GTGSTGCAYGGYTGTCGTCA | 8 | 0 | 60 | 146 | B,C | Maeda et al., 2003 |
| | | Eub1194R | ACGTCRTCCMCACCTTCCTC | 4 | 0 | 60 | | | |
| EUK | Eukaryotic 18s rRNA | EUK345f | AAGGAAGGCAGCAGGCG | 0 | 0 | 64.7 | 149 | B,C | Zhu et al., 2005 |
| | | EUK499r | CACCAGACTTGCCCTCYAAT | 2 | 0 | 52.5 | | | |
| nagAC | β-Proteobacteria napthalene dioxygenase | nagAc-like-F | GGCTGTTTTGATGCAGA | 0 | 0 | 47.1 | 107 | C | Dionisi et al., 2004 in (Debruyn et al., 2007) in Powell, 2009 |
| | | nagAc-like-R | GGGCCTACAAGTTCCA | 0 | 0 | 56.3 | | | |
| NAH | Gram negative napthalene dioxygenase | NAH-F | CAAAARCACCTGATTYATGG | 4 | 0 | 40 | 377 | A,B,C | Baldwin et al., 2003 |

*(Continued)*

**TABLE 1** | Continued

| Primer pair | Target | Primer | Sequence | Degeneracy score | Inosines | GC content (%) | Amplicon size (bp) | Set[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| | | NAH-R | AYRCGRGSGACTTCTTTCAA | 16 | 0 | 47.5 | | | |
| napA14 | Nitrate reductase | napA14-F | ATGTGGGTGGAGAAGGA | 0 | 0 | 52.9 | 130 | C | This study |
| | | napA14-R | TGAAGCGCTTGGAGAATT | 0 | 0 | 44.4 | | | |
| narG | Membrane-bound nitrate reductase | narG1960m2F | TAYGTSGGGCAGGARAAACTG | 4 | 0 | 52.4 | 110 | B,C | López-Gutiérrez et al., 2004 in Hallin et al., 2009 |
| | | narG2050m2R | CGTAGAAGAAGCTGGTGCTGTT | 0 | 0 | 50 | | | |
| NidA[c] | Pyrene dioxygenase | Nid A-forward | TTCCCGAGTACGAGGGATAC | 0 | 0 | 55 | 141 | C | (Debruyn et al., 2007) in Powell, 2009 |
| | | Nid A-reverse | TCACGTTGATGAACGACAAA | 0 | 0 | 40 | | | |
| nifD23 | Nitrogenase | nifD23-F | TCATCGGCGACTACAACAT | 0 | 0 | 47.4 | 167 | C | This study |
| | | nifD23-R | GTTCATCGAGCGGTAGCA | 0 | 0 | 55.6 | | | |
| nifD33 | Nitrogenase | nifD33-F | TGCCGTTCCGCCAGATGCA | 0 | 0 | 63.2 | 69 | C | This study |
| | | nifD33-R | AGATGGCGAAGCCGTCATAGC | 0 | 0 | 57.1 | | | |
| nifH[b] | Nitrogenase reductase | IGK3 | GCIWTHTAYGGIAARGGIGGIATHGGIA | 72 | 5 | 69.2 | 390 | A | Ando et al., 2005 in Gaby and Buckley, 2012 |
| | | DVV | ATIGCRAAICCICCRCAIACIACRTC | 8 | 5 | 71.3 | | | |
| nifH3[b] | Nitrogenase reductase | nifH-2F | GMRCCIGGIGTIGGYTGYGC | 16 | 3 | 73.1 | 214 | B | Fedorov et al., 2008 in Gaby and Buckley, 2012 |
| | | nifH-3R | TTGTTGGCIGCRTASAKIGCCAT | 8 | 2 | 71.5 | | | |
| nifH32 | Nitrogenase reductase | nifH32-F | GGCGTCATCACCTCGATCA | 0 | 0 | 57.9 | 173-176 | C | This study |
| | | nifH32-R | GCATAGAGCGCCATCATCTC | 0 | 0 | 55 | | | |
| nifH66 | Nitrogenase reductase | nifH66-F | CGCTCTATGCCGCCAACAACA | 0 | 0 | 57.1 | 173-206 | C | This study |
| | | nifH66-R | GTTGTCGCGCGGCACGAA | 0 | 0 | 66.7 | | | |
| nirK | Copper nitrite reductase | nirK876 | ATYGGCGGVCAYGGCGA | 12 | 0 | 68.6 | 165 | B,C | Henry et al., 2004 in Hallin et al., 2009 |
| | | nirK1040 | GCCTCGATCAGRTTRTGGTT | 4 | 0 | 50 | | | |
| nirS | Cytochrome nitrite reductase | nirSCd3aFm | AACGYSAAGGARACSGG | 16 | 0 | 58.8 | 425 | A | Throbäck et al., 2004 in Hallin et al., 2009 |
| | | nirSR3cdm | GASTTCGGRTGSGTCTTSAYGAA | 32 | 0 | 52.2 | | | |
| nirS1 | Cytochrome nitrite reductase | nirS1F | CCTAYTGGCCGCCRCART | 8 | 0 | 63.9 | 256 | B,C | Braker et al., 1998 in Levy-Booth and Winder, 2010 |

*(Continued)*

**TABLE 1** | Continued

| Primer pair | Target | Primer | Sequence | Degeneracy score | Inosines | GC content (%) | Amplicon size (bp) | Set[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| | | nirs3R | GCCGCCGTCRTGVAGGAA | 6 | 0 | 67.6 | | | |
| norB79 | Nitrate reductase | norB79-F | GAATACTGGCGTTGGT | 0 | 0 | 50 | 55 | C | This study |
| | | norB79-R | ATACTTCAAAGAAGCCTTC | 0 | 0 | 36.8 | | | |
| norB153 | Nitrate reductase | norB153-F | CACCAAGGTTACGAATAC | 0 | 0 | 44.4 | 84 | C | This study |
| | | norB153-R | CATCAGGAACAGCCACA | 0 | 0 | 52.9 | | | |
| nosZ1 | Nitrous oxide reductase subunit | nosZ1-F | AAGGGCGAGAAGGT | 0 | 0 | 57.1 | 143 | C | This study |
| | | nosZ1-R | AGGAAGCGGTCCTT | 0 | 0 | 57.1 | | | |
| nosZ2 | Nitrous oxide reductase subunit | nosZ2F | CGCRACGGCAASAAGGTSMSSGT | 64 | 0 | 65.2 | 267 | A,B,C | Henry et al., 2006 |
| | | nosZ2R | CAKRTGCAKSGCRTGGCAGAA | 32 | 0 | 57.1 | | | |
| P450[c] | Cytochrome P450 (alkane oxidisers) | P4501RTf | GAGAATTTACCGACGAAGATG | 0 | 0 | 42.9 | 211 | C | Wang and Shao, 2012 |
| | | P4501RTr | CCAACGATAAGCAGAGCC | 0 | 0 | 55.6 | | | |
| rpoB | RNA polymerase beta subunit | 1698F | CAACATCGGTTTGATCAA | 0 | 0 | 38.9 | 343 | A,B | Dahllöf et al., 2000 |
| | | 2041R | CGTTGCATGTTGGTACCCAT | 0 | 0 | 50 | | | |

[a]*Primer pairs were trialed across three different sets of MFQPCR assay conditions; Sets A, B & C. Assay conditions used in each Set are summarized in* ***Table 2****.* [b]*Primers failed to amplify under MFQPCR conditions* [c]*Non-specific amplification observed in majority of samples.*

**TABLE 2 |** MFQPCR assay conditions varied across the three experimental sets.

| Set | IFC[a] | N° of IFCs | Reaction volume (nL) | Primer con-centration (nM) | Extension time (s) | Standard method | Sample Source | Total Samples | Total Assays[b] |
|---|---|---|---|---|---|---|---|---|---|
| A | 48.48 | 1 | 10.1 | 500 | 20 | PCR | Macquarie Island | 35 | 15 |
| B | 48.48 | 3 | 10.1 | 700 | 20 | PCR | Macquarie Island | 99 | 16 |
| C | 96.96 | 1 | 6.7 | 700 | 25 | gBlock | Casey Station | 83 | 30 + 2[C] |

[a]*IFC, Integrated Fluidic Circuit.* [b]*All assays were run in triplicate.* [c]*Two assays (nifH66 & amoA2) were run twice, with and without the additive T4 gene 32.*

## Traditional qPCR

Traditional qPCR was conducted with three primer pairs; amoA, narG, and bamA, for comparison with MFQPCR results obtained for Set B. Reactions were carried out using optimized qPCR conditions; in 20 μL volumes containing 1× QuantiFast SYBR Green PCR Master Mix (Qiagen), 500 nM each primer, and 1.25 μL template gDNA. Each 96-well plate consisted of a 7-point standard curve ($10^2$-$10^8$ copies/μL), no-template control (NTC), inter-plate calibrator sample (IPC) and 23 randomly selected samples, all in triplicate. Thermocycling was conducted with a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad) with a hot start of 95°C for 5 min, followed by 40 cycles of 94°C for 20 s and 60°C for 50 s, and melt curve analysis from 50 to 95°C at a ramp rate of 0.5°C/5 s. Analysis of qPCR data were conducted with the CFX manager software (Bio-Rad). Replicates with >0.5 $C_t$ variation were examined, and outliers discarded. Specificity was confirmed with melt peak analysis and reactions were discarded if non-specific amplification was evident. The average $C_t$ values across replicates were determined and copy numbers were calculated based on linear regression of the standard curve. Standard curve efficiencies and copy numbers were converted to copies/g of soil for subsequent analysis.

## Analysis of Reaction Efficiencies

Mean reaction efficiencies (percentage increase of template in each round of thermocycling) of samples and standards were calculated from observed increases in fluorescence using the LinRegPCR program (version 2015.3) (Ramakers et al., 2003). For qPCR data, non-baseline corrected data were exported from the CFX manager software and the raw fluorescence values imported into LinRegPCR. For microfluidic data, as the Fluidigm software does not allow for the export of raw fluorescence data, a constant baseline was first applied in the Real-Time PCR Analysis software version 4.1.2 (Fluidigm), and the data reanalyzed. Normalized fluorescence intensity values for all samples or standards, to 20 decimal places, were exported gene by gene, which does not allow for the identification of individual reactions but allows for group analysis of all samples or standards for each assay. Computation of efficiencies in LinRegPCR was conducted as per the program instructions (Ramakers et al., 2003). Noisy samples, where a continuous increase could not be identified, were excluded from "Window of Linearity" calculations and "strictly continuous log-linear phase" criteria was applied to baseline estimations. Samples were also excluded from mean efficiency calculations if they had no plateau. In accordance with qPCR best practice, we considered reaction efficiencies over 90% to be optimal (Rodríguez et al., 2015).

# RESULTS

## Evaluation of MFQPCR Assay Quality

In this study, we used three different sets of assay conditions (Sets A, B, and C), to evaluate variations in primer concentrations, extension times, soil samples, methods of standard generation, and the size and number of IFC chips used (**Table 2**). In total 41 qPCR pairs were evaluated, 29 of which were selected from the literature as they had been used previously in qPCR (**Table 1**). Additionally, 12 nitrogen cycle primer pairs with no degeneracy and amplicons of < 200 bp in length were designed here. Overall, primer degeneracy ranged from 0 to 96, and amplicon sizes from 55 to 544 bp. Other factors, such as GC content, melt temperature, homo-, and hetero- dimer complementarity, were not considered in the selection of primers but were also found to be variable (**Table S1**).

MFQPCR reaction efficiencies (**Figure 1**), were highly variable, ranging from 25.9% (Cu1, Set A) to 100.1% (EUK, Set B), with five assays failing completely to amplify the target sequence under MFQPCR conditions (18s, alkH, BED, nifH, nifH3). All five failed assays contained at least one inosine residue in either the forward or reverse primer, and exclusion of primers containing inosines in Set C eradicated further assay failure. Moderate negative correlations between efficiency and amplicon size ($r = 0.45$) and degeneracy ($r = 0.38$) were observed when data from the three Sets were pooled (**Figures 2A,B**). Of the other primer characteristics examined, weak, non-linear relationships with reaction efficiency were detected (**Figures 2C–E**). While the GC content of gBlock standards, analyzed in Set C, had a moderate negative correlation with reactionefficiency ($r = -0.66$, **Figure 2F**).

Reaction efficiencies exhibited a strong Set effect, reflecting a combination of both assay conditions and primer selection, with the highest median efficiency observed in Set B (**Figure 1**). Increased primer concentrations in Set B (700 nM) compared to Set A (500 nM) improved efficiency of all assays used in both Sets. However, results varied widely from an improvement of 5% for amoA2 through to 29.1% nirK indicating that in Set A, primer concentration was limiting the reaction. In Set C, efficiencies of several assays were lower than in Set B, likely due to the smaller reaction volumes (6.7 nl vs. 10.1 nl) and the GC content of the gBlock standards used. The addition of T4 gene 32 to a subset of six samples and two assays (amoA2

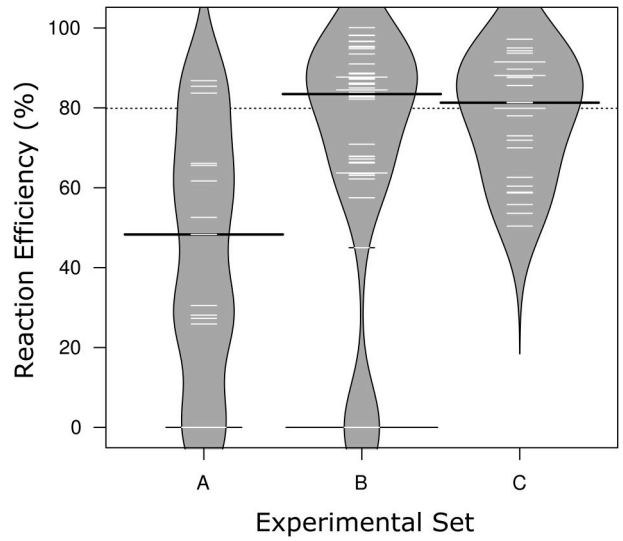


**FIGURE 1 |** Distribution of reaction efficiencies across the three Sets of MFQPCR assay conditions. Higher primer concentrations in Sets B & C corresponded with improved efficiencies, and exclusion of primers with inosines corresponds with the elimination of failed/zero efficiencies in Set C. Further differences in assay conditions are outlined in **Table 2**. Black lines show the medians; white lines represent mean efficiency score of individual assays (n = 15, 48, 27, respectively); polygons represent the estimated density of the data. Efficiencies are based on standards only.

and nifH66) in Set C did not produce a detectable effect on efficiencies or copy numbers compared to samples without this additive. Across the three experiments, all assays exhibited similar reaction efficiencies for standards and samples, except for four assays in Set C (AlkB, nirS1, nifH66, and nifD23; **Table S2**). Efficiencies in these four assays were 21–28% lower in standards compared to samples, reflecting a discordance between the GC rich representative sequences used in the gBlock, and those present in the environmental samples. Characteristics of MFQPCR assays displaying optimal efficiencies ($\geq$ 90%) are summarized in **Box 1**.

Standard curves were linear over between 4 and 7 orders of magnitude, with sensitivity ranging from $10^1$ to $10^4$ copies/$\mu$L and $R^2$ values from 0.919 to 1.000. In Set A, 13 samples exceeded detection limits for 16S, and in one chip of Set B the maximal value of accurate detection for AlkB, amoA2, Cu1bac, Eub, and narG was $10^6$ copies/$\mu$L, and $10^5$ for EUK. Amplification in all other standards and samples fell within the maximal range of detection. Low levels of non-specific amplification in the NTC was observed for 16S in Set A and Bprot and Eub in Set C, but in all cases amplification was $> 5$ $C_t$ below all standards and samples analyzed. Non-specific amplification, determined by melt curve analysis, was observed in most samples for BssA, NidA, SRB-BssA, and P450, suggesting these assays were lacking specificity. An increase in primer concentration in Sets B & C did not appear to be associated with any increase in non-specific amplification. The intra-run coefficient of variance ranged from 0% to 26.5%, and the inter-run coefficient of variance ranged from 2.3% to 24.1% (**Figure S1**).

## Comparison of MFQPCR and qPCR

Three primer pairs; amoA2, narG, and bamA, were used in a comparison between traditional and microfluidic qPCR. Mean efficiencies of standards and samples were higher with MFQPCR for amoA2 and narG, and slightly lower for bamA (**Figure S2**). BamA had very low efficiencies in both qPCR and MFQPCR assays and most samples failed melt-curve analysis due to the presence of an additional peak that did not correspond to that observed for the standards (**Figure S3**). MFQPCR and qPCR copy number estimates for amoA2 and narG were within one order of magnitude of each other for samples diluted $\geq$ 4-fold (**Figure 3**), while MFQPCR estimates were substantially lower than those of qPCR for samples at dilution factors of 3 or less. These results indicated that MFQPCR was more sensitive to the presence of inhibitors compared to traditional qPCR, and samples should be diluted accordingly.

# DISCUSSION

Analyzing the efficiencies of a wide range of published qPCR primer pairs under various run conditions and against over 200 soil gDNA extracts allowed us to determine which parameters were most essential to MFQPCR assay success (**Box 1**). The combination of nanoliter reaction volumes, rapid thermocycling and one-size-fits-all reaction conditions of MFQPCR required more stringent primer selection criteria, compared to common practices in environmental qPCR studies. In the case of assays targeting functional genes the most problematic requirement outlined here was for primers with degeneracy scores of $\leq$ 8. These constraints excluded many broad-coverage primers, which rely on degeneracy to achieve adequate coverage of taxonomically-diverse functional communities (Gaby and Buckley, 2012).

In addition to assay conditions and primer parameters, we trialed the use of artificially synthesized gBlock standards using sequences randomly selected from databases. This use of gBlock standards increased the accuracy of standard dilutions, produced greater inter-assay correlation, and reduced preparation time by eliminating the need to source representative cultures or generate amplicons from the environment. However, as the sequences used to design the gBlock standards were selected randomly, several amplicons exhibited different melt peaks and significant discrepancies in efficiencies compared to environmental samples. This introduces new inaccuracies, as differences in standard and sample efficiencies lead to an under- or over-estimation of copy numbers and hence invalidate estimation of absolute copy numbers (Ramakers et al., 2003; Bru et al., 2008).

It is important to emphasize that these guidelines are based on observed efficiencies under specific assay conditions, and alterations of thermocycling times, reagent concentrations or the use of additives could be manipulated to relax the stringent constraints outlined here (**Box 1**). Indeed, we found that increased primer concentrations in Sets B and C drastically improved assay efficiencies, ostensibly as it remedied the reduced primer/template ratio that results from using degenerate mixtures of primers (**Figure 1**) (Rose et al., 1998; Gaby and

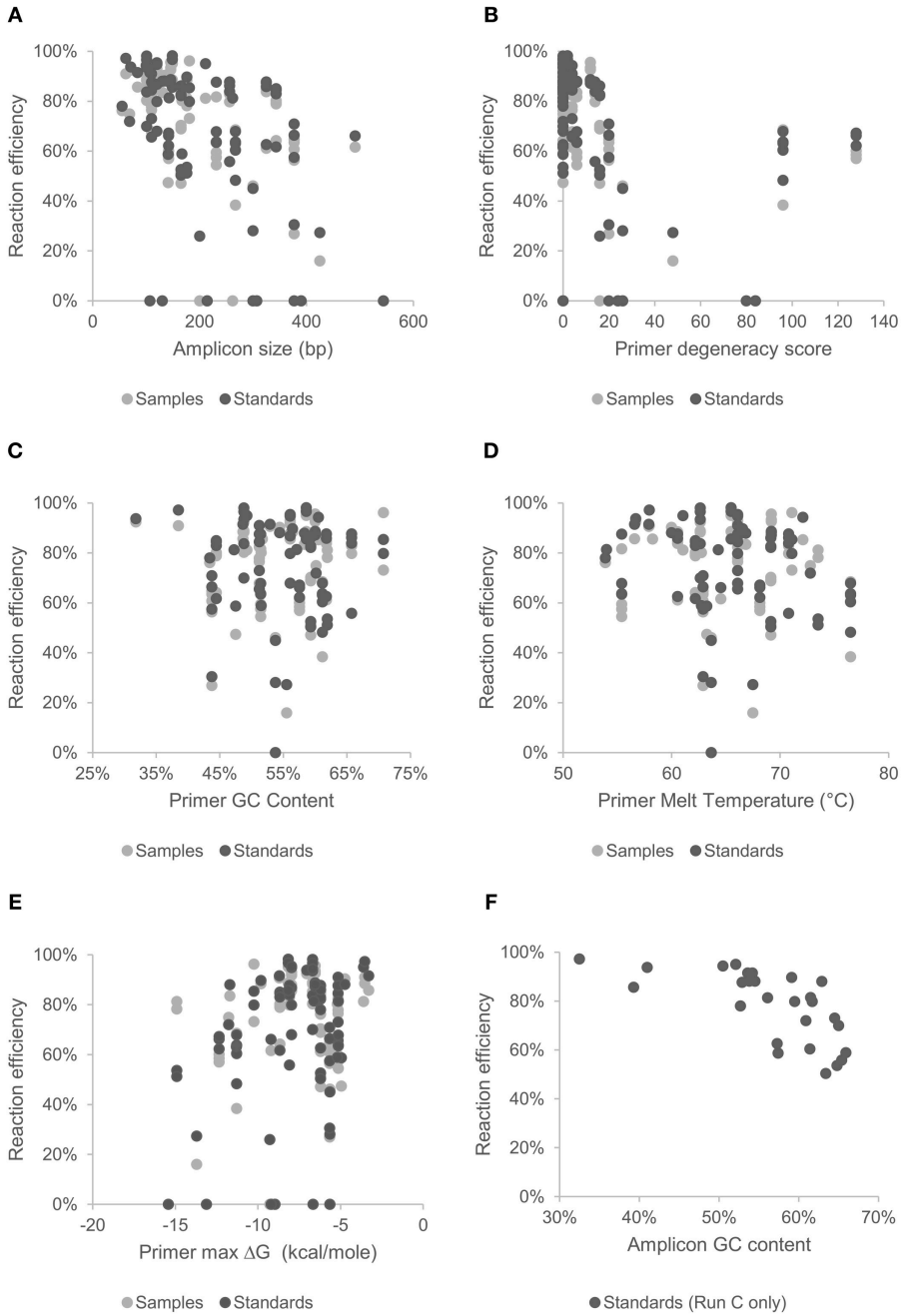


**FIGURE 2 |** Effect of amplicon size **(A)**, and primer characteristics; degeneracy **(B)**, GC content **(C)**, melt temperature **(D)**, and hetero-dimer ΔG **(E)**, on mean reaction efficiency of samples and standards across all chips and assays. Effect of GC content of gBlock artificially synthesized standards (Set C) on reaction efficiency are shown in **(F)**.

Buckley, 2017). Similarly, employing additives such as T4 gene 32 (Dandie et al., 2007) or an alternative soil DNA extraction kit (Mahmoudi et al., 2011) could potentially alleviate the inhibition observed in undiluted samples (**Figure 3**). The selection of chemistry is also clearly important; while we observed unusual sensitivity to inosine residues compared to those reported for qPCR (Zheng et al., 2008), this phenomenon has not been reported for inosine-containing MFQPCR assays that use TaqMan chemistry (Ishii et al., 2014a) or for Evagreen® chemistry with the Access Array™ (Oshiki et al., 2018).

While previous environmental studies using MFQPCR have examined correlations between MFQPCR and qPCR copy number estimates (Ishii et al., 2014a; Byappanahalli et al., 2015), our study is one of the first to explicitly examine individual MFQPCR assay efficiencies. In addition to the discrepancies between sample and standard efficiencies discussed

**Box 1** | What makes an optimal MFQPCR assay?

The following characteristics defined assays in this study with efficiencies greater than 90%:

- ☐ Amplicon length of 62 - 211 bp
- ☐ Individual degeneracy score of 0 - 8
- ☐ Combined degeneracy score (F+R) of 0 - 12
- ☐ No inosine residues
- ☐ GC content of 32 - 61%
- ☐ Predicted melt temperature of 57 - 71°C
- ☐ Hetero-dimer ΔG of (-9.8) – (-3.5)
- ☐ GC content of standards 33 - 59%
- ☐ Primer concentration of 700 nM
- ☐ Soil gDNA extracts diluted ≥ 5-fold

above, we also observed major variation in the efficiencies of unoptimized assays (25.9- 100.1%). Neither of these sources of inaccuracy would have been detected without analysis in LinRegPCR (Ramakers et al., 2003). This finding highlights the need for MFQPCR uptake to be accompanied by uniform and transparent reporting of experimental data including efficiencies, in accordance with MIQE guidelines (Bustin et al., 2009, 2013). Efficiency analysis is currently hampered by the inability of regular users of Real-Time PCR Analysis (Fluidigm) software to export raw fluorescence data without a cumbersome work-around, which may explain the lack of efficiency reporting in most MFQPCR studies. We anticipate that with increased adoption of MFQPCR, greater emphasis will be placed on the establishment of MFQPCR 'best-practice' protocols, similar to the Digital MIQE guidelines for digital PCR (Huggett et al., 2013), and that qPCR efficiency analysis software like LinRegPCR will allow data importation from MFQPCR programs (Ramakers et al., 2003).

To ensure MFQPCR assays are of high-efficiency while capitalizing on the increased throughput that the platform affords, we suggest that selection of both primers and representative sequences for gBlock standards be informed by knowledge of which gene variants are present in the environment in question. This approach could be taken further, by harnessing recent advances in high-resolution melting curve (HRM) analysis, which allows the identification and quantification of multiple gene variants based on differences in melt temperatures (Hjelmso et al., 2014). In our study, assays were excluded from analysis if there was severe separation of standard and sample melt peaks. In the case of bamA, early samples and standards had a different melt peak profile to later samples (**Figure S3**), but the inability to confirm the identity of this new peak meant that such data were considered to be non-specific and were thus discarded. Such shifts in the abundance of different gene variants potentially reflect not non-specific amplification, but adaptation of the community. By designing gBlock standards that cover a range of gene variants, in combination with HRM, such diversity could be quantified reliably. This capability is not far off, with a Single Nucleotide Polymorphism (SNP) melting curve analysis method already available on the Fluidigm BioMark platform [e.g., (Kim et al., 2017)].

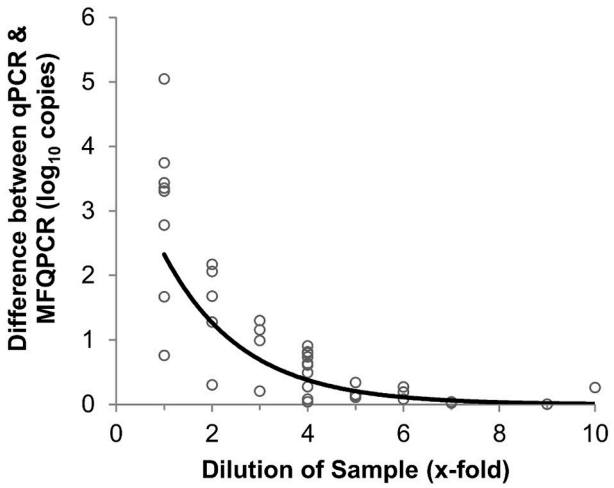


**FIGURE 3 |** Effect of sample dilution on accuracy of MFQPCR copy number estimates. Large discrepancies between qPCR and MFQPCR estimates are evident when gDNA soil extracts are diluted 4-fold or less. Data points are a combination of estimates for amoA2 and narG under Set B assay conditions (summarized in **Table 2**).

## CONCLUSION

MFQPCR is a valuable tool for quantifying microbial functional communities in soil, provided primer pair and assay conditions are stringently curated. When optimal conditions were met, MFQPCR allowed reliable, simultaneous quantification of taxonomic, nitrogen-cycling and hydrocarbon degradation genes in over 200 gDNA extracts from subantarctic and Antarctic soils. MFQPCR will be of greatest utility if multiple low-degeneracy, clade-specific primers can be designed, or if primer selection is guided by prior knowledge of the specific environment, such as that revealed by metagenomic or predictive metagenomic surveys (Bonilla-Rosso et al., 2016; Mukherjee et al., 2017). Employed in such a fashion, MFQPCR will fill a valuable niche between high-throughput taxonomic sequencing and low-throughput functional gene qPCR, allowing accurate quantification of microbial ecosystem services, such as clade-specific resolution of microbial functional guilds involved in biogeochemical nutrient cycling. We propose that MFQPCR will be particularly useful in monitoring the response of sensitive functional groups such as ammonia-oxidizers to environmental disturbances (Van Dorst et al., 2014), or for the accurate prediction of biogeochemical process rates (Breuillin-Sessoms et al., 2017).

## AUTHOR CONTRIBUTIONS

All authors contributed to the study design. BF coordinated the study. SC and JvD carried out the experimental procedure. SC conducted the data analysis and drafted the manuscript. All authors finalized, read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2018.00145/full#supplementary-material

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