RESEARCH ARTICLE

Dye-based qPCR assays for the separate quantification of total bacterial, fungal, arthropod, and plant DNA

Teresa M. Tiedge[1], **Kim R. Love**[2], **Kelly A. Meiklejohn**[1*]

1 Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States of America, **2** K. R. Love Quantitative Consulting and Collaboration, Athens, Georgia, United States of America

* kameikle@ncsu.edu

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Abstract

Characterization of bacterial, fungal, arthropod, and plant communities from bulk environmental samples can enhance our understanding of the biodiversity on Earth and aid in biomonitoring of various biomes. An important step in any molecular biology protocol is DNA quantification. While fast methods that determine the total amount of DNA in a sample are widely available, quantitative polymerase chain reaction (qPCR) is often preferred as it permits quantification of specific DNA molecules of interest. Currently, commercial qPCR assays are only available to quantify total bacterial and fungal DNA; limited studies describe the use of qPCR to quantify total arthropod and plant DNA. Here we describe the rigorous validation of four dye-based qPCR assays to separately quantify total bacterial, fungal, arthropod, and plant DNA following the MIQE guidelines. Validation experiments included primer annealing, primer concentration, specificity (*in vitro* and *in silico*), sensitivity, and reproducibility. Standard curves were created using synthetic double stranded DNA (gBlocks™) that were designed to quantify the DNA molecules of interest. Melt curve temperatures were identified for each taxon for specificity confirmation. These four assays were found to be specific, sensitive, and reproducible and can quantify DNA from single source specimens and mixed DNA samples, such as environmental DNA.

Introduction

DNA quantification is a key step in most molecular biology workflows or protocols. Most biological approaches require specific amounts of input to ensure optimal results. Quantitative polymerase chain reaction (qPCR) is a molecular biology tool that utilizes PCR to make copies of specific region(s) of DNA for quantification. Quantification can be performed using either probe-based or dye-based assays and are run using a real time PCR instrument, that allows the user to monitor the reaction as it is progressing. For qPCR using hydrolysis probes, the probe anneals to the template DNA and as

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synthesis occurs the DNA polymerase releases the reporter dye from the quencher. During dye-based qPCR, a fluorescent dye binds to the double stranded DNA copies that are generated through target-specific PCR. As amplification continues for both assay types, the amount of fluorescence increases proportionally to the number of DNA copies that are present. To determine the amount of target-specific DNA that is present in unknown samples, these assays incorporate the use of a standard curve, which is a series of DNA samples with a known concentration. Other quantification methods such as spectrophotometers and fluorometers, can only provide total DNA quantification whereas qPCR is able to quantify specific DNA molecules of interest.

The assessment of environmental DNA (eDNA) from bacteria, fungi, arthropods, and plants has broad implications across multiple disciplines in understanding biodiversity and can assist in the monitoring of numerous environments [1,2]. Accurate and reliable DNA quantification of taxa recovered in environmental samples is critical in assessing the taxonomic communities present [3]. Various molecular biology manufacturers have developed commercial dye-based qPCR assays (typically SYBR Green) for the quantification of bacterial and fungal DNA, but there are no available assays that quantify total plant and arthropod DNA; rather species-specific assays can be purchased from commercial vendors (*e.g., Arabidopsis*) or have been described within the literature. Additionally, for the bacterial, fungal, and plant qPCR assays that have been published, these assays may not have either undergone testing with bulk environmental samples [4–7] or described their validation experiments in detail [1,7,8].

Forensic crime laboratories that perform traditional human DNA analysis are required by the FBI Quality Assurance Standards (QAS) to quantify the amount of human and/or male DNA present within a sample prior to processing, typically completed using a commercially available probe-based qPCR kit. Information gleaned from DNA quantification will a) dictate if the sample has DNA of sufficient quantity and quality to proceed with DNA analysis, and b) how much of the sample will be used as input for testing [9]. The forensic science research community is exploring how the human microbiome, as well as environmental DNA (eDNA) derived from plants, fungi, bacteria and arthropods associated with soil and dust can be used to assist in criminal investigations [10–17]. Additionally, with the advent of newer sequencing technologies, microbiome research has become an attractive area in understanding topics such as soil dynamics [18–22], human health [23–25], and other ecological applications [1,26–31]. To adhere to the FBI QAS, it is likely that accurate quantification of biological taxa would most likely be required if these methodologies were implemented into casework. Thus, the goal of this study was to develop four dye-based qPCR assays for the separate quantification of total bacterial, fungal, plant, and arthropod DNA and describe detailed validation studies using these primer pairs with single-source and mixed DNA samples.

Materials and methods

Primer selection

A review of DNA metabarcoding and qPCR literature identified numerous universal primer pair options for bacteria, fungi, plants, and arthropods. These pairs were

examined initially based on amplicon size, as it is an important consideration for qPCR given: 1) qPCR assays typically have optimal amplicon sizes of 200 bp or less, and 2) smaller amplicon sizes could potentially capture degraded DNA. Prior to testing primer pairs that met the desired amplicon size *in vitro*, primers were screened for specificity *in silico* using CLC Genomics Workbench 20.0.2 (Qiagen, Hilden, Germany). This was completed by 1) downloading gene and taxon-specific sequences from GenBank (S1 File), 2) creating an alignment using the 'Create Alignment' tool with default gap cost settings and a 'very accurate (slow)' setting for the alignment, and 3) using the 'Find Binding Sites and Create Fragments' tool with default concentration settings with an exact match selected for match criteria. If a primer exactly matched a non-specific sequence (*e.g.,* 16S bacteria forward primer matched to 16S plant sequences), the primer was removed from consideration. Primers that were deemed specific via *in silico* testing and subsequently used for *in vitro* specificity testing were as follows: 1) bacteria – 1369F and 1519R targeting a ~170 bp region of 16S ribosomal rRNA gene [32], 2) fungi – 18S-Uni-FW and 18S-Uni-RV targeting a ~130 bp region of 18S ribosomal rRNA gene [33], 3) arthropods – Ins16S_9F and Ins16S_9R targeting a ~180 bp region of 16S ribosomal rRNA gene [34], and 4) plants – psbA2Fand psbA2R targeting a ~110 bp region of *psbA* gene (photosystem II protein D1) (this study). Notably, numerous plant primer pairs targeting ribosomal genes often had non-specific binding, leading to a change in focus to chloroplast genes. The plant primers for the *psbA* gene were designed using Integrated DNA Technologies' (IDT) PrimerQuest Tool (IDT, Coralville, IA, USA) using the 'qPCR 2 primers Intercalating Dyes' design. One nucleotide in both the forward and reverse *psbA* primers was changed to a degenerate base to accommodate sequence heterogeneity in that position. Primers used in this study are listed in S1 Table and S2 File lists the scientific papers which contained primers that were screened for utility.

Single source specimen curation and DNA isolation

Bacteria and fungi. To test the specificity of each qPCR assay, reference material was obtained to cover broad taxonomic orders within each target group (S3 File). A total of 23 bacterial and 23 fungal species were provided from the U.S. Department of Agriculture (USDA) Agricultural Research Service Culture Collection (Peoria, IL, USA) either as live or lyophilized specimens. Live cultures, provided as agar slants, were scraped with loops, and submerged into tubes containing trypticase soy broth. Bacteria were plated onto both trypticase soy agar and blood agar, and fungi were plated on cornmeal agar. Lyophilized cultures were first submerged in trypticase soy broth and swirled with a loop to assist in rehydration. After the pellet dissolved, cultures were plated as above. Plates and broth tubes were incubated at room temperature for approximately 14 days. DNA was isolated from the culture plates and broth tubes using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol [35]. For broth cultures, the broth was thoroughly mixed with a pipette and 1 mL was transferred to a 1.5 mL tube and centrifuged at max speed for 3 min using a Centrifuge 5415 C (Eppendorf, Hamburg, Germany). A volume of 250 µL from the bottom of the tube was pipetted into the ZR BashingBead™ Lysis Tubes for DNA isolation. Visible colonies from cultures grown on agar were swabbed with BBL™ CultureSwab™ EZ swabs (Becton Dickinson, Franklin Lakes, NJ, USA) and swab heads were cut directly into DNA lysis tubes. Samples underwent bead beating for 40 min using speed 7 with the Vortex-Genie 2T vortex (Scientific Industries, Inc. Bohemia, NY, USA). DNA was eluted in 100 µL of ZymoBIOMICS™ DNase/RNase Free water and steps 11–13 of the manufacturer's protocol were repeated for a second 100 µL elution.

Plants and arthropods. Forty-four plant specimens (single leaf for each plant) were collected from live plants at the JC Raulston Arboretum in Raleigh, North Carolina and 22 arthropods were collected live from various locations in Raleigh. Subsamples of plant leaves (~10–35 mg) and single arthropod legs were placed into 1.5 mL microcentrifuge tubes and subsequently bleached in a 5% bleach solution for 5 min and washed three times with sterile water to minimize co-extraction of non-specific DNA [36]. After washing, specimens were placed onto to a thermomixer at 40°C for 20 min to facilitate drying. Tubes were left open and allowed to dry in a biosafety cabinet overnight. DNA from plant leaves was subsequently isolated using the DNeasy Plant Pro Kit (Qiagen) following the manufacturer's protocol with a minor modification that included a second 50 µL elution. Plant leaves that were extremely tough (*e.g.,* pine needles) were

crushed with sterilized plastic pestles (Carolina Biological Supply Co., Burlington, NC, USA) prior to DNA isolation to ensure sufficient cell lysis. The DNeasy Blood and Tissue Kit (Qiagen) was used to isolate arthropod DNA following the manufacturer's protocol for insects. Arthropod legs or whole arthropods (mealybugs only) were ground using sterile BioMasher® II Micro Tissue Homogenizers (Kimble Chase, Vineland, NJ, USA) and after the addition of lysis reagents, incubated at 56°C in a ThermoMixer C (Eppendorf) for 2 hours at 300 rpm. DNA was subsequently eluted in two 50 µL elutions.

DNA quantification. DNA isolated from single source bacteria, fungi, plants, and arthropods was quantified with the dsDNA High Sensitivity Assay (Invitrogen, Waltham, MA, USA) using the Qubit™ Fluorometer and subsequently diluted to 0.5 ng/µL with sterile water. DNA was stored at −20°C until use.

qPCR assay validation

Each primer pair underwent annealing temperature and primer concentration testing to identify optimal parameters for subsequent validation experiments. Assay validation experiments were designed following the MIQE guidelines [37]. All primer pairs were tested for specificity, reproducibility, and sensitivity. Linear dynamic range was assessed using a standard curve. Four pools containing DNA from 10 single source species (covering 10 different orders) were created; one each for bacteria, fungi, arthropods, and plants, and pools were used in all experiments. Diluted DNA (0.5 ng/µL) for each single source species were combined in equal volumes to create each pool. These DNA pools were used to permit efficient screening across broader taxonomic orders, rather than using single sample reactions as well as mitigating the consumption of limited samples. All qPCR experiments were performed on the QuantStudio™ 5 (Applied Biosystems, Waltham, MA, USA) using the PowerTrack™ SYBR™ Green Master Mix [38] with standard cycling conditions and the dissociation step (melt curve analysis) as described in the manual, unless noted otherwise. For each 20 µL reaction, 10 µL of master mix was combined with 2 µL of diluted DNA (0.5 ng/µL), 6 µL of sterile water, and 1 µL of each primer (8 µM starting concentration). No template controls, consisting of sterile water, were included in each experiment. The DNA tracking dye included in the master mix kit was omitted from all reactions. The quantification cycle (C_q) and melting temperature were recorded for each reaction.

Annealing temperature optimization. For each primer pair, a total of seven different sources of DNA were tested to determine the optimal annealing temperature: a) four individual single source species within the target taxon (*e.g.,* four taxonomically diverse plant species for the plant *psbA* primers), and b) each non-target DNA pool (*e.g.,* fungal primers were tested against bacterial, plant, and arthropod pools). Duplicate reactions for each primer pair and DNA source were completed at 57°C, 58°C, 59°C, and 60°C. These temperatures were selected as: 1) the manufacturer recommends an annealing temperature range of 55–65°C for this master mix, and 2) considering these primers were selected to amplify across broad taxa, annealing should be slightly lower than the standard 60°C but not too low to introduce non-specific amplification.

Primer concentration optimization. Nine primer concentrations were tested with each primer pair using an annealing temperature of 57°C. For these experiments, only the specific DNA pool for a given primer pair was used (*e.g.,* only plant DNA pool was tested with plant *psbA* primers). Final concentrations tested were as follows with the forward primer listed first and the reverse primer listed second: 300/300 nM, 300/500 nM, 300/800 nM, 500/300 nM, 500/500 nM, 500/800 nM, 800/300 nM, 800/500 nM, and 800/800 nM [38].

Specificity. Each primer pair was tested with a) individual single source DNA from species within the target taxon, b) each non-target DNA pool (*e.g.,* fungal primers were tested against bacterial, plant, and arthropod pools), and c) individual single source DNA from seven vertebrate species (black bear, alligator, sheep, rhesus monkey, dog, mourning dove, northern water snake). For all four primer pairs, duplicate reactions for each DNA source were completed at an annealing temperature of 57°C with a final primer concentration of 300 nM for both forward and reverse primers. The number of taxon-specific single source species screened for each primer pair was 47 for plants, 24 for bacteria, 30 for fungi, and

23 for arthropods. Additionally, DNA isolated from 15 surface soil and 15 dust samples were tested with each primer pair. Fungal, plant, and arthropod DNA pools that were utilized in a separate project were also included as a source of mixed starting template, as some of the taxa used in these pools were not included in the list of single source species being tested. These pools consisted of two fungi (baker's yeast and a yard mushroom), two bacteria (*E. coli* and a *Salmonella* species), ten plants (basil, pine, moss, Boston fern, white wild indigo, Columbian monkshood, red bitterberry, henbane, hemlock, and purging croton), and ten arthropods (cockroach, moth, tick, beetle, fly, wasp, cricket, silverfish, spider, and pill bug). Additionally, the ZymoBIOMICS™ Microbial Community DNA Standard (Zymo Research), which includes eight bacteria and two fungi, was incorporated to test the bacterial primers.

Reproducibility. Reproducibility experiments encompassed two separate phases: 1) analyst, and 2) master mix. Notably for both phases, different qPCR instruments were used to additionally assess instrument reproducibility. For phase 1, two analysts completed matching experiments (*i.e.,* same reagents and DNA samples) across three qPCR instruments: the QuantStudio 5 (Applied Biosystems; used for the initial experiments previously outlined) and two different Step-One Plus (Applied Biosystems) instruments. Each primer pair was tested with a) four individual single source DNA from species within the target taxon (*e.g.,* four plants for the plant *psbA* primers), and b) each non-target DNA pool (*e.g.,* fungal primers were tested against the bacterial, plant, and arthropod pools). For phase 2, three different SYBR-based qPCR master mixes were evaluated: PowerTrack™ SYBR™ Green Master Mix (Applied Biosystems; used for the initial experiments previously outlined), Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA), and GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA). Experiments to evaluate master mix were completed on both a QuantStudio 5 and a Step-One Plus qPCR instrument. DNA pools were only used for phase 2 and individual species testing was not performed. For experiments completed in both phase 1 and 2, duplicate reactions were performed for each DNA sample, and an annealing temperature of 57°C with a final primer concentration of 300 nM each were used.

Standard curve generation & linear dynamic range. Complete bacterial 16S, fungal 18S, arthropod 16S, and plant *psbA* sequences were downloaded from NCBI and aligned in CLC Genomics Workbench 20.0.2 (Qiagen) using the alignment tool with the default settings (S4 File). The region to be amplified by each primer pair was first identified in the alignment, then the consensus sequence for each target amplicon was subsequently extracted from each alignment and provided to IDT for synthesis into four separate gBlocks™ (*i.e.*, synthetic double stranded DNA fragments). To provide some flexibility with primer binding, 15 nucleotides upstream of the forward primer binding site and 15 nucleotides downstream of the reverse primer binding site were included in each consensus sequence for gBlock™ synthesis at a yield of 250 ng. IDTE (IDT) was used to resuspend each gBlock™ with a volume of 25 µL for a final concentration of 10 ng/µL and were stored at 4°C. Copy number was determined for each gBlock™ following an online copy number calculator [39] using the mass of each gBlock™ and a 1:10 serial dilution was subsequently performed (10^1–10^8). Each dilution (n = 8) was tested with all primer pairs using 57°C as the annealing temperature with a final primer concentration of 300 nM for both the forward and reverse primers. Each gBlock™ was also used as DNA template (10^5 copy number dilution only) for all primer pairs to further assess cross-reactivity and specificity.

Sensitivity. Sensitivity was assessed using the smallest gBlock standard (10 copies) with 10 replicates to determine if 10 DNA copies could be consistently detected. Each primer pair was tested against each replicate with a final primer concentration of 300 nM each and annealing temperature of 57°C.

Inhibition. Inhibition experiments were completed to assess the impact of three commonly encountered PCR inhibitors likely to be associated with environmental samples on qPCR [40]. Ethylenediaminetetraacetic acid (EDTA; 0.5 M, pH 8.0; Fisher Scientific, Hampton, NH, USA), humic acid (Fisher Scientific), and tannic acid (Fisher Scientific) were prepared and diluted following Kavlick 2019 which describes the development of a mitochondrial DNA quantification assay for forensic DNA analysis [41]. Stock EDTA was diluted to three concentrations for qPCR testing: 5000 µM, 2500 µM, and 1250 µM. Humic acid was reconstituted using 4 mg of stock powder and 1 mL of nuclease-free water and was subsequently diluted

to 40 µg/mL, 20 µg/mL, and 10 µg/mL for qPCR. Tannic acid was reconstituted using 10 mg of stock powder and 10 mL of nuclease-free water and was diluted to four concentrations: 75 µg/mL, 50 µg/mL, 25 µg/mL, and 12.5 µg/mL. All 20 µL qPCR reactions used 2 µL of the diluted inhibitor. Each inhibitor was tested in triplicate using the gBlock™ standards at both 10^3 and 10^5 copy numbers for each primer pair. Triplicate reactions were also performed for each primer pair using each inhibitor concentration containing no DNA (inhibitor control).

In silico analysis

The testing of these four qPCR assays *in vitro* with numerous species is by no means exhaustive and required an additional method to screen more taxa to characterize amplification specificity. Sequences within the "RefSeq" database on GenBank for *psbA*, 16S eukaryote, and 18S sequences were downloaded in August 2023. Whole RefSeq bacterial genomes were downloaded from NCBI in February 2024. The ecoPCR function within OBITools3 (version 3.0.1b24) was used to perform *in silico* analysis of the four primer pairs using a mismatch value of three [42]. Since ecoPCR can only execute the analysis using primers that are 31 nucleotides or less, four nucleotides were removed from the 5' end of the 18S forward primer, and two nucleotides were removed from the 5' end of the 18S reverse primer to accommodate this restriction. Nucleotides at the 5' end were removed rather than the 3' end as the 3' end is imperative for the polymerase to bind to for synthesis and thus, ensuring the primer can efficiently anneal at the 3' end is critical to the analysis.

Statistical analysis

C_q values. Replicates were averaged together for each individual DNA sample in each experiment and used to generate the summary statistics (mean, standard deviation, median, minimum, and maximum) of the C_q values. The C_q values from the specificity experiments were calculated for each taxon's primer pair for the: 1) "in" group (*e.g.,* plant primers tested against plant single source species), 2) "out" group (*e.g.,* plant primers used with non-plant DNA pools), and 3) "mixed" group (*e.g.,* plant primers amplifying plant DNA within soil and dust samples). Normality was tested using the Shapiro-Wilk test and found not to hold. The Kruskal-Wallis test was used to compare means between all three groups and Dunn tests with a Bonferroni correction were performed to determine which group differences were statistically significant. For reproducibility testing, summary statistics were determined for each instrument by analyst. Analyst's C_q values for each instrument were compared using the Wilcoxon signed rank test or paired t-test depending on whether the data was non-normally or normally distributed, respectively. The intraclass correlation coefficient (ICC) based on a single rater and on absolute agreement was utilized to assess reliability [43]. C_q values between instruments (averaged over raters) were compared using Wilcoxon signed rank tests and the ICC was calculated for the three instruments. Summary statistics were calculated for the master mix reproducibility experiments. C_q values from each instrument within each master mix were compared using the Wilcoxon signed rank test (non-normally distributed) or paired t-test (normally distributed) and the ICC across the instruments within each master mix was determined. Following this, master mix C_q values were within each instrument were compared to each other using Wilcoxon signed rank tests and the ICC across master mixes within each instrument was calculated. For each standard curve, the regression equation ($C_q = \beta_0 + \beta_1 X_1$, where the value of X_1 is the power of the dilution) and standard errors were calculated. Summary statistics and reference intervals were determined for sensitivity experiments. Kruskal-Wallis test and Dunn post-hoc tests were also used for the inhibition studies.

Melt curve temperatures. For specificity experiments, each taxon's primer pair melt curve temperatures were summarized per group (*i.e.,* in, out, and mixed) as described above. Normality was assessed with Shapiro-Wilk tests and found not to hold, therefore Kruskal-Wallis tests were used to compare the melt curve temperatures and Dunn tests with a Bonferroni correction were applied to determine which of the three groups were significantly different when required. Reference intervals were also calculated for each group including 90% confidence intervals for the locations of the reference internal endpoints. Summary statistics were calculated for reproducibility experiments, and 1) differences

between analysts for each instrument were assessed using Wilcoxon signed rank tests and the ICC was calculated, and 2) instruments were compared to each other averaging over analysts using Wilcoxon signed rank tests and the ICC was calculated. Master mix reproducibility was assessed by first determining the summary statistics and comparing the instruments within each master mix using Wilcoxon signed rank tests or paired t-tests, followed by the ICC. Master mixes were then compared within each instrument using paired t-tests and the ICC. Summary statistics and reference intervals were determined for sensitivity experiments. Kruskal-Wallis test and Dunn post-hoc tests were also used for the inhibition studies.

Results and discussion

Two key data components were collected for analysis: C_q values and melt curve temperatures. For C_q, there is an inverse relationship between DNA quantity and C_q, whereas melt curve temperatures indicate where 50% of amplified DNA is double stranded and 50% is single stranded, and this temperature is dependent on length of the amplicon and GC content. Melt curve analysis is performed after PCR and a single peak indicates specificity, whereas multiple peaks could indicate primer dimer (often at lower temperatures than the target amplicon) and/or non-specific amplification. For multi-locus genes, such as bacterial 16S rRNA, it is possible to have the gene located at multiple regions throughout the genome with variation in size as well as in the underlying sequence [44]. Based on this, it is therefore feasible that multiple peaks may be generated during the melt curve analysis for a single species for such genes/targets.

qPCR assay validation

Annealing temperature optimization. Four target single source species amplified in duplicate for each primer pair were used to determine the optimal annealing temperature (S2 Table). For fungi and plants, the average quantification cycle (C_q) value increased as temperature increased, however this trend was not observed with bacteria and arthropods. With increasing temperature, this trend indicated that less DNA was able to be amplified which can have an impact on determining the true copy number in each sample. The average melt curve temperature and standard deviation for each primer pair across all temperatures tested (57°C, 58°C, 59°C, and 60°C) were as follows (four species each performed in duplicate for each temperature): 1) bacteria – 89.02°C ± 2.14°C, 2) fungi – 79.69°C ± 0.98°C, 3) arthropods – 79.83°C ± 1.79°C, and 4) plants – 81.92°C ± 0.28°C. Considering the temperature consistency of the melt curves, and that fungi and plants had the lowest C_q value at the lowest temperature, 57°C was selected as the annealing temperature for all primer pairs and used in all remaining experiments. If a lower temperature was selected there is an increased risk for non-specific amplification and if a higher temperature had been chosen there is the potential for reduced amplification of true template.

Primer concentration optimization. Nine primer concentration combinations were tested in duplicate using taxon-specific DNA pools. Average and standard deviation C_q values for each primer pair were as follows (one pool performed in duplicate for each concentration combination): 1) bacteria – 22.47 ± 0.40, 2) fungi – 18.62 ± 0.44, 3) arthropods – 19.61 ± 0.11, and 4) plants – 21.66 ± 0.33. Negative controls were assessed to see if increasing primer concentration had an impact in obtaining a C_q, as negative controls should be undetermined (no DNA present). A total of 18 negative controls were included for each primer pair, and the number with "undetermined" C_q (those with C_q greater than the 40 cycles of PCR) were as follows: bacteria 1/18, fungi –16/18, plants –17/18, and arthropods – 6/18. For those negative controls in which a C_q value was generated, all C_q values for negative controls exceeded 30, with a median C_q of 35.54 for bacteria and 36.55 for arthropods (which exceeded the limit of detection; see *Sensitivity*). Bacteria's one undetermined occurred when both primers were 300 nM. The instances of undetermined for arthropods happened when both primers were 300 nM for both replicates, one replicate for 300/500 nM, both replicates for 500/300 nM, and one replicate at 800/500 nM. Since a) the standard deviation between C_q values was low between the primer concentrations tested, and b) that each primer pair negative control for 300/300 nM were undetermined (except for one negative for bacteria), 300 nM for each primer was selected for all targets.

Specificity. Specificity of each primer pair was assessed using single source species, pools of single source DNA, and mixed soil and dust DNA samples (Fig 1 and S1–S8 Figs). *Bacteria* – All but one single source sample for bacteria generated C_q values. Notably, multiple melt curve peaks were identified; of 24 single source species screened, 15 had a single melt curve peak, whereas the remaining eight species had double melt curve peaks and one species had a triple melt curve peak. The phyla that encompass the multi-peak melt curves were Actinomycetota (n = 6), Pseudomonadota (n = 1), Verrucomicrobiota (n = 1), and Bacteroidota (n = 1). Species within these phyla have been reported to have multiple 16S rRNA genes within the genome [44] and thus the observation of multiple melt curve peaks could be attributed to gene duplication. The bacterial standard, eight soil samples, and six dust samples produced single melt curve peaks, whereas the remaining seven soil and six dust samples had double melt curve peaks, and even three dust samples had triple melt curve peaks. *Fungi* – All fungi samples and replicates had generated C_q values, and all melt curve analyses resulted in single peaks except for one fungal species (*Coemansia nantahalensis* [Kickxellales order]) that had a double melt curve peak for one replicate, but not the second replicate. *Arthropods* – All arthropod samples and replicates had determined C_q values except one single source sample (mealybug), one soil sample and two dust samples. Single melt curve peaks were produced for all single source species except for a mealybug species (that did not result in a melt curve peak), 11 soil samples, and nine dust samples. Four soil samples and five dust samples had double melt curve peaks and one dust sample had triple melt curve peaks. *Plants* - C_q values were generated for each sample and its replicate, as well as a single melt curve peak. Only one plant species (*Selaginella braunii* [Selaginellales order]) had a double melt curve peak at

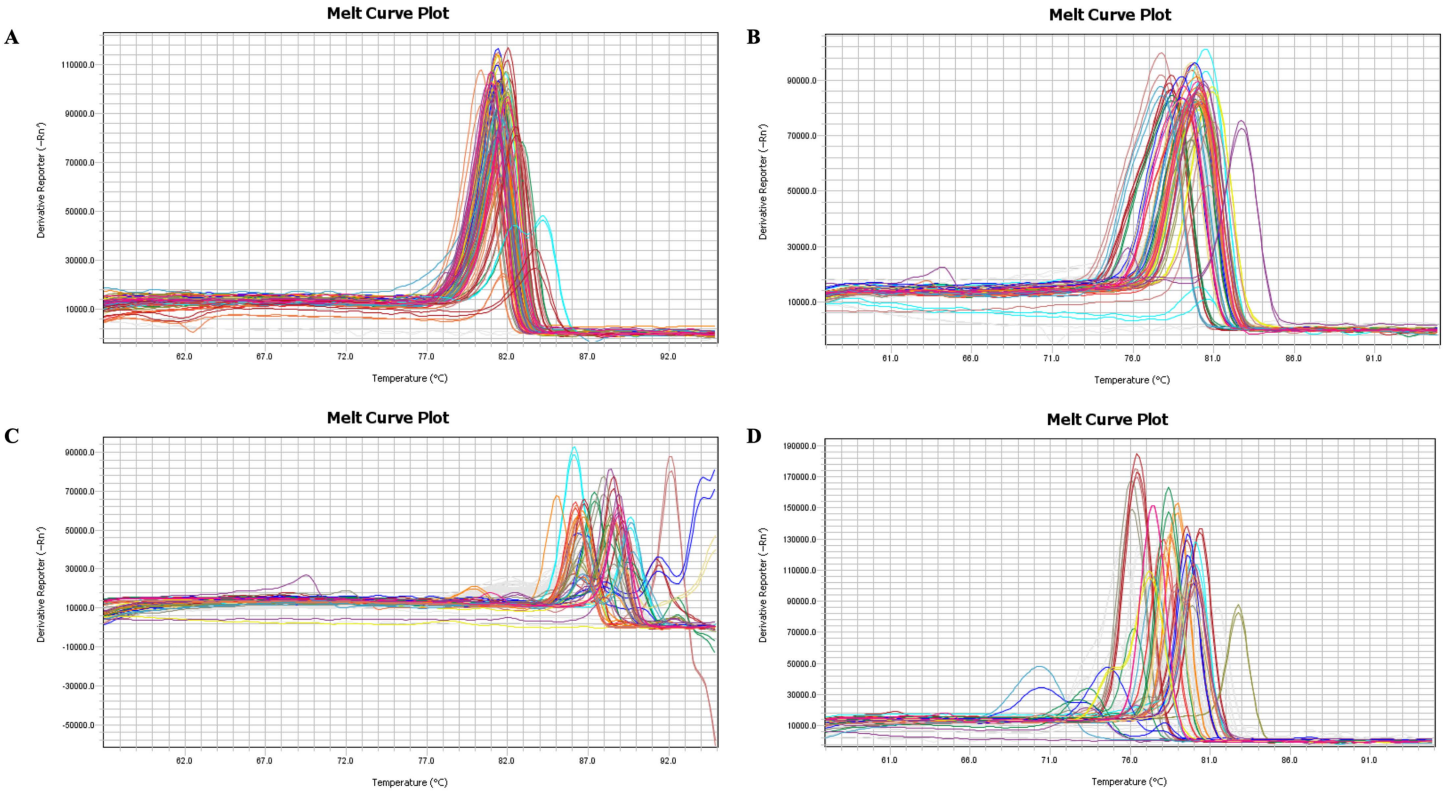

Fig 1. Melt curve temperature plots for all in group samples. Single source specimens for plants **(A)**, fungi **(B)**, bacteria **(C)**, and arthropods **(D)** underwent melt curve analysis for specificity testing of each primer pair. Each trace is a different sample. The y-axis is the derivative reporter fluorescence (-Rn') and the x-axis is temperature (°C). The peak of the melt curve is the temperature at which 50% of DNA is double stranded and 50% is single stranded.

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82.41°C and 84.20°C. Table 1 summarizes the mean, standard deviation, median, minimum, and maximum of C_q values for each primer pair and group.

Mean C_q values were compared for all groups (in, out, and mixed) for each primer pair and were found to be statistically different (Table 1). For bacteria, fungi, arthropods, and plants the in groups for each taxon were statistically different from the mixed groups (soil and dust) (Table 1). When comparing in *vs.* out (*e.g.,* plant primers used with non-plant DNA pools) groups within plants, fungi, and arthropods, the C_q values were significantly different, but were not for bacteria, and only arthropods were significantly different between the out *vs.* mixed group. It is important to recognize that the out groups should have n = 0; however, these assays are designed to quantify total taxon DNA, and it is possible that DNA from non-target species may be present in target samples (*i.e.,* bacterial species that were co-isolated with plant leaf tissue). S3 and S4 Tables provide summary statistics and comparisons, respectively, of the associated melt curve temperatures. The only statistically significant differences between groups were for plants and bacteria, where plant in *vs.* mixed groups were different, and bacterial in *vs.* mixed and in *vs.* out groups were different. The sample sizes for the out group samples were generally small, which may have been responsible for the lack of statistically significant differences between the out group and in or mixed groups. This is expected as a larger sample size of out groups would indicate the assays are not specific. Additionally, Sanger sequencing was used to confirm that primer pairs were amplifying the intended target if non-specific amplification occurred. For instance, when screening the plant *Oxalis crassipes* with bacterial primers, amplification occurred. Upon searching the resulting Sanger sequence data against GenBank, the best hit was to the bacterium, *Serratia marcescens*, likely co-isolated despite completing a 5% bleach wash of the plant tissue prior to DNA isolation. Reference intervals were also calculated for both in and mixed groups for each primer pair, but intervals were unable to be calculated for out groups given small sample sizes (Table 2). These intervals provided an estimate for expected melt curve peaks for each primer pair and whether the sample is single source or mixed.

Of note, mixed DNA samples (soil and dust) that were targeted with bacterial primers often had melt curve peaks that do not follow normal peak morphology for dye-based qPCR (*i.e.,* multiple peaks, short and broad) (S1D Fig). Considering many bacteria contain different copy numbers of the 16S rRNA gene [44], we hypothesize that the combination of numerous bacterial species and a range of 16S copy numbers present in environmental samples, impacted the melt curve analysis. Melt curve analysis was key in identifying primer dimer as the temperature was found in the low 60s°C and morphology of these peaks were short and wide compared to true amplicon signal (tall and narrow). We therefore identified

Table 1. Summary Statistics Using C_q Values for Each Primer Pair.

Primer	Group	n	mean	sd	median	min	max	statistic	In *vs.* out	In *vs.* mixed	Out *vs.* mixed
Bacteria	In	25	25.61	5.33	27.25	16.59	33.50	W = 9.694, p = 0.008	Z = −1.06, p = 0.290	Z = −3.11, p = 0.002	Z = 0.810, p = 0.418
	Mixed	30	20.47	2.46	19.01	18.01	26.08				
	Out[a]	6	22.96	5.58	20.61	18.08	30.20				
Fungi	In	31	22.02	5.28	20.29	15.55	39.63	W = 15.094, p = 0.001	Z = 3.35, p = 0.001	Z = 3.35, p = 0.001	Z = 1.09, p = 0.277
	Mixed	30	25.56	3.44	27.08	17.63	30.24				
	Out[a]	4	28.87	3.36	28.76	25.83	32.11				
Arthropods	In	24	23.22	7.03	19.94	15.74	37.82	W = 17.991, p < 0.001	Z = 4.10, p = < 0.001	Z = 2.10, p = 0.017	Z = 2.71, p = 0.007
	Mixed	30	27.79	4.05	28.23	16.81	34.18				
	Out[a]	6	34.98	1.58	34.48	33.27	37.21				
Plant	In	48	26.56	4.74	25.72	19.04	37.82	W = 13.698, p = 0.001	Z = 2.73, p = 0.019	Z = 2.81, p = 0.015	Z = 1.81, p = 0.213
	Mixed	30	30.38	5.15	29.48	22.07	37.33				
	Out[a]	2	38.7	0.30	38.70	38.49	38.92				

[a]Out groups should not have a C_q value, thus for out group samples that did have a reportable C_q, having a low n with a high C_q is appropriate.

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Table 2. Reference Intervals for Each Primer Pair Based on Melt Curve Temperatures (°C).

Primer	Group	95% RI		90% CI Lower		90% CI Upper	
Bacteria	In	82.87	93.22	81.42	84.31	91.64	94.94
	Mixed	81.64	92.10	80.67	83.22	91.62	93.30
Fungi	In	77.23	82.36	76.24	78.08	81.60	83.20
	Mixed	79.16	80.31	79.03	79.29	80.19	80.45
Arthropods	In	73.67	83.45	71.66	75.41	81.61	85.31
	Mixed	74.50	82.58	73.36	75.47	81.73	83.58
Plants	In	80.13	83.02	79.72	80.50	82.64	83.43
	Mixed	81.69	82.68	81.47	81.87	82.49	82.90

RI denotes reference interval; CI denotes confidence interval.

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that the arthropod 16S primers had a higher incidence of primer dimer, which fit the primer dimer peak morphology, (Fig 1D) compared to the other three assays that had minimal to no primer dimer.

Additionally, seven vertebrate species were also screened using each of the four primer pairs in duplicate. For bacteria, at least one replicate for each species had amplification (as observed by a recorded C_q value), but were all below the sensitivity threshold (see 3.1.8), except the sheep with an average C_q of 18.73. To confirm specificity, each vertebrate species was amplified via standard PCR with the bacterial primers, underwent Sanger sequencing, and the resulting sequences searched against NCBI's BLAST. The PCR product amplified using sheep DNA returned an uncultured Ruminobacter species and the amplicon product from the alligator DNA was identified as an uncultured Gammaproteobacteria species. The remaining sequences were too messy to query against BLAST, however since these are not sterilized specimens, it is likely that the PCR product contains multiple bacterial species. Surprisingly, the sequence generated from dog DNA returned a BLAST hit for a ncRNA belonging to *Canis lupis dingo* and mRNA for *Canis lupus familiaris*; this sequence was however ~35 bp longer than what was visualized on a 2.2% agarose Flash gel (Lonza, Basel, Switzerland). For arthropods, three of the seven vertebrate species did not have any amplification (alligator, sheep, and mourning dove), whereas only one replicate amplified for the northern water snake, and both replicates amplified for the bear, monkey and dog. However, the C_q values were below the sensitivity threshold for bear and snake, but within range for the monkey (31.09) and dog (30.81). As with the bacteria, standard PCR was performed and the resulting amplicons were Sanger sequenced and searched against BLAST for the black bear, rhesus monkey, and dog samples. The black bear sequence was too messy to search most likely due to its low quantity. The dog sequence returned a match to a giraffe species, *Giraffa tippelskirchi tippelskirchi*, and the rhesus monkey sequence matched to *Macaca mulatta*; both matched the mitochondrial genome. It is unclear how the dog sample was identified as a giraffe, but notably the generated Sanger sequence was not pristine *(i.e.,* some higher background noise), which may have impacted the BLAST results as the top hit had a percent identity of 93.44%, query coverage of 98%, and e-value of 6e-15. While giraffe was the best match, according to the Organization of Scientific Area Subcommittee's GenBank Standard, the reported BLAST statistics would not result in species identification at that level [45]. When comparing the melt curves, the melt curve temperatures fall in the range identified in Table 2 (82.85°C for rhesus monkey and 81.32°C for dog). Amplification did not occur for any of the seven vertebrate species with the fungal and plant primers.

Reproducibility – analyst. Analyst reproducibility was assessed for each instrument using mean C_q value and found to not be significantly different for all three instruments. The intraclass correlation coefficient (ICC) between analysts was determined for each instrument and found to have ICC values of 0.86 [StepOne Plus 1], 0.99 [QuantStudio 5], and 0.97 [StepOne Plus 2]. The ICC of 0.86 is considered good reliability and the ICC of 0.99 and 0.97 are excellent reliability, indicating that the two analysts' C_q values were in agreement. The mean melt curve temperatures between

the two StepOne Plus instruments were also not significantly different, however the mean melt curve temperatures were statistically different for the two analysts (83.16 *vs.* 83.10°C) using the QuantStudio 5 ($V = 273$, $p < 0.001$). This is possible when one analyst consistently produced values that were smaller compared to the other analyst. The statistics applied here indicated that the analysts were consistently different from each other, but the difference was not large (0.06°C). The resulting ICC between analysts for each instrument was 1, demonstrating excellent reliability, despite the minor melt curve temperature differences between analysts when using the QuantStudio 5.

Reproducibility – instrument. When comparing C_q values, these were found to be significantly different between StepOne Plus 1 and the QuantStudio 5 ($V = 50$, $p < 0.001$) and the StepOne Plus 2 and the QuantStudio 5 ($V = 364$, $p < 0.001$), but not for both StepOne Plus instruments ($V = 289$, $p = 0.253$). The ICC was also calculated across all three instruments and was 0.94. Despite the disagreement between the actual values between the QuantStudio 5 and both StepOne Plus instruments, the ratings are very highly correlated across the three instruments to where any single instrument reliably differentiates among samples in the same way. Mean and standard deviation melt curve temperatures are 83.37°C ± 3.94°C for the StepOne Plus 1, 83.50°C ± 3.95°C for the QuantStudio 5, and 83.30°C ± 3.97°C for the StepOne Plus 2. Instrument reproducibility was found to be significantly different across all three instruments when comparing mean melt curve temperatures: StepOne Plus 1 *vs*. QuantStudio 5 ($V = 76$, $p = 0.019$), StepOne Plus 1 *vs.* StepOne Plus 2 ($V = 389$, $p = < 0.001$), and QuantStudio 5 *vs.* StepOne Plus 2 ($V = 325$, $p = 0.004$). Despite the disagreement between values, the ICC was 1 across all three instruments, demonstrating that any single instrument reliably differentiates among samples in the same way.

Reproducibility – master mix. Master mix reproducibility was evaluated across two instruments, the QuantStudio 5 and StepOne Plus 1, and three master mixes. Luna® Universal qPCR Master Mix was found to be significantly different between instruments ($V = 192$, $p = 0.033$), however the ICC was determined to be 0.94, indicating that despite the differences in C_q values, the correlation between measures was high. The C_q values obtained using the GoTaq® qPCR Master Mix were not different between instruments ($t(17) = -1.40$, $p = 0.178$) and had an ICC of 0.89 and was considered good reliability. Lastly, the PowerTrack™ SYBR™ Green Master Mix was determined to be significantly different between the two instruments ($V = 22$, $p = 0.016$), however as seen with the Luna® Universal qPCR Master Mix, the ICC was 0.85, indicating that despite the differences in C_q values there was good reliability between the two instruments. When comparing the master mixes to each other, they were not significantly different when used on the StepOne Plus 1 and had an ICC of 0.83, which was considered good reliability. For the QuantStudio 5, the GoTaq master mix was significantly different from the Luna ($V = 6$, $p < 0.001$) and PowerTrack master mixes ($V = 127$, $p = 0.015$). The ICC was calculated across the three master mixes for QuantStudio 5, and was 0.79, demonstrating good reliability despite differences in C_q values.

When comparing melt curve temperatures across the different master mixes, the samples amplified with Luna had a slightly higher mean temperature when using the QuantStudio 5 (82.67°C) compared to the StepOne Plus (82.55°C) ($V = 5$, $p = 0.001$). The ICC between the two instruments was 1, indicating that the correlation between the two instruments was high with the Luna master mix. When using the GoTaq master mix, the mean melt curve temperature was higher on the QuantStudio 5 (81.91°C) compared to the StepOne Plus (81.72°C). These results were significant ($t(13) = -2.60$, $p = 0.022$), however the ICC of the two instruments when using GoTaq was 0.99 where the correlation between the two instruments was high. The PowerTrack master mix showed no significant differences between the QuantStudio 5 (82.85°C) and StepOne Plus (82.82°C) and the two instruments also had an ICC of 1. When comparing master mixes when using the StepOne Plus, the Luna master mix (81.79°C) was significantly different from the GoTaq master mix (81.43°C; $t(12) = 4.91$, $p < 0.001$), but not compared to PowerTrack (81.98°C; $t(13) = -1.43$, $p = 0.176$). GoTaq and PowerTrack were also significantly different from each other ($t(13) = -3.85$, $p = 0.002$). GoTaq had a lower mean melt curve temperature compared to the other two master mixes. The ICC was 0.98, signifying excellent reliability. Similar observations were seen for the QuantStudio 5, where GoTaq had a lower mean melt curve temperature (82.02°C) compared to

Luna (82.36°C) and PowerTrack (82.55°C) master mixes. GoTaq was significantly different from Luna (V = 84, p = 0.08) and PowerTrack (V = 12, p = 0.007), but Luna and PowerTrack are not statistically different (V = 29, p = 0.149). The ICC was also 0.98, showing excellent reliability despite significant differences.

Standard curve generation & linear dynamic range. Consensus sequences for each gBlock™ standard are provided in S5 Table with associated length information. Copy number was calculated for each gBlock™ using the mass of each dsDNA standard, the total amount of DNA in ng, and Avogadro's constant. A serial dilution starting with 10^7 copies and ending with 10^1 copies were prepared for each gBlock™ standard (S9 Fig). The highest standard, 10^8 copies, contained too much template resulting in reaction failure, and thus was not included in the standard curve. Regression analysis was used to determine the slope and R^2 values for each standard (Table 3). A range in slopes from −3.58 and −3.10 indicate an ideal reaction, with a slope of −3.3 signifying 100% amplification efficiency [46]. The slopes for these four assays were found to be higher than −3.3 indicating that PCR is less than 100% efficient. The slopes for the bacterial and arthropod assays fall within the acceptable slope range, however the plant and fungal assays have slopes that fall outside of this range (Table 3). Radwan and colleagues, who published the fungal primers for probe-based qPCR, reported their slopes as −3.0472 (single-plex), −3.3034 (multiplex with bacteria), and −4.202 (multiplex with bacteria and archaea) [33]. This disparity could potentially be due to the use of a dye-based assay compared to probe-based, additionally the cycling parameters employed in this research were different compared to those previously reported [33]. Specificity was also confirmed for each gBlock™ standard using the 10^5 dilutions with all primer pairs, in which all primer pairs were found to be specific to their gBlock™ standard.

Sensitivity. Sensitivity was assessed using 10 replicates of the lowest gBlock™ standard (10^1) for each primer pair. All 40 reactions successfully generated a C_q value and summary statistics can be found in Table 4. The lowest mean C_q was 29.43 for bacteria, followed by 30.26 for arthropods, 34.23 for plants, and the highest C_q of 34.46 for fungi. Mean melt curve temperatures for each primer pair were determined, with arthropods having the lowest temperature at 78.6°C and bacteria the highest at 87.94°C (Table 4). Reference intervals (95%) were also calculated for each standard with respect to melt curve temperature (Table 4). Importantly, the median, minimum, and maximum for each standard were within the reference interval described in Table 2, demonstrating their specificity. These assays exhibit sensitivity down to 10^1 copies and have minimal variation in their melt curve analysis. Importantly, the limit of detection is equivalent to the median C_q values obtained for each primer pair and samples generating C_q values that are higher than those reported should be considered inconclusive or below the limit of detection.

Inhibition. EDTA, humic acid, and tannic acid at varying concentrations were used to evaluate inhibition for each primer pair using two gBlock™ concentrations, 10^3 and 10^5 copies of DNA. Increases in C_q values indicated that the inhibitor used in the qPCR reaction had a negative impact on amplification when compared to the controls. Melt curve temperatures were also analyzed to determine if inhibition increased or decreased the temperature compared to the controls. EDTA did not impact qPCR when using the bacterial and arthropod primers, however C_q values were significantly different for the fungal and plant primers (S6 Table). Specifically, for the fungal primer pair with both gBlock™ quantities,

Table 3. Regression Analysis of gBlock™ Standard Curves.

Primer Pair	Slope	R^2	Standard Error
Bacteria	−3.56	0.999	0.031
Fungi	−4.11	0.998	0.050
Plants	−3.97	0.996	0.064
Arthropods	−3.52	0.993	0.074

Values calculated from three runs.

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Table 4. Sensitivity Assessment Utilizing the Lowest Standard of Each Primer Pair.

Standard	n	C_q Values					Melt Curve Temperatures (°C)						
		mean	sd	median	min	max	mean	sd	median	min	max	95% RI	
Bacteria	10	29.43	0.16	29.41	29.19	29.73	87.94	0.09	87.94	87.80	88.09	87.74	88.15
Fungi	10	34.46	0.40	34.30	34.19	35.55	80.04	0.05	80.05	79.98	80.15	79.91	80.16
Plant	10	34.23	0.27	34.23	33.82	34.63	81.72	0.11	81.72	81.61	81.90	81.44	81.99
Arthropod	10	30.62	0.75	30.63	29.62	32.18	78.60	0.07	78.59	78.52	78.74	78.43	78.76

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the 5000 μM EDTA concentration had C_q values that were significantly higher compared to the control. This was also observed for the plant primer pair when using 10^5 copies of DNA, and for the 10^3 copy number samples, statistical significance was observed, but following adjustment for multiple testing, the post-hoc tests could not identify any specific group differences (S6 Table). As for the melt curves for samples treated with EDTA, only the arthropod primer pair with 10^5 DNA copies was statistically significant, with the 5000 μM samples having a slightly lower temperature compared to the 1250 μM samples. The addition of humic acid resulted in a statistical difference for all primer pairs and gBlock™ concentrations with the exception of the fungal primers using 10^5 copies of DNA. Post-hoc testing identified that the C_q values were higher for 40 μg/mL treated samples compared to the control for 1) both gBlock™ concentrations when using the bacterial primers, 2) the 10^3 copies of DNA for the fungal primers, and 3) the 10^5 copies of DNA for the arthropod primer pair. The 40 μg/mL humic acid treated samples were significantly different from the 10 μg/mL treated samples for the arthropod primers at 10^3 copies and for the plant primers at 10^5 copies. There were not specific differences identified for the plant primers using 10^3 copy numbers (S6 Table). With respect to melt curve temperatures, all primer pairs and gBlock™ concentrations were significantly different. Post-hoc testing identified that the 40 μg/mL treated humic acid samples were slightly lower compared to the 10 μg/mL treated samples for the bacterial primers (10^3 copies) and arthropod primers (10^5 copies). The 40 μg/mL treated samples were also slightly lower compared the control (0 μg/mL of humic acid) for the fungal primers (10^3 copies), arthropod primers (10^3 copies), and plant primers (10^5 copies). No specific differences were identified for 10^5 copy number samples using the bacterial and fungal primers as well as the 10^3 copy number samples with the plant primers (S6 Table). Lastly, qPCR reactions with the addition of tannic acid were statistically significant for all gBlock™ concentrations and primer pairs except for the fungal primers. The 75 μg/mL tannic acid treated samples had higher C_q values compared to the controls when using the bacterial primers (10^3 copies) and arthropod primers (10^5 copies). Similar observations were seen for the 50 μg/mL and 25 μg/mL tannic acid treated samples compared to the control for the plant primers at 10^3 copies and 10^5 copies, respectively. Specific differences were unable to be identified via post-hoc testing for the bacterial primers (10^5 copies) and arthropod primers (10^3 copies) (S6 Table). The only statistical difference observed for melt curve temperatures with tannic acid was the arthropod primer pair using the 10^5 copy gBlock™, where the 75 μg/mL tannic acid samples had a slightly lower temperature compared to the 25 μg/mL samples (S6 Table).

When looking across each inhibitor, there was not a consistent pattern that was apparent (*e.g.,* all high inhibitor concentrations have higher C_q values compared to controls for all primer pairs), nor does a specific DNA copy number appear to be affected more by inhibition than the other. Interestingly, not all reactions that had significant C_q values had significant differences observed for melt curve temperatures. It is evident that these assays were able to tolerate various levels of inhibitor, particularly 1250 μM and 2500 μM of EDTA, 10 μg/mL and 20 μg/mL of humic acid, and 12.5 μg/mL of tannic acid. While not all reactions with each primer pair were affected by high inhibitor concentrations, a large proportion resulted in delayed C_q values. If inhibition is suspected, additional DNA purification may be needed. Additionally, other inhibitors that may be encountered (*e.g.*, melanin) should undergo testing to characterize the level of inhibition that may be observed. While advances to DNA isolation kits to remove inhibitors and polymerases to be tolerant to inhibitors have been made, there could be some scenarios in which inhibition could negatively impact quantification results.

***In silico* analysis.** Each qPCR primer pair was assessed with the *in silico* primer tool, ecoPCR, for specificity. A total of 14,710 *psbA*, 22,652 18S, and 26,152 16S eukaryotic RefSeq sequences were downloaded for analysis. Additionally, 38,138 RefSeq bacterial assemblies were downloaded which contained 86,817 complete genome and plasmid sequences. Table 5 describes the results from each *in silico* analysis. For plants, 81.9% of plant sequences were amplified and had a median forward and reverse primer mismatch of 0 and 2, respectively. A mismatch of 0 is perfect annealing of primer to template DNA, whereas a mismatch of 2 is an imperfect annealing, where two nucleotides of template will not be able to anneal to the primer, which may impact amplification. Only 3.7% of non-plant sequences were amplified and had a median of three mismatches for both the forward and reverse primers. The taxa comprising the 3.7% non-specific amplification at the highest taxonomic ranking included two clades (Sar [n = 1] and Discoba [n = 2]) and seven phyla within the bacteria kingdom (Rhodophyta [n = 5], Pseudomonadota [n = 5], Fusobacteriota [n = 1], Verrucomicrobiae [n = 1], Cyanobacteriota [n = 58], Bacillota [n = 3], Actinomycetota [n = 15]). With bacteria, each assembly downloaded from NCBI may have had more than one genome or plasmid present and thus the number of genomes/plasmids differed from the number of assembly entries. Therefore, amplification success was determined based on number of assemblies, where 97.6% of bacterial assemblies had at least one sequence amplified using ecoPCR. The median number of mismatches was zero for the forward primer and one for the reverse. The ecoPCR output also provided the melting temperature for primers that did not have any degenerate bases. Given the reverse bacteria primer did not contain degenerates, the tool calculated median melting temperature was 50.1°C. The bacterial primers were also tested against the 16S eukaryotic sequences, where 23,427 sequences had been amplified *in silico*, belonging to 12,002 accession numbers, none of which were arthropod sequences. The median amplicon size was 133 bp, the median melting temperature was 48.64°C, and median forward and reverse primer mismatches were one and two, respectively. Fungi had an amplification success rate of only 29.1% and a median of zero mismatches for both primers. Fungal primers had non-specific amplification at 0.08%, where the phylum Streptophyta (n = 4), kingdom Metazoa (n = 1), and clade Sar (n = 5) were included. Lastly, 97.1% of arthropod sequences were amplified *in silico* with zero median mismatches for both primers and a median melting temperature of 50.06°C. The non-specificity rate against 16S eukaryotic sequences is 37.2% with a median of

Table 5. Summary of *in silico* analysis using ecoPCR.

	16S (Bacteria)	18S (Fungi)	*psbA* (Plant)	16S (Arthropods)
No. of RefSeq Sequences	Assemblies: 38,138 Genomes & Plasmids: 86,817	Fungi: 10,655 Non-fungi: 11,997 ***TOTAL***: 22,652	Plant: 12,233 Non-plant: 2,477 ***TOTAL***: 14,710	Arthropods: 5,083 Non-arthropods: 21,069 ***TOTAL***: 26,152
No. of Sequences Returned from ecoPCR	204,450 (39,158[a] from 37,223 unique assembly numbers)	Fungi: 3,105 (2,819)[a] Non-fungi: 24 (10)[a] ***TOTAL***: 3,129	Plant: 10,239 (10,021)[a] Non-plant: 101 (91)[a] ***TOTAL***: 10,444	Arthropods: 4,974 (4,936)[a] Non-arthropods: 7,830 (7,828)[a] ***TOTAL***: 12,804
Median Forward Primer Mismatch	0	Fungi: 0 Non-fungi: 3	Plant: 0 Non-plant: 3	Arthropods: 0 Non-arthropods: 2
Median Reverse Primer Mismatch	1	Fungi: 0 Non-fungi: 3	Plant: 0 Non-plant: 3	Arthropods: 0 Non-arthropods: 2
Median Amplicon Size[b]	133 bp	Fungi: 64 bp Non-fungi: 63 bp	Plant: 68 bp Non-plant: 67 bp	Arthropods: 142 bp Non-arthropods: 142 bp
Reverse T_m	50.1°C	*NA*	*NA*	Arthropods: 50.06°C Non-arthropods: 37.41°C

[a]Number in parentheses indicates the number of unique accession numbers from ecoPCR analysis.

[b]Median amplicon size is the number of base pairs without the primer sequences.

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two mismatches for both primers, however median melting temperature is 37.41°C. Arthropod primers were also tested against the bacterial genomes and plasmids, where only two sequences were amplified: one from *Mycoplasma tauri* (3 mismatches for both primers and amplicon size of 143 bp) and the other from a *Wolbachia* endosymbiont of *Corcyra cephalonica* (rice moth; 3 mismatches for the forward and 0 for the reverse primers and an amplicon size of 142 bp).

The plant, bacterial, and arthropod primer pairs demonstrate a high amplification success and high specificity. Since ecoPCR is unable to determine melting temperatures for sequences when using primers with degenerate bases, median temperatures were not calculated for plants and fungi. Considering the higher percentage of non-specific amplification of arthropod primers (37.2%), the median melting temperature was 37.41°C, which is well below the annealing temperature of 57°C, and thus this rate would drastically reduce *in vitro*, as the annealing temperature should be a few degrees lower than melting temperature. With the fungal primers, only 29.1% of sequences were amplified, which was lower than expected. However, *in vitro* experiments indicated that these primers amplified all single source species successfully as observed in Radwan and colleagues [33].

As with most scientific studies, limitations are often present. One notable challenge when working with environmental samples is the inability to detect all species that may be present, or the potential of detecting non-target species. The primers selected for these assays were found to be the best performers compared to many others identified from the scientific literature. It is impossible to screen primers in wet-laboratory experiments against every species on Earth to demonstrate 100% specificity. Therefore, there is the possibility that the primer pairs validated in this paper could amplify non-target taxa. Notably, results from *in vitro* and *in silico* experiments have indicated that each of the four primer pairs are highly specific, and non-specificity occurrence is low. We have identified low level cross-reactivity with the arthropod primers, which amplified mitochondrial DNA from two vertebrate species, however since there is currently not a qPCR assay on the market to quantify total arthropod DNA, this assay is still an important starting point for quantification. Given the broad range of taxa screened during this validation, the specificity of the arthropod primer pair is still considered high, however researchers should take caution if they suspect cross-reactivity. Implementation of these assays into a laboratory should consider the laboratory's own validation studies, including specificity, especially if bulk environmental sources other than soil and dust are intended for use. Furthermore, since the plant and fungal assays had standard curve slopes that fell outside of the ideal range, additional validation studies exploring primer annealing temperature effect on PCR efficiency should be undertaken. Given this study only assessed inhibition with target DNA, validations should also include an assessment of non-target DNA in the presence of inhibitors. Future work is needed to modify the arthropod primer pairs to reduce this cross-reactivity, noting that *in silico* screening of all arthropods and vertebrates is not feasible. Given the 1 ng total input of DNA and low-level amplification observed here (indicated by higher C_q values), it is hypothesized that non-target species would contribute to a lower signal compared to the overall sample concentration of arthropods in a mixed DNA sample of unknown origin, however further investigation is required to support this hypothesis.

Conclusions

We have developed four dye-based SYBR Green qPCR assays that separately quantify the total amount of bacterial, fungal, plant, and arthropod DNA. These assays have undergone rigorous validation testing employing both *in silico* and *in vitro* methods while utilizing single source and mixed source environmental sample types. The primer pairs selected yielded short amplicons to permit quantification of samples containing degraded DNA templates and are broad enough to be highly specific to the taxon of interest, while also minimizing non-specificity. Melt curve temperatures have also been identified for each in and mixed groups to be used as a range for specificity confirmation. Reproducibility was achieved between analysts, and although instrumentation did differ, reliability was still observed. These assays were able to consistently detect 10^1 copies of template and the development of gBlock™ standards provided the ability to perform absolute quantification with a linear dynamic range of 10^7 down to 10^1 copy numbers.

Supporting information

S1 Table. Primers and Associated Sequences Selected for *in vitro* Testing.
(DOCX)

S2 Table. Average (n = 4) C_q Value for Each Annealing Temperature Assessed Using Four Species for Each Taxon.
(DOCX)

S3 Table. Summary Statistics Using Melt Curve Temperatures (°C) for Each Primer Pair.
(DOCX)

S4 Table. Statistical Analysis of Groups Using Melt Curve Temperatures for Each Primer Pair.
(DOCX)

S5 Table. gBlock™ Standard Sequences. Highlighted sequences are primer binding regions and red nucleotides are bases that were degenerate in the alignment/consensus but changed to a singular base for synthesis.
(DOCX)

S6 Table. Inhibition Summary Statistics Using C_q Values and Melt Curve Temperatures (°C) for Each Primer Pair and Inhibitor.
(DOCX)

S1 Fig. Amplification Curve Plots for Samples Tested with the Bacterial Primers. Amplification curves are displayed for the gBlock™ standards (A), single source bacterial samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the baseline-corrected normalized reporter (ΔRn) and the x-axis is quantification cycle (C_q). Samples in panels B and C were amplified on the same plate and therefore have the same negative control (NC). Samples in panels D and E were amplified on the same plate and therefore have the same negative control. Negative controls were included on every plate.
(DOCX)

S2 Fig. Amplification Curve Plots for Samples Tested with the Fungal Primers. Amplification curves are displayed for the gBlock™ standards (A), single source fungal samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the baseline-corrected normalized reporter (ΔRn) and the x-axis is quantification cycle (C_q). Samples in panels B and C were amplified on the same plate and therefore have the same negative control (NC). Samples in panels D and E were amplified on the same plate and therefore have the same negative control. Negative controls were included on every plate and the absence of a control in a panel indicates there was no amplification.
(DOCX)

S3 Fig. Amplification Curve Plots for Samples Tested with the Plant Primers. Amplification curves are displayed for the gBlock™ standards (A), single source plant samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the baseline-corrected normalized reporter (ΔRn) and the x-axis is quantification cycle (C_q). Samples in panels C, D and E were amplified on the same plate and therefore have the same negative control (NC). Negative controls were included on every plate and the absence of a control in a panel indicates there was no amplification.
(DOCX)

S4 Fig. Amplification Curve Plots for Samples Tested with the Arthropod Primers. Amplification curves are displayed for the gBlock™ standards (A), single source arthropod samples [in group] (B), soil samples [mixed] (C), dust

samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the baseline-corrected normalized reporter (ΔRn) and the x-axis is quantification cycle (C_q). Samples in panels B, C and D were amplified on the same plate and therefore have the same negative control (NC). Negative controls were included on every plate and the absence of a control in a panel indicates there was no amplification.
(DOCX)

S5 Fig. Melt Curve Temperature Plots for Samples Tested with Arthropod Primers. Melt curve peaks are displayed gBlock™ standards (A), single source arthropod samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the derivative reporter fluorescence (-Rn') and the x-axis is temperature (°C). The peak of the melt curve is the temperature at which 50% of the DNA is double stranded and 50% is single stranded. Samples in panels B and C were amplified on the same plate and therefore have the same negative control (NC) colored in teal. Samples in panels D and E were amplified on the same plate and therefore have the same negative control colored yellow. Negative controls were included on every plate and the absence of a control in a panel indicates there was no amplification.
(DOCX)

S6 Fig. Melt Curve Temperature Plots for Samples Tested with Bacterial Primers. Melt curve peaks are displayed gBlock™ standards (A), single source bacterial samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the derivative reporter fluorescence (-Rn') and the x-axis is temperature (°C). The peak of the melt curve is the temperature at which 50% of the DNA is double stranded and 50% is single stranded. Samples in panels B and C were amplified on the same plate and therefore have the same negative control (NC) colored light yellow. Samples in panels D and E were amplified on the same plate and therefore have the same negative control colored light red. Negative controls were included on every plate.
(DOCX)

S7 Fig. Melt Curve Temperature Plots for Samples Tested with Fungal Primers. Melt curve peaks are displayed gBlock™ standards (A), single source fungal samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the derivative reporter fluorescence (-Rn') and the x-axis is temperature (°C). The peak of the melt curve is the temperature at which 50% of the DNA is double stranded and 50% is single stranded. Samples in panels B and C were amplified on the same plate and therefore have the same negative control (NC). Samples in panels D and E were amplified on the same plate and therefore have the same negative control. Negative controls were included on every plate and the absence of a control in a panel indicates there was no amplification.
(DOCX)

S8 Fig. Melt Curve Temperature Plots for Samples Tested with Plant Primers. Melt curve peaks are displayed gBlock™ standards (A), single source plant samples [in group] (B), soil samples [mixed] (C), dust samples [mixed] (D), and non-target DNA pools [out group] (E). Each trace is an individual sample. The y-axis is the derivative reporter fluorescence (-Rn') and the x-axis is temperature (°C). The peak of the melt curve is the temperature at which 50% of the DNA is double stranded and 50% is single stranded. Samples in panels C, D, and E were amplified on the same plate and therefore have the same negative control (NC). Negative controls were included on every plate and the absence of a control in a panel indicates there was no amplification.
(DOCX)

S9 Fig. Standard Curves for Each Primer Pair. Standard curves were developed for each qPCR assay using a linear dynamic range of 10^7 and 10^1 copies. An example of the standard curve for bacteria (A), fungi (B), arthropods (C), and plants (D) using gBlocks™ is provided. Red points on each curve are the standards plotted in duplicate. Blue points are the

negative controls that recorded a C_q value. The negative control for the fungal and plant example did not have a C_q value, and thus could not be plotted.
(DOCX)

S1 File. Taxa used for Primer Screening.
(XLSX)

S2 File. List of Scientific Papers Containing Screened Primers.
(XLSX)

S3 File. Reference material.
(XLSX)

S4 File. Consensus alignment.
(XLSX)

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Author contributions

Conceptualization: Teresa M. Tiedge, Kelly A. Meiklejohn.

Data curation: Teresa M. Tiedge.

Formal analysis: Teresa M. Tiedge, Kim R. Love.

Funding acquisition: Teresa M. Tiedge, Kelly A. Meiklejohn.

Investigation: Teresa M. Tiedge.

Methodology: Teresa M. Tiedge, Kelly A. Meiklejohn.

Project administration: Kelly A. Meiklejohn.

Resources: Kelly A. Meiklejohn.

Software: Kim R. Love.

Supervision: Kelly A. Meiklejohn.

Validation: Teresa M. Tiedge.

Visualization: Teresa M. Tiedge.

Writing – original draft: Teresa M. Tiedge, Kelly A. Meiklejohn.

Writing – review & editing: Teresa M. Tiedge, Kim R. Love, Kelly A. Meiklejohn.

References

1. van der Heyde M, Bunce M, Wardell-Johnson G, Fernandes K, White NE, Nevill P. Testing multiple substrates for terrestrial biodiversity monitoring using environmental DNA metabarcoding. Mol Ecol Resour. 2020;20(3). https://doi.org/10.1111/1755-0998.13148 PMID: 32065512

2. Langlois VS, Allison MJ, Bergman LC, To TA, Helbing CC. The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. Environmental DNA. 2020;3(3):519–27. https://doi.org/10.1002/edn3.164
3. Manzari C, Oranger A, Fosso B, Piancone E, Pesole G, D'Erchia AM. Accurate quantification of bacterial abundance in metagenomic DNAs accounting for variable DNA integrity levels. Microb Genom. 2020;6(10):mgen000417. https://doi.org/10.1099/mgen.0.000417 PMID: 32749951
4. Liu CM, Aziz M, Kachur S, Hsueh P-R, Huang Y-T, Keim P, et al. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. BMC Microbiol. 2012;12:56. https://doi.org/10.1186/1471-2180-12-56 PMID: 22510143
5. Liu CM, Kachur S, Dwan MG, Abraham AG, Aziz M, Hsueh P-R, et al. FungiQuant: a broad-coverage fungal quantitative real-time PCR assay. BMC Microbiol. 2012;12:255. https://doi.org/10.1186/1471-2180-12-255 PMID: 23136846
6. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology (Reading). 2002;148(Pt 1):257–66. https://doi.org/10.1099/00221287-148-1-257 PMID: 11782518
7. Kikkawa HS, Tsuge K, Sugita R. Real-Time PCR Quantification of Chloroplast DNA Supports DNA Barcoding of Plant Species. Mol Biotechnol. 2016;58(3):212–9. https://doi.org/10.1007/s12033-016-9918-1 PMID: 26800992
8. Beule L, Lehtsaar E, Corre MD, Schmidt M, Veldkamp E, Karlovsky P. Poplar Rows in Temperate Agroforestry Croplands Promote Bacteria, Fungi, and Denitrification Genes in Soils. Front Microbiol. 2020;10:3108. https://doi.org/10.3389/fmicb.2019.03108 PMID: 32038551
9. Federal Bureau of Investigation FBI. Quality Assurance Standards for Forensic DNA Testing Laboratories. 2020. https://www.swgdam.org/_files/ugd/4344b0_d73afdd0007c4ed6a0e7e2ffbd6c4eb8.pdf
10. Young JM, Weyrich LS, Cooper A. Forensic soil DNA analysis using high-throughput sequencing: a comparison of four molecular markers. Forensic Sci Int Genet. 2014;13:176–84. https://doi.org/10.1016/j.fsigen.2014.07.014 PMID: 25151602
11. Young JM, Weyrich LS, Breen J, Macdonald LM, Cooper A. Predicting the origin of soil evidence: High throughput eukaryote sequencing and MIR spectroscopy applied to a crime scene scenario. Forensic Sci Int. 2015;251:22–31. https://doi.org/10.1016/j.forsciint.2015.03.008 PMID: 25839677
12. Young JM, Weyrich LS, Cooper A. High-throughput Sequencing of Trace Quantities of Soil Provides Reproducible and Discriminative Fungal DNA Profiles. J Forensic Sci. 2016;61(2):478–84. https://doi.org/10.1111/1556-4029.12996 PMID: 27404621
13. Boggs LM, Scheible MKR, Machado G, Meiklejohn KA. Single Fragment or Bulk Soil DNA Metabarcoding: Which is Better for Characterizing Biological Taxa Found in Surface Soils for Sample Separation? Genes (Basel). 2019;10(6):431. https://doi.org/10.3390/genes10060431 PMID: 31174412
14. Fierer N, Lauber CL, Zhou N, McDonald D, Costello EK, Knight R. Forensic identification using skin bacterial communities. Proc Natl Acad Sci U S A. 2010;107(14):6477–81. https://doi.org/10.1073/pnas.1000162107 PMID: 20231444
15. Metcalf JL, Xu ZZ, Bouslimani A, Dorrestein P, Carter DO, Knight R. Microbiome Tools for Forensic Science. Trends Biotechnol. 2017;35(9):814–23. https://doi.org/10.1016/j.tibtech.2017.03.006 PMID: 28366290
16. Tiedge TM, Meiklejohn KA. Assessing three soil removal methods for environmental DNA analysis of mock forensic geology evidence. J Forensic Sci. 2024;69(1):52–9. https://doi.org/10.1111/1556-4029.15399 PMID: 37839019
17. Kodama WA, Xu Z, Metcalf JL, Song SJ, Harrison N, Knight R, et al. Trace Evidence Potential in Postmortem Skin Microbiomes: From Death Scene to Morgue. J Forensic Sci. 2019;64(3):791–8. https://doi.org/10.1111/1556-4029.13949 PMID: 30408195
18. Hemkemeyer M, Dohrmann AB, Christensen BT, Tebbe CC. Bacterial Preferences for Specific Soil Particle Size Fractions Revealed by Community Analyses. Front Microbiol. 2018;9:149. https://doi.org/10.3389/fmicb.2018.00149 PMID: 29527192
19. Hemkemeyer M, Christensen BT, Tebbe CC, Hartmann M. Taxon-specific fungal preference for distinct soil particle size fractions. European Journal of Soil Biology. 2019;94:103103. https://doi.org/10.1016/j.ejsobi.2019.103103
20. Fierer N, Jackson JA, Vilgalys R, Jackson RB. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol. 2005;71(7):4117–20. https://doi.org/10.1128/AEM.71.7.4117-4120.2005 PMID: 16000830
21. Fox A, Ikoyi I, Torres-Sallan G, Lanigan G, Schmalenberger A, Wakelin S, et al. The influence of aggregate size fraction and horizon position on microbial community composition. Applied Soil Ecology. 2018;127:19–29. https://doi.org/10.1016/j.apsoil.2018.02.023
22. Lauber CL, Strickland MS, Bradford MA, Fierer N. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biology and Biochemistry. 2008;40(9):2407–15. https://doi.org/10.1016/j.soilbio.2008.05.021
23. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559–63. https://doi.org/10.1038/nature12820 PMID: 24336217
24. Dimitriu PA, Iker B, Malik K, Leung H, Mohn WW, Hillebrand GG. New Insights into the Intrinsic and Extrinsic Factors That Shape the Human Skin Microbiome. mBio. 2019;10(4):e00839–19. https://doi.org/10.1128/mBio.00839-19 PMID: 31266865
25. Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI, et al. The vaginal microbiome and preterm birth. Nat Med. 2019;25(6):1012–21. https://doi.org/10.1038/s41591-019-0450-2 PMID: 31142849
26. Ariza M, Fouks B, Mauvisseau Q, Halvorsen R, Alsos IG, de Boer HJ. Plant biodiversity assessment through soil eDNA reflects temporal and local diversity. Methods Ecol Evol. 2022;14(2):415–30. https://doi.org/10.1111/2041-210x.13865
27. Dopheide A, Tooman LK, Grosser S, Agabiti B, Rhode B, Xie D, et al. Estimating the biodiversity of terrestrial invertebrates on a forested island using DNA barcodes and metabarcoding data. Ecol Appl. 2019;29(4):e01877. https://doi.org/10.1002/eap.1877 PMID: 30811075

28. Dunn RR, Fierer N, Henley JB, Leff JW, Menninger HL. Home life: factors structuring the bacterial diversity found within and between homes. PLoS One. 2013;8(5):e64133. https://doi.org/10.1371/journal.pone.0064133 PMID: 23717552
29. Yoccoz NG, Bråthen KA, Gielly L, Haile J, Edwards ME, Goslar T, et al. DNA from soil mirrors plant taxonomic and growth form diversity. Mol Ecol. 2012;21(15):3647–55. https://doi.org/10.1111/j.1365-294X.2012.05545.x PMID: 22507540
30. Hassan S, Sabreena, Poczai P, Ganai BA, Almalki WH, Gafur A, et al. Environmental DNA Metabarcoding: A Novel Contrivance for Documenting Terrestrial Biodiversity. Biology (Basel). 2022;11(9):1297. https://doi.org/10.3390/biology11091297 PMID: 36138776
31. Roger F, Ghanavi HR, Danielsson N, Wahlberg N, Löndahl J, Pettersson LB, et al. Airborne environmental DNA metabarcoding for the monitoring of terrestrial insects—A proof of concept from the field. Environmental DNA. 2022;4(4):790–807. https://doi.org/10.1002/edn3.290
32. Dorn-In S, Bassitta R, Schwaiger K, Bauer J, Hölzel CS. Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. J Microbiol Methods. 2015;113:50–6. https://doi.org/10.1016/j.mimet.2015.04.001 PMID: 25863142
33. Radwan O, Gunasekera TS, Ruiz ON. Robust Multiplex Quantitative Polymerase Chain Reaction Assay for Universal Detection of Microorganisms in Fuel. Energy Fuels. 2018;32(10):10530–9. https://doi.org/10.1021/acs.energyfuels.8b02292
34. Clarke LJ, Soubrier J, Weyrich LS, Cooper A. Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. Mol Ecol Resour. 2014;14(6):1160–70. https://doi.org/10.1111/1755-0998.12265 PMID: 24751203
35. Zymo R. ZymoBIOMICS™ DNA Miniprep Kit ver. 1.5.2. 2021. https://files.zymoresearch.com/protocols/_d4300t_d4300_d4304_zymobiomics_dna_miniprep_kit.pdf
36. Meiklejohn KA, Jackson ML, Stern LA, Robertson JM. A protocol for obtaining DNA barcodes from plant and insect fragments isolated from forensic-type soils. Int J Legal Med. 2018;132(6):1515–26. https://doi.org/10.1007/s00414-018-1772-1 PMID: 29423711
37. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611–22. https://doi.org/10.1373/clinchem.2008.112797 PMID: 19246619
38. Applied Biosystems. PowerTrack™ SYBR™ Green Master Mix User Guide. 2020. https://www.thermofisher.com/document-connect/document-connect.html?url= https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0018825_PowerTrackSYBRGreenMasterMix_UG.pdf
39. Staroscik A. Copy number calculator for realtime PCR. Science Primer. https://scienceprimer.com/copy-number-calculator-for-realtime-pcr. 2011–Accessed 2022. Accessed 2024 March 15.
40. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors - occurrence, properties and removal. J Appl Microbiol. 2012;113(5):1014–26. https://doi.org/10.1111/j.1365-2672.2012.05384.x PMID: 22747964
41. Kavlick MF. Development of a triplex mtDNA qPCR assay to assess quantification, degradation, inhibition, and amplification target copy numbers. Mitochondrion. 2019;46:41–50. https://doi.org/10.1016/j.mito.2018.09.007 PMID: 30261278
42. Ficetola GF, Coissac E, Zundel S, Riaz T, Shehzad W, Bessière J, et al. An in silico approach for the evaluation of DNA barcodes. BMC Genomics. 2010;11:434. https://doi.org/10.1186/1471-2164-11-434 PMID: 20637073
43. Koo TK, Li MY. A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. J Chiropr Med. 2016;15(2):155–63. https://doi.org/10.1016/j.jcm.2016.02.012 PMID: 27330520
44. Větrovský T, Baldrian P. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS One. 2013;8(2):e57923. https://doi.org/10.1371/journal.pone.0057923 PMID: 23460914
45. The Organization of Scientific Area Committees for Forensic Science. ANSI/ASB Standard 180 Standard for the Use of GenBank for Taxonomic Assignment of Wildlife. 2024. https://www.aafs.org/asb-standard/standard-selection-and-evaluation-genbankr-results-taxonomic-assignment-wildlife
46. Life Technologies. Real-Time PCR Handbook. 2014.