

Using metagenomic methods to detect organismal contaminants in microbial materials.

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ABSTRACT

High sensitivity methods such as next generation sequencing and PCR are adversely impacted by organismal and DNA contaminants. Current methods for detecting contaminants in microbial materials (genomic DNA and cultures) are not sensitivity enough and require either a known or culturable contaminant. Therefore, higher sensitivity methods not requiring *a priori* assumptions about the contaminant or that the organism is culturable are needed. We demonstrate the use of whole genome sequencing data and a metagenomic taxonomic classification algorithm for assessing the organismal purity of a microbial material. Using whole genome sequencing and a taxonomic classification algorithm we characterized the types of false positive contaminants reported by the method and how the detectable contaminant concentration varies with material and contaminant genome and contaminant proportion using simulated whole genome sequencing data. The application of this method to characterizing the purity of a microbial material will help to ensure that the materials used to validate pathogen detection assays, generate genome assemblies for database submission, or benchmarking sequencing methods are free of contaminants that would adversely impact measurement results.

Keywords: Biodetection, Microbial Material, Reference Material, Purity, Bioinformatics

INTRODUCTION

Contaminants interfering with measurements is a common problem. The required purity level changes with measurement method sensitivity. High sensitivity methods such as PCR and next generation sequencing require higher material and reagent purity than traditional microbiology methods such as culturing, biochemical tests, and microscopy. Issues related to reagent contaminants have been well documented and addressed with improved methods for removing contaminants (Woyke et al., 2011; Motley et al., 2014), negative controls (Jervis-Bardy et al., 2015), and post processing of sequence data (Mukherjee et al., 2015). However, contaminants in microbial materials such as non-axenic cellular materials (Shrestha et al., 2013) and genomic materials with foreign DNA contaminants have only been addressed in data processing (Tennessen et al., 2015). High sensitivity methods to detect and characterize contaminants in microbial materials are needed.

Shotgun metagenomic sequencing is used to characterize environmental samples and detect pathogens in clinical samples. Shotgun metagenomic sequencing can also be used to detect contaminants in microbial materials. Microbial materials free of contaminants are needed; to populate sequence databases (Parks et al., 2015), for mock communities used to validate metagenomic methods (Bokulich et al., 2016), biodetection assay validation (Ieven et al., 2013; Coates et al., 2011), basic research using model systems (Shrestha et al., 2013). Current methods for detecting contaminants in microbial materials use traditional methods such as culture, microscopy, and polymerase chain reaction (PCR). Culture and microscopy-based methods lack the required sensitivity for NGS and PCR applications, are not appropriate for genomic DNA materials, and assumes the contaminants are phenotypically distinct from the material isolate it is contaminating. While PCR-based methods can detect contaminants in genomic DNA, the methods are limited as they can only detect targeted contaminants and not amenable to high-throughput applications (Heck et al., 2016; Marron et al., 2013). In contrast to these methods, shotgun metagenomic methods can be used to detect contaminants in both cell cultures and genomic DNA materials while only requiring the contaminant has sequencing reads that differentiate it from the material

46 strain.

47 Shotgun metagenomics consists of two main steps, whole genome sequencing of genomic DNA,
48 and analyzing the resulting sequencing data, most commonly using a taxonomic assignment algorithm
49 (Thomas et al., 2012). For genomic DNA materials, the material itself is sequenced, whereas genomic
50 DNA must be extracted from cell cultures prior to sequencing. After sequencing, a taxonomic assignment
51 algorithm is used to characterize the sequencing data. There is a variety of classification algorithms with
52 varying accuracy and computational performance (Bazinet and Cummings, 2012; Menzel et al., 2016).
53 All methods require a reference database. In order to detect a contaminant in a microbial material, the
54 contaminating organism (or an organism more closely related to the contaminant than the material) is in
55 the database. As taxonomic classification algorithms are constantly improving, reference databases are
56 expanding, and the cost of sequencing drops, shotgun metagenomic sequencing provides an alternative
57 to current methods for detecting contaminants in microbial materials.

58 In this work, we present the results from an *in-silico* assessment method to evaluate the suitability
59 of whole genome sequencing data combined with a taxonomic assignment algorithm for detecting
60 contaminant DNA. This work provides a baseline assessment of the method using simulated sequencing
61 data from single microorganisms characterizing the types of false positive contaminants the method
62 may report. Then, the method was challenged for the ability to detect organismal contaminants in mi-
63 crobial material strains using sequencing data simulated to replicate microbial materials with different
64 organismal contaminants at a range of concentrations.

65 METHODS

66 Simulated whole genome sequence data was used to evaluate the suitability of using whole genome
67 sequence data and metagenomic taxonomic classification methods for detecting foreign DNA in micro-
68 bial materials. Simulated data from individual prokaryotic genomes was used to characterize the rate at
69 which the method correctly classifies reads to the material species. To evaluate contaminant detection
70 we used datasets comprised of pairwise combinations of simulated reads from individual genomes.

71 Simulating Sequencing Data

72 To approximate real sequencing data reads were simulated using an empirical error model and insert size
73 distribution. Whole genome sequencing data was simulated using the ART sequencing read simulator
74 (Huang et al., 2012). Reads were simulated with ART simulator using the Illumina MiSeq error model
75 for 2×230 base pair (bp) paired-end reads with an insert size of 690 ± 10 bp (average \pm standard
76 deviation) and 20 X mean coverage. The insert size parameters were defined based on the observed
77 average and standard deviation insert size of the NIST RM8375-MG002 MiSeq sequencing data (Olson
78 et al., 2016).

79 Assessing Taxonomic Composition

80 The taxonomic composition of simulated datasets was assessed using the Pathoscope sequence taxo-
81 nomic classifier (Francis et al., 2013). Pathoscope was selected for two reasons: (1) it uses a large
82 reference database reducing potential biases due to contaminant sequences not present in the database
83 and (2) it leverages efficient whole genome read mapping algorithms. This method uses an expecta-
84 tion maximization algorithm where the sequence data are first mapped to a database comprised of all
85 sequence data in the Genbank nt database. Then, through an iterative process, it re-assigns ambigu-
86 ously mapped reads based on the proportion of reads mapped unambiguously to individual taxa in the
87 database. The Pathoscope 2.0 taxonomic read classification pipeline has three steps; (1) PathoQC -
88 read quality filtering and trimming using the PRINSEQ algorithm (Schmieder and Edwards, 2011),
89 (2) PathoMap - mapping reads to a reference database using the bowtie2 algorithm (Langmead and
90 Salzberg, 2012), (3) PathoID - expectation-maximization classification algorithm. The annotated Gen-
91 bank nt database provided by the PathoScope developers was used as the reference database (`ftp:`
92 `//pathoscope.bumc.bu.edu/data/nt_ti.fa.gz`).

93 Baseline Assessment Using Individual Genomes

94 Simulated sequencing data from individual genomes was used to characterize the false positive con-
95 taminants reported by Pathoscope. Sequence data was simulated for 406 strains, from 9 genera (Table

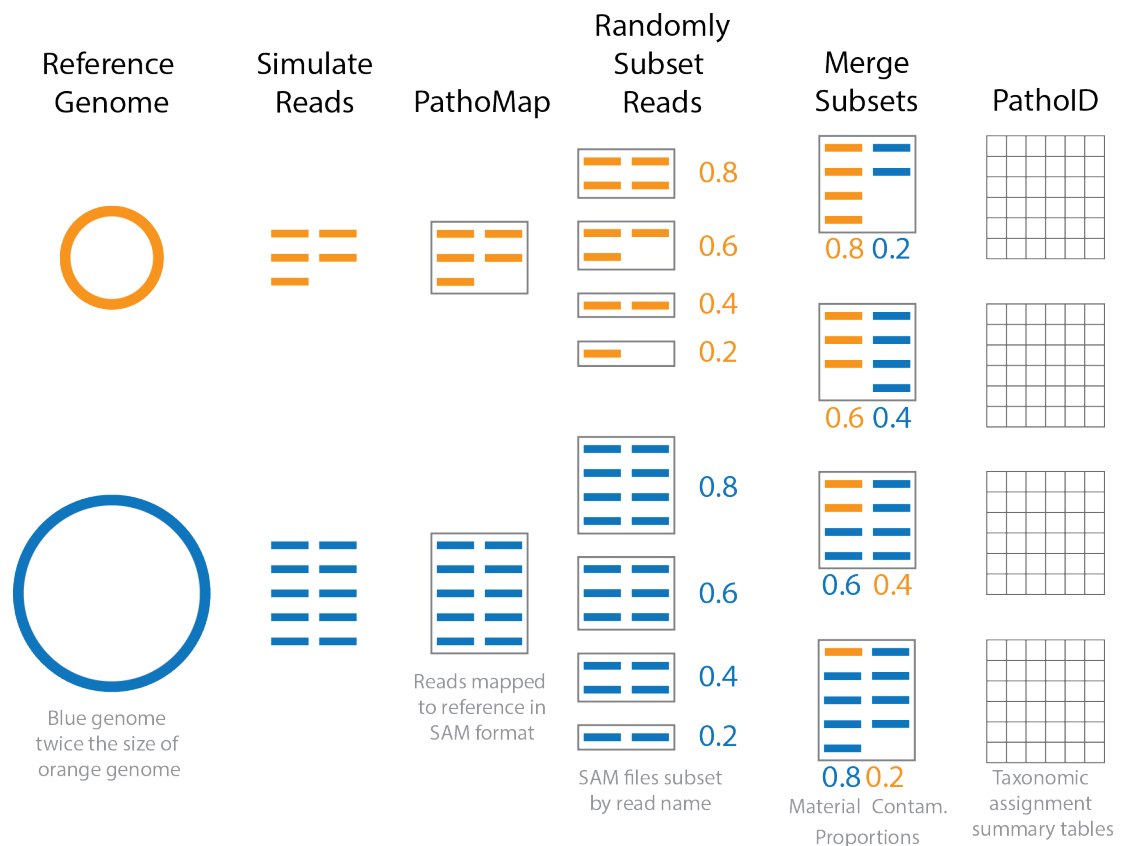


Figure 1. Diagram of the simulated contaminant dataset workflow for two individual genomes. Contaminant proportions 0.2 and 0.4 are used for demonstration purposes. The reads were initially simulated from individual genomes. The blue genome is twice the size of the orange genome and twice as many reads are simulated for the blue genome compared to the orange in order to obtain the same coverage. The simulated reads were aligned to the reference database using PathoMap. The resulting alignment file, in SAM file format, was randomly subset based on the desired proportions. Complementary subsets of SAM files (e.g. 0.2 contaminant and 0.8 material) from the two genomes were merged to create individual simulated contaminant datasets. Due to the different sized genomes, the simulated contaminant datasets have different numbers of reads. Taxonomic assignment summary tables were generated from simulated contaminant datasets using PathoID.

1). We will refer to the genome used to generate the reads as the material genome. The genomes included in the simulation study were limited to the number of closed genomes in the Genbank database (<http://www.ncbi.nlm.nih.gov/genbank/>, accessed 10/18/2013) belonging to the genera of interest (Table 1). Due to the large number of closed genomes from the genera *Bacillus*, *Escherichia*, and *Salmonella*, genomes from these genera were limited to the species *Bacillus cereus*, *Escherichia coli*, and *Salmonella enterica*. The taxonomic hierarchy for the material genome and simulated read assignment match levels were determined using the R package, Taxize (Scott Chamberlain and Eduard Szocs, 2013; Chamberlain et al., 2016).

Contaminant Detection Assessment

Simulated contaminated datasets were used to evaluate how contaminant detection varied by material and contaminant strain over a range of contaminant concentrations. Representative genomes for 8 of the 9 genera were used to generate the simulated contaminant datasets (Table 2). An *Escherichia coli* strain was selected as a representative of both and *Shigella* as the genus *Shigella* phylogenetically resides within the species *Escherichia coli* (Lan and Reeves, 2002). For each pairwise combination of representative genomes, the simulated contaminant dataset was comprised of a randomly selected subset of reads from the material and contaminant simulated single genome sequence dataset (Fig. 1). The simulated datasets

were randomly subsampled at defined proportions, with p representing the proportion of reads from the contaminant single genome dataset, and $1 - p$ representing the proportion of reads from the material genome simulated dataset. A range of contaminant proportions at 10-fold increments was simulated with p ranging from 10^{-1} to 10^{-8} , resulting in 512 simulated contaminant datasets. This approach simulates the proportions of cells in a contaminated material and not the amount of DNA, assuming unbiased DNA extraction. This results in organisms with larger genomes having more simulated reads.

To generate the simulated contaminant datasets single organism simulated datasets were first generated for the 8 representative genomes using the same methods as used in baseline assessment. The resulting simulated sequencing data was first processed using the PathoQC and PathoMap steps in the Pathoscope pipeline. The output from the PathoMap step (sam file, sequence alignment file <https://samtools.github.io/hts-specs/SAMv1.pdf>) for the material and contaminant datasets were subsampled as described above then combined. The resulting SAM file was processed by PathoID, the third step in the Pathoscope pipeline (Fig. 1). Subsampling the sam files instead of the simulated sequence files greatly reduces the computational cost of the analysis as the simulated reads were only processed by the first two steps in Pathoscope pipeline once rather than for every simulated contaminant dataset.

Bioinformatic Pipeline

To facilitate repeatability and transparency, a Docker (www.docker.com) container is available with pre-installed pipeline dependencies (www.registry.hub.docker.com/u/natedolson/docker-pathos). The script used to run the simulations are available at https://github.com/nate-d-olson/genomic_purity. Additionally, seed numbers for the random number generator were randomly assigned and recorded for each dataset so the simulated datasets used in the study could be regenerated. Pathoscope results were processed using the statistical programming language R (R Core Team, 2016), and intermediate analysis and data summaries were organized using ProjectTemplate (White, 2014) and archived in a GitHub repository (https://github.com/nate-d-olson/genomic_purity_analysis) along with the source files for this manuscript.

RESULTS

Baseline Assessment Using Individual Genomes

We first assessed baseline performance of the proposed method for detecting contaminant DNA in microbial materials. Our analysis included taxonomic classification results for simulated sequencing data from 388 genomes, representing 9 different genera (Table 1). For 105 out of 388 genomes, Pathoscope estimated that less than 99% of the material was the same species as the genome the sequencing data was simulated from (Fig. 2). The estimated proportion of the material identified as the correct species varied by genus. None of the *Shigella* genomes and five of the 49 *Staphylococcus* genomes had estimated proportions greater than 0.9 for the correct species. 87 of the 105 genomes with estimated match proportions less than 0.99 at the species level come from *Shigella*, *Staphylococcus*, or *Escherichia*. Excluding *Shigella*, *Escherichia*, and *Staphylococcus* the median estimated proportion matching at the species level or higher is 0.9995. We characterized false positive contaminants detected in genomes from the genera *Shigella*, *Escherichia*, and *Staphylococcus*, as well as genomes of other species, match proportions less than 0.9. Two types of false positive contaminants were identified (1) contaminants that were genomically indistinguishable from the material and (2) contaminants due to errors in the reference database.

Two genome sequences can be genomically indistinguishable as they are either phylogenetically closely related or share parts of their genome. Phylogenetic similarity is at least partially responsible for the low species level match proportions for *Shigella* and *Escherichia*, as *Shigella* is not phylogenetically distinct from *E. coli* (Lan and Reeves, 2002). When including matches to *E. coli* as species level matches, the median match proportions for *Shigella* genomes increase from 0.66 to 0.92. Another example of false positives at the species level due to phylogenetic similarity was low match percentage for *Clostridium autoethanogenum* strain DSM10061 which was due to *Clostridium ljungdahlii* strain DSM13528 assigned the top proportion (0.998) instead of *C. autoethanogenum*. False positive contaminants due to phylogenetic similarity are not limited to closely related species or genus. *Escherichia coli* strain UMNK88 low match proportions, was due to two bacteria in the same family as *E. coli* (Enterobacteriaceae) *Providencia stuartii* and *Salmonella enterica* subsp. *enterica* serovar Heidelberg with estimated proportions of 0.11 and 0.03 respectively.

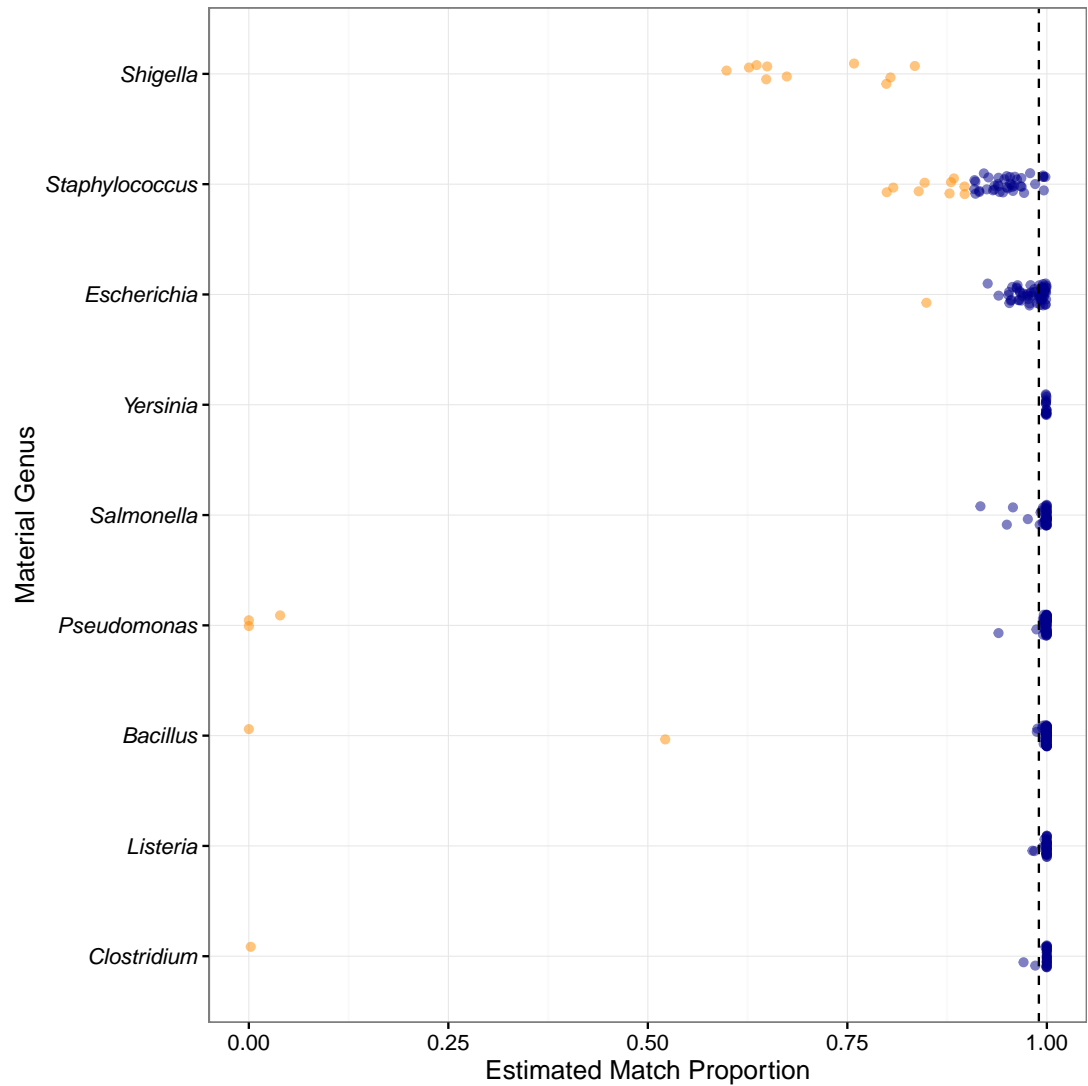


Figure 2. Species level estimated match proportion varies by material genus. The proportion of the material, simulated sequence data from individual genomes, was estimated by Pathoscope. The estimated match proportion is the total proportion of the material with taxonomic assignments to the genome species, subspecies, strain, or isolate levels. The vertical dashed line indicates the 0.99 match proportion. Orange points are genomes with species level match proportions less than 0.90 and blue points greater than 0.90

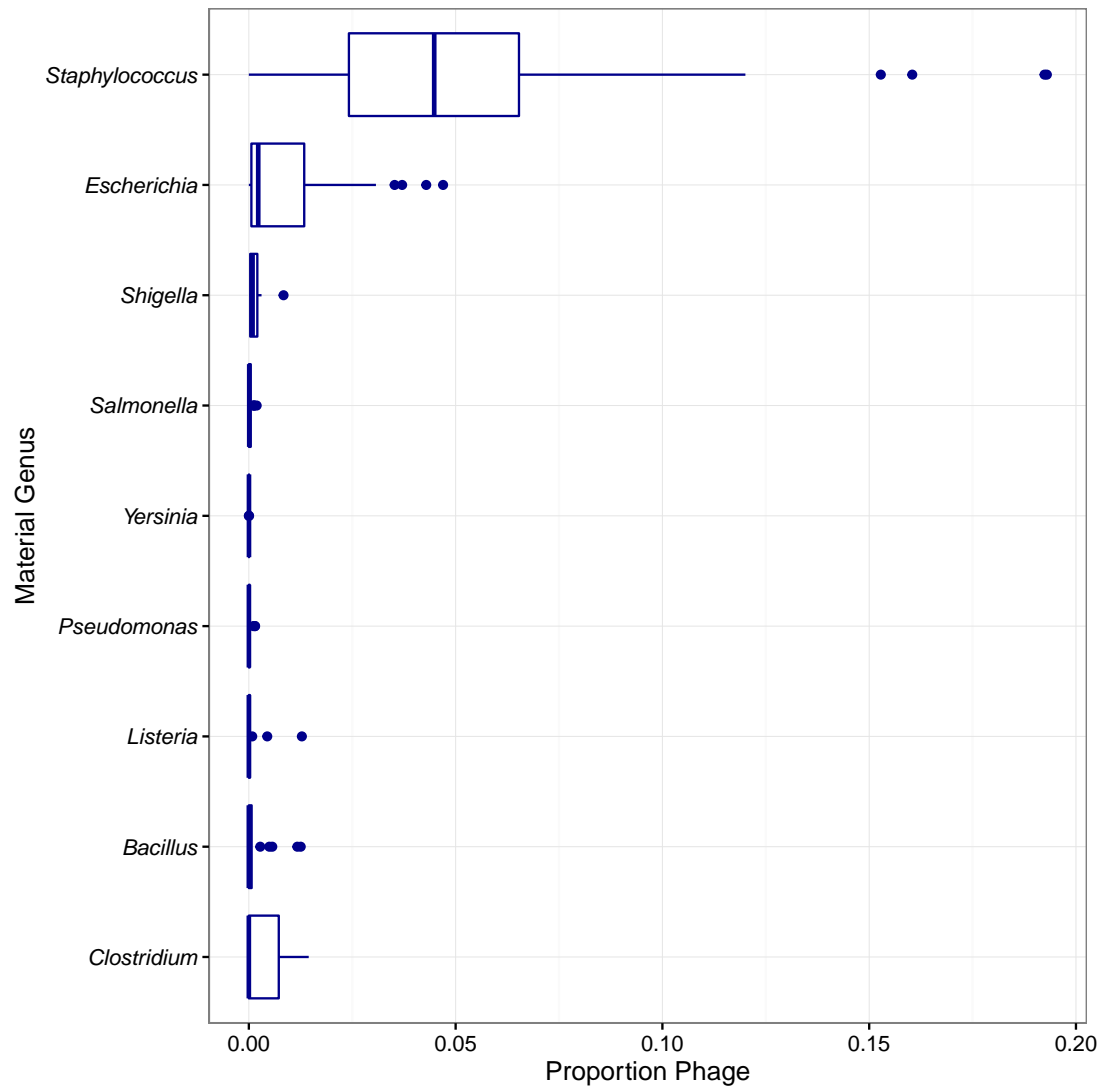


Figure 3. Estimated proportion of phage in the simulated single genome datasets by genera. Proportions based on the final estimated proportions for all phage.

Genus	N	Genome Size (Mb)
<i>Bacillus</i>	76	5.05 (3.07-7.59)
<i>Escherichia</i>	62	5.11 (3.98-5.86)
<i>Pseudomonas</i>	57	6.18 (4.17-7.01)
<i>Staphylococcus</i>	49	2.82 (2.69-3.08)
<i>Salmonella</i>	44	4.88 (4.46-5.27)
<i>Listeria</i>	39	2.97 (2.78-3.11)
<i>Clostridium</i>	32	4.02 (2.55-6.67)
<i>Yersinia</i>	19	4.73 (4.62-4.94)
<i>Francisella</i>	18	1.89 (1.85-2.05)
<i>Shigella</i>	10	4.74 (4.48-5.22)

Table 1. Breakdown of the number of genomes by genus used to generate single genome simulated datasets. N indicates the number of genomes, and Genome Size is presented as the median and range (minimum to maximum) genome size

False positives were also due to sharing of genetic material between organisms. An example of this type of false positive contaminant was phage. Phage was identified as false positive contaminants at varying proportions for genomes from all 9 genera investigated (Fig. 3). Most notably, the low proportions of species level matches for *E. coli* and *Staphylococcus* are partly due to relatively higher proportions of matches to phage, compared to the other genera investigated. Based on phage names all of the false positive phage contaminants were specific to the taxonomy of the genome the sequence data was simulated from.

False positive contaminants were also due to potential errors in the database such as misclassified or unclassified sequences in the database, genome assemblies in the database including sequence data from organismal or reagent contaminants. *Bacillus subtilis* BEST7613 genome had low estimated species level match proportion due to *Synechocystis* sp. PCC 6803 substr. PCC-P being estimated as comprising 47% of the material (Kanesaki et al., 2012). *Synechocystis* is in a different phylum compared to *Bacillus*, cyanobacteria versus firmicutes. The high match proportion is potentially due to an error in the database. Low species level match proportions can also be due to the database containing unclassified sequence data for organisms highly similar to the material genome. For example, the low match proportion for *Pseudomonas* strain FGI182 was due to matches to unclassified bacteria, bacterium 142412, and unclassified *Pseudomonas* species, *Pseudomonas* sp. HF-1. The low species proportion of species level matches for *Pseudomonas* strain TKP was also due to misclassified sequences (*Thioalkalivibrio sulfidophilus* strain HL-EbGr7 match proportion 0.0648).

The genome sequences used to populate the reference database can contain contaminants themselves. These database contaminants are responsible for additional false positive contaminants. The eukaryotic false positive contaminants are likely due to contaminants in the material or reagents used to generate the sequencing data used in the assembly (Parks et al., 2015). The low species proportion of species level matches for *Pseudomonas* strain TKP was partially due to contaminated genome sequences in the database (wheat - *Triticum aestivum* match proportion 0.087).

Contaminant Detection Assessment

Finally, contaminant detection was assessed using simulated sequencing data from individual genomes. Contaminant datasets were developed by combining subsets of simulated data from two organisms at defined proportions, with the larger proportion representing the microbial material and smaller proportion the contaminant (Fig. 1). We simulated contaminant datasets as pairwise combinations of representative genomes from 8 of the genera used in the baseline assessment section of the study (Table 2). For all of the genomes selected for the detection assessment study, the estimated proportion of material assigned to the correct species was greater than 0.98 (Table 2).

The minimum contaminant proportion detected was 10×10^{-3} and 10×10^{-4} for most pairwise comparisons with a few notable exceptions. When *Yersinia* was the simulated contaminant the minimum detected proportion was 0.1 for all material strains (Table 3). Conversely, a few contaminants were detected at lower proportions, 10×10^{-8} , when *Yersinia* was contaminated with *E. coli* as well as when *S. enterica* and *E. coli* contaminated with *B. anthracis*.

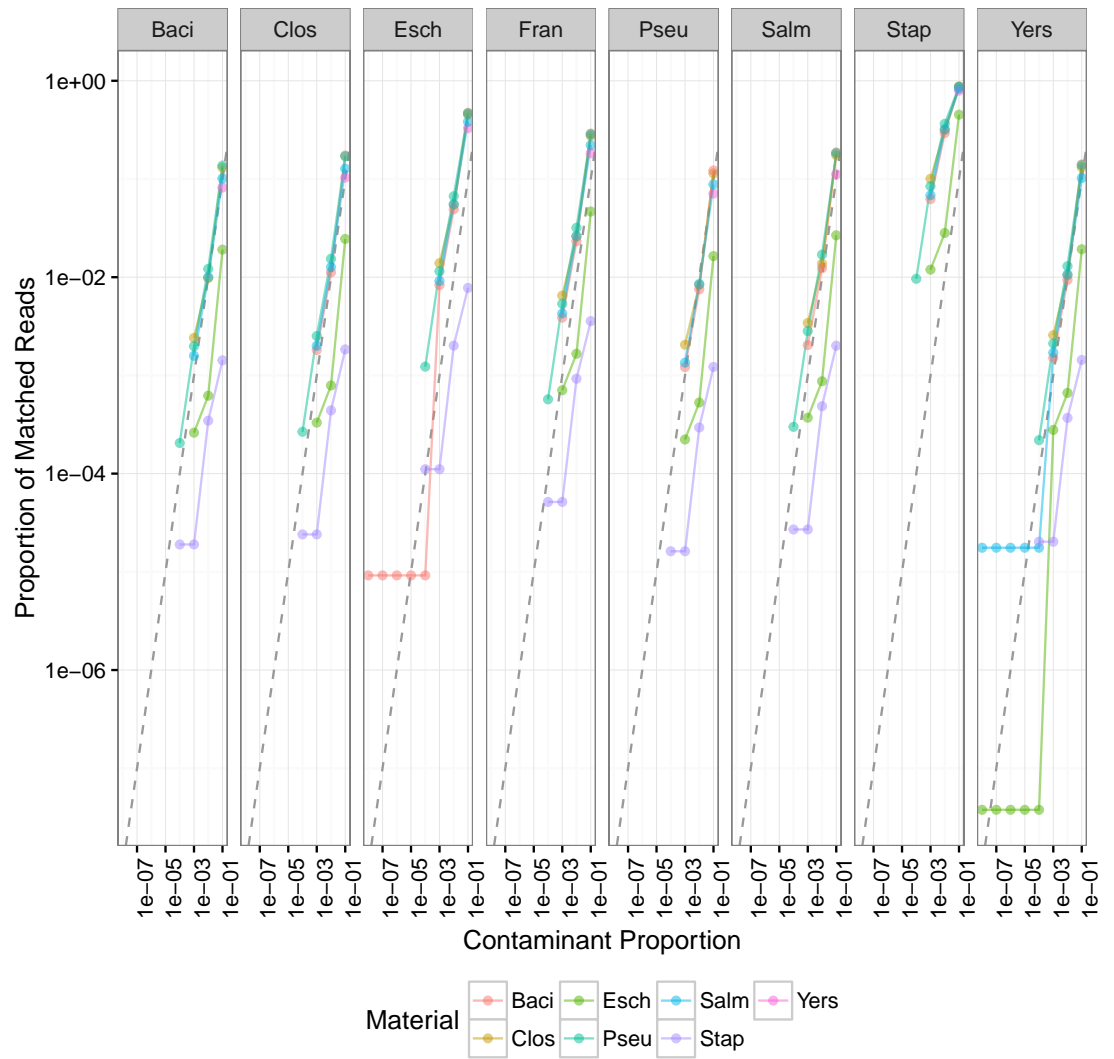


Figure 4. Relationship between the proportion of contaminant reads simulated per dataset and the proportion of reads matched to the contaminant genus.

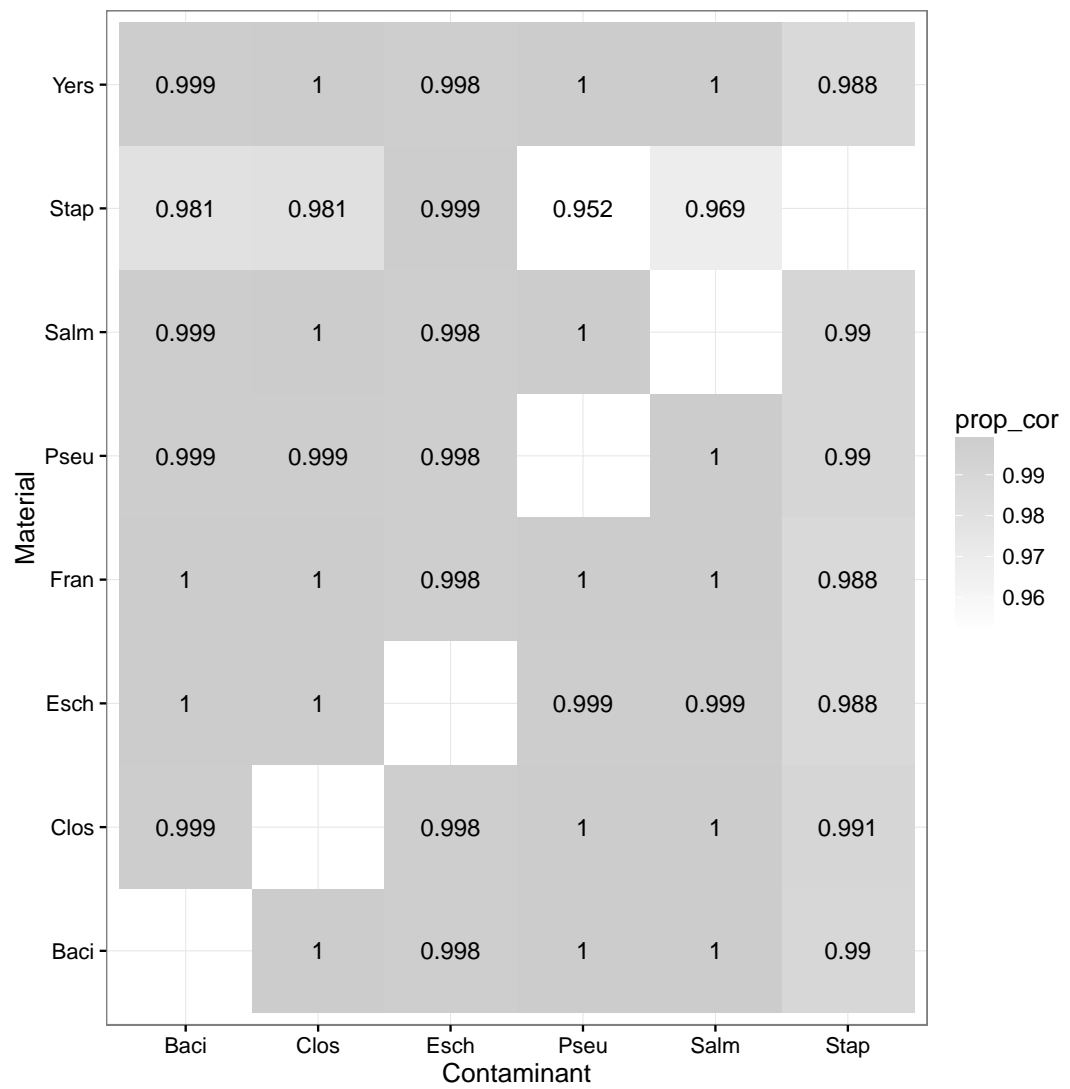


Figure 5. Pearson correlation coefficients for estimated and true contaminant proportions.

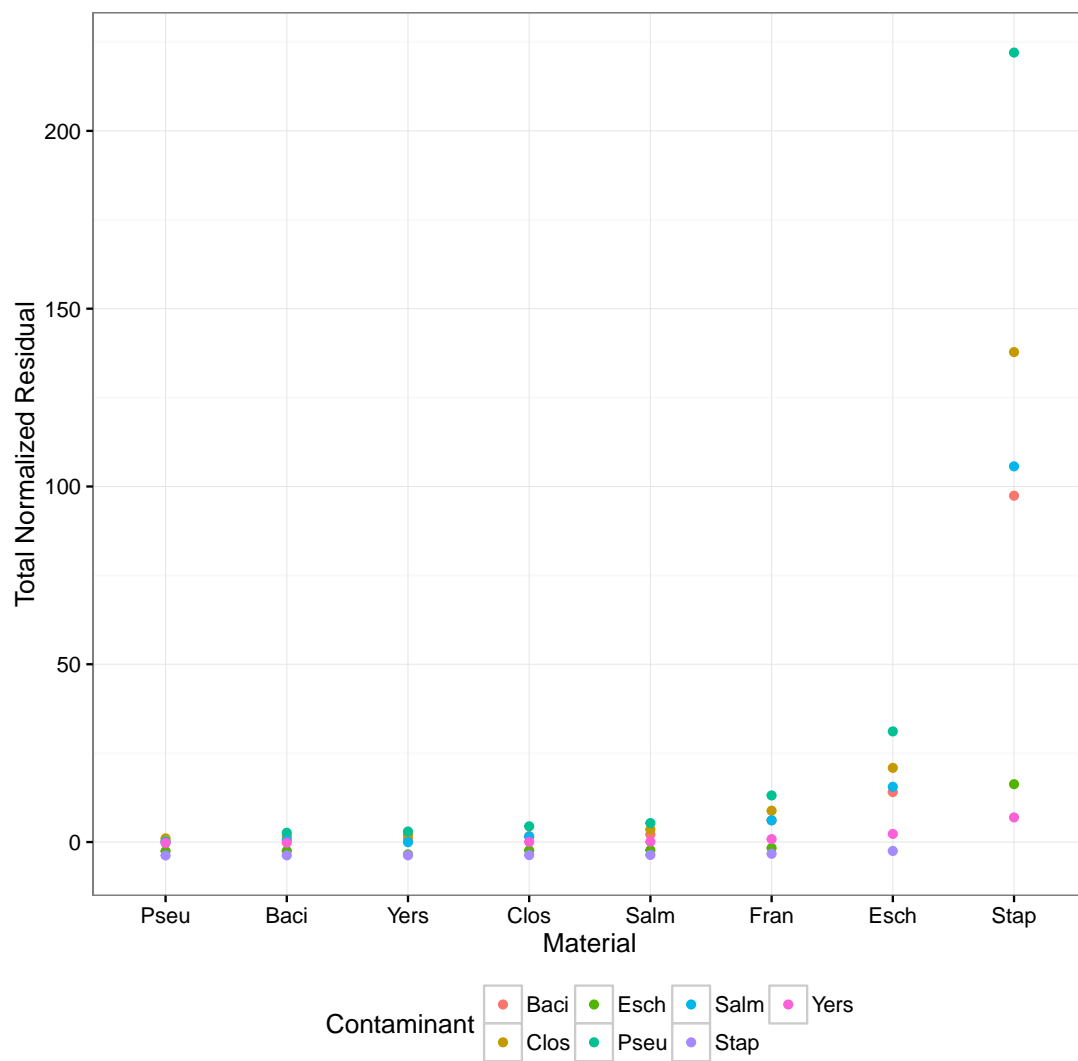


Figure 6. Total normalized residuals for pairwise combinations of material and contaminants.

Representative Strain	Species	C Mb	C Acc	P Mb	P Acc
Bacillus anthracis str. Ames	1.00	5.23	AE016879.1		
Clostridium botulinum A str. Hall	1.00	3.76	CP000727.1		
Escherichia coli O157:H7 str. EC4115	0.98	5.57	CP001164.1	0.13	CP001163.1, CP001165.1
Francisella tularensis subsp. tularensis SCHU S4	1.00	1.89	AJ749949.2		
Pseudomonas aeruginosa PAO1	1.00	6.26	AE004091.2		
Salmonella enterica subsp. enterica serovar Typhimurium str. D23580	1.00	4.88	FN424405.1		
Staphylococcus aureus subsp. aureus ED133	0.98	2.83	CP001996.1		
Yersinia pestis CO92	1.00	4.65	AL590842.1	0.18	AL109969.1, AL117189.1, AL117211.1

Table 2. Representative strains used in simulated contaminant datasets. When available type strains were selected as the representative genome. Species indicates the proportion of the material assigned to the correct species. DNA size (Mb) and Genbank accession numbers (Acc) are indicated for chromosomes (C) and plasmids (P). *Escherichia coli* O157:H7 str. EC4115 and *Yersinia pestis* CO92 have two and three plasmids respectively.

contam_label	Baci	Clos	Esch	Fran	Pseu	Salm	Stap	Yers
Bacillus anthracis Ames		1.0E-03	1.0E-08	1.0E-03	1.0E-03	1.0E-03	1.0E-03	1.0E-03
Clostridium botulinum A Hall	1.0E-03		1.0E-03	1.0E-03	1.0E-03	1.0E-03	1.0E-03	1.0E-03
Escherichia coli O157 H7 EC4115	1.0E-03	1.0E-03		1.0E-03	1.0E-03	1.0E-03	1.0E-03	1.0E-08
Pseudomonas aeruginosa	1.0E-04	1.0E-04	1.0E-04	1.0E-04		1.0E-04	1.0E-04	1.0E-04
Salmonella enterica serovar Typhimurium	1.0E-03	1.0E-03	1.0E-03	1.0E-03	1.0E-03		1.0E-03	1.0E-08
Staphylococcus aureus ED133	1.0E-04	1.0E-04	1.0E-04	1.0E-04	1.0E-04	1.0E-04		1.0E-04
Yersinia pestis CO92	1.0E-01	1.0E-01	1.0E-01	1.0E-01	1.0E-01	1.0E-01	1.0E-01	

Table 3. Lowest proportion of contaminant in each pairwise combination of representative genomes detected.

The Pearson's correlation coefficient was used to measure the correlation between the estimated contaminant and true contaminant proportions. The estimated and true proportions were strongly correlated for all pairwise comparisons, with an overall median and 95% confidence interval of 0.99945 (0.96943 - 0.99999 (Fig. 4). Eight of the pairwise comparisons have correlation coefficients below 0.99, all of which have *S. aureus* as either the contaminant or the material strain. Two coefficients were below 0.98, *S. aureus* contaminated with *P. aeruginosa* and *S. enterica*, 0.952 and 0.969 respectively. Normalized contaminant proportion residuals, $(estimated - true)/true$, were used to assess the accuracy of the Pathoscope contaminant proportion estimates (Fig. 6). The material genome strongly influenced the total normalized residuals with *E. coli* and *S. aureus* having consistently higher total normalized residuals compared to the other genomes.

DISCUSSION

The potential for using whole genome sequencing data and taxonomic sequence classification algorithm *Pathoscope* to detect contaminant DNA in microbial materials using whole genome sequencing data was evaluated. A baseline assessment of the contaminant DNA detection method using simulated sequencing data generated from individual genomes to characterize the types of false positive contaminants identified by the method was initially performed. The false positive contaminants were split into two categories (1) those due to an inability of the method to differentiate the material genome from the contaminant genome and (2) those due to errors in the reference database. Variation in contaminant detection was characterized by varying the material, the contaminant, and level of contamination. Overall the method was able to identify contaminant proportions at 10×10^{-3} for most pairwise contaminant-material combinations. However, the accuracy of the estimated proportion of the contaminant in the simulated contaminated material varied by contaminant and material strain.

A primary limitation of the proposed method is the observed false positive contaminants for single genome simulated sequencing data. Performing a baseline assessment of false positive contaminant using simulated sequence data from the microbial material's genome sequence, and choosing the appropriate database and taxonomic assignment algorithm can help reduce the impact of false positive on the method's ability to detect contaminant DNA. Additionally, false positive contaminants are likely database and taxonomic assignment algorithm dependent.

Removing sequences from the database for irrelevant contaminants, such as phage, plasmids, vectors, and multicellular eukaryotes can help reduce the proportion of false positives. Though the relevance of these contaminants is application specific. Similarly, using a curated database free of missclassified and

unclassified sequence data would further help to reduce the proportion of false positive contaminants (Tennessen et al., 2015). Pathoscope was used for this proof of concept study as the method uses the full reads and paired-end information for taxonomic classification rather than shorter sequence fragment, k -mers. Our assumption is that the longer sequence allows for better discrimination between highly similar sequences. As there are numerous taxonomic classification algorithms, evaluating multiple methods using sequence data simulated from the material genome of interest can help to determine the optimal contaminant detection method for a specific microbial material.

Identification and characterization of low abundance contaminants are critical for when the material is used for high sensitivity assays such as PCR. The minimum contaminant proportion was consistent for most simulated contaminant datasets, *Yersinia* was only detected at a proportion of 0.1. The quantitative accuracy of the method varied by material and contaminant, but for all material-contaminant pairs, the Pathoscope estimated and true contaminant proportions were highly correlated. While not necessarily relevant to organismal contaminant detection, quantitative accuracy is relevant if the contaminant proportion is included in the report of analysis characterizing a material (Olson et al., 2016). Similar to the false positive contaminant baseline assessment. Simulated data can be used to evaluate the minimal detectable contaminant proportion for specific contaminants of interest using different taxonomic assignment algorithms and databases. Additionally, sequencing at a higher depth would theoretically result in lower minimum detectable contaminant proportions and potentially increased quantitative accuracy due to the larger sample size (number of sequences).

CONCLUSIONS

With the continual decline in the cost of sequencing, advances in sequence analysis methods, whole genome sequencing combined with taxonomic assignment algorithms provides a viable alternative to commonly used organismal contaminant detection methods such as culturing, microscopy, and PCR. The method presented here is suitable for detecting organismal contaminants in both genomic DNA and whole cell microbial materials with the only *a priori* assumptions about the contaminant are that it is present in the reference database. Furthermore, the method was shown to detect contaminants making up 10×10^{-3} proportion of cells in a high-throughput manner. Even with the rapid decrease in sequencing cost, whole genome sequencing is more expensive than culture and PCR-based contaminant detection methods. However, unlike culture and PCR-based contaminant detection methods, the data generated when whole genome sequencing data is used for organismal contaminant detection can also be used to further characterize the material's genome and potentially identify other characteristics of interest such as the presence of virulence or antibiotic resistance genes.

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276 REFERENCES

- 277 Bazinet, A. L. and Cummings, M. P. (2012). A comparative evaluation of sequence classification pro-
278 grams. *BMC Bioinformatics*, 13(1):92.
- 279 Bokulich, N. A., Rideout, J. R., Mercurio, W. G., Shiffer, A., Wolfe, B., Maurice, C. F., Dutton, R. J.,
280 Turnbaugh, P. J., Knight, R., and Caporaso, J. G. (2016). mockrobiota: a public resource for micro-
281 biome bioinformatics benchmarking. *mSystems*, 1(5).
- 282 Chamberlain, S., Szocs, E., Boettiger, C., Ram, K., Bartomeus, I., Baumgartner, J., Foster, Z., and
283 O'Donnell, J. (2016). *taxize: Taxonomic information from around the web*. R package version 0.7.4.
- 284 Coates, S. G., Brunelle, S. L., and Davenport, M. G. (2011). Development of standard method perfor-
285 mance requirements for biological threat agent detection methods. *Journal of AOAC International*,
286 94(4):1328–37.
- 287 Francis, O. E., Bendall, M., Manimaran, S., Hong, C., Clement, N. L., Castro-Nallar, E., Snell, Q.,
288 Schaalje, G. B., Clement, M. J., Crandall, K. a., and Johnson, W. E. (2013). Pathoscope: Species
289 identification and strain attribution with unassembled sequencing data. *Genome research*.
- 290 Heck, K., Machineski, G. S., Alvarenga, D. O., Vaz, M. G. M. V., de Mello Varani, A., and Fiore,
291 M. F. (2016). Evaluating methods for purifying cyanobacterial cultures by qpcr and high-throughput
292 illumina sequencing. *Journal of Microbiological Methods*.
- 293 Huang, W., Li, L., Myers, J. R., and Marth, G. T. (2012). ART: a next-generation sequencing read
294 simulator. *Bioinformatics (Oxford, England)*, 28(4):593–4.
- 295 Ieven, M., Finch, R., and van Belkum, a. (2013). European quality clearance of new microbiological
296 diagnostics. *Clinical microbiology and infection : the official publication of the European Society of*
297 *Clinical Microbiology and Infectious Diseases*, 19(1):29–38.
- 298 Jervis-Bardy, J., Leong, L. E., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy,
299 E., Morris, P. S., OLeary, S., Rogers, G. B., et al. (2015). Deriving accurate microbiota profiles from
300 human samples with low bacterial content through post-sequencing processing of illumina miseq data.
301 *Microbiome*, 3(1):1.
- 302 Kanesaki, Y., Shiwa, Y., Tajima, N., Suzuki, M., Watanabe, S., Sato, N., Ikeuchi, M., and Yoshikawa, H.
303 (2012). Identification of substrain-specific mutations by massively parallel whole-genome resequenc-
304 ing of synechocystis sp. pcc 6803. *DNA research*, 19(1):67–79.
- 305 Lan, R. and Reeves, P. R. (2002). Escherichia coli in disguise: molecular origins of shigella. *Microbes*
306 *and infection*, 4(11):1125–1132.
- 307 Langmead, B. and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods*,
308 9(4):357–9.
- 309 Marron, A. O., Akam, M., and Walker, G. (2013). A Duplex PCR-Based Assay for Measuring the
310 Amount of Bacterial Contamination in a Nucleic Acid Extract from a Culture of Free-Living Protists.
311 *PloS one*, 8(4):e61732.
- 312 Menzel, P., Ng, K. L., and Krogh, A. (2016). Fast and sensitive taxonomic classification for metage-
313 nomics with kaiju. *Nature communications*, 7.
- 314 Motley, S. T., Picuri, J. M., Crowder, C. D., Minich, J. J., Hofstadler, S. A., and Eshoo, M. W. (2014). Im-
315 proved multiple displacement amplification (imda) and ultraclean reagents. *BMC genomics*, 15(1):1.
- 316 Mukherjee, S., Huntemann, M., Ivanova, N., Kyrpides, N. C., and Pati, A. (2015). Large-scale con-
317 tamination of microbial isolate genomes by illumina phix control. *Standards in genomic sciences*,
318 10(1):1.

319 Olson, N. D., Zook, J. M., Samarov, D. V., Jackson, S. A., and Salit, M. L. (2016). Pepr: pipelines for
 320 evaluating prokaryotic references. *Analytical and bioanalytical chemistry*, 408(11):2975–2983.
 321 Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015). Checkm:
 322 assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes.
 323 *Genome research*, 25(7):1043–1055.
 324 R Core Team (2016). *R: A Language and Environment for Statistical Computing*. R Foundation for
 325 Statistical Computing, Vienna, Austria.
 326 Schmieder, R. and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets.
 327 *Bioinformatics*, 27(6):863–864.
 328 Scott Chamberlain and Eduard Szocs (2013). taxize - taxonomic search and retrieval in r.
 329 *F1000Research*.
 330 Shrestha, P. M., Nevin, K. P., Shrestha, M., and Lovley, D. R. (2013). When Is a Microbial Culture Pure
 331 ? Persistent Cryptic Contaminant Escapes Detection Even with Deep Genome Sequencing.
 332 Tennessen, K., Andersen, E., Clingenpeel, S., Rinke, C., Lundberg, D. S., Han, J., Dangl, J. L., Ivanova,
 333 N., Woyke, T., Kyrpides, N., et al. (2015). Prodege: a computational protocol for fully automated
 334 decontamination of genomes. *The ISME journal*.
 335 Thomas, T., Gilbert, J., and Meyer, F. (2012). Metagenomics - a guide from sampling to data analysis.
 336 *Microbial informatics and experimentation*, 2(1):3.
 337 White, J. M. (2014). *ProjectTemplate: Automates the creation of new statistical analysis projects*. R
 338 package version 0.6.
 339 Woyke, T., Sczyrba, A., Lee, J., Rinke, C., Tighe, D., Clingenpeel, S., Malmstrom, R., Stepanauskas, R.,
 340 and Cheng, J.-F. (2011). Decontamination of mda reagents for single cell whole genome amplification.
 341 *PloS one*, 6(10):e26161.