

1 Evaluating Metagenome Assembly on a Simple
2 Defined Community with Many Strain Variants

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5 **Abstract**

6 We evaluate the performance of three metagenome assemblers, IDBA,
7 MetaSPAdes, and MEGAHIT, on short-read sequencing of a defined
8 “mock” community containing 64 genomes (Shakya et al. (2013)). We
9 update the reference metagenome for this mock community and detect
10 several additional genomes in the read data set. We show that strain
11 confusion results in significant loss in assembly of reference genomes
12 that are otherwise completely present in the read data set. In agree-
13 ment with previous studies, we find that MEGAHIT performs best
14 computationally; we also show that MEGAHIT tends to recover larger
15 portions of the strain variants than the other assemblers.

16 Introduction

17 Metagenomics refers to sequencing of DNA from a mixture of organisms,
18 often from an environmental or uncultured sample. Unlike whole genome
19 sequencing, metagenomics targets a mixture of genomes, which introduces
20 metagenome-specific challenges in analysis [1]. Most approaches to analyz-
21 ing metagenomic data rely on mapping or comparing sequencing reads to
22 reference sequence collections. However, reference databases contain only
23 a small subset of microbial diversity [2], and much of the remaining diver-
24 sity is evolutionarily distant and reference-based search techniques may not
25 recover it [3].

26 As sequencing capacity increases and sequence data is generated from
27 many more environmental samples, metagenomics is increasingly using *de*
28 *novo* assembly techniques to generate new reference genomes and metagenomes
29 [4]. There are a number of metagenome assemblers that are widely used -
30 see [5] for an overview of the available software, and [1] for a review of the
31 different assembler methodologies. However, evaluating the results of these
32 assemblers is challenging due to the general lack of good quality reference
33 metagenomes.

34 Moya et al. in [6] evaluated metagenome assembly using two simulated
35 454 viral metagenome and six assemblers. The assemblies were evaluated
36 based on several metrics including N50, percentages of reads assembled,
37 accuracy when compared to the reference genome. In addition to these met-
38 rics, the authors evaluated chimeras per contigs and the effect of assembly
39 on taxonomic and functional annotations.

40 Mavromatis et al. in [7] provided a benchmark study to evaluate the
41 fidelity of metagenome processing methods. The study used simulated
42 metagenomic data sets constructed at different complexity levels. The datasets
43 were assembled using Phrap v3.57, Arachne v.2 [8] and JAZZ [9]. This study
44 evaluates assembly, gene prediction, and binning methods. However, the
45 study did not evaluate the assembly quality against a reference genome.

46 Rangwala et al. in [10] presented an evaluation study of metagenome
47 assembly. The study used a de Bruijn graph based assembler ABYSS [11] to
48 assemble simulated metagenome reads of 36 bp. The data set is classified at
49 different complexity levels. The study compared the quality of the assembly
50 of the data sets in terms of contig length and assembly accuracy. The
51 study also took into consideration the effect of kmer size and the degree of
52 chimericity. However, the study evaluated the assembly based on only one
53 assembler. Also, these previous studies used simulated data, which may lack

54 confounders of assembly such as sequencing artifacts and GC bias.

55 In a landmark study, Shakya et al. (2013) constructed a synthetic com-
56 munity of organisms by mixing DNA isolated from individual cultures of 64
57 bacteria and archaea, including a variety of strains across a range of average
58 nucleotide distances [12]. In addition to performing 16s amplicon analy-
59 sis and doing 454 sequencing, the authors shotgun-sequenced the mixture
60 with Illumina. While the authors concluded that this metagenomic sequenc-
61 ing generally outperformed amplicon sequencing, they did not conduct an
62 assembly based analysis. This data set was also used in several other eval-
63 uation studies, including gbtools for binning [13] and benchmarking of the
64 MEGAHIT and metaSPAdes assemblers [14, 15]. Importantly, the authors
65 of the MEGAHIT benchmarking paper noted the presence of unexpected
66 sequence in this data set.

67 More recently, several benchmark studies systematically evaluated metagenome
68 assembly of short reads. The Critical Assessment of Metagenome Interpre-
69 tation (CAMI) collaboration benchmarked a number of metagenome as-
70 semblers on several simulated data sets of varying complexity, evaluating
71 recovery of novel genomes and multiple strain variants [3]. Notably, CAMI
72 concluded that “The resolution of strain-level diversity represents a substan-
73 tial challenge to all evaluated programs.” Another recent study evaluated
74 eight assemblers on nine environmental metagenomes and three simulated
75 data sets and provided a workflow for choosing a metagenome assembler
76 based on the biological goal and computational resources available [16]. [5]
77 explored metagenome assembler performance on a pair of real data sets,
78 again concluding that the biological goal and computational resources de-
79 fined the choice of assembler. Also see [17] for an analysis of a previously
80 generated HMP benchmark data set; however, the Illumina reads used for
81 this study are much shorter than current sequencing and are arguably not
82 relevant to future studies.

83 In this study, we extend previous work by delving into questions of
84 chimeric misassembly and strain recovery in the Shakya et al. (2013) data
85 set. This data set is the most complex synthetic community for which bulk
86 sequencing data is available, and has been used for several independent as-
87 sembly benchmarking efforts [13, 14, 15]. However, while previous efforts
88 have noted the presence of unexpected sequence data in the data set [14],
89 no further analysis has been done to characterize this sequence or its likely
90 origins.

91 Below, we first update the list of reference genomes for Shakya et al.
92 to include the latest GenBank assemblies along with plasmids. We then

93 compare IDBA [18], MetaSPAdes [19], and MEGAHIT [20] performance on
94 assembling this short-read data set, and explore concordance in recovery
95 between the three assemblers. We describe the effects of “strain confusion”
96 between multiple strains. We also detect and analyze several previously
97 unreported strains and genomes in the Shakya et al. data set. We find that
98 in the absence of closely related genomes, all three metagenome assemblers
99 recover 95% or more of known reference genomes. However, in the presence
100 of closely related genomes, these three metagenome assemblers vary widely
101 in their performance and, in extreme cases, can fail to recover the majority
102 of some genomes even when they are completely present in the reads. Our
103 report provides strong guidance on choice of assemblers and extends previous
104 analyses of this low-complexity metagenome benchmarking data set.

105 Datasets

106 We used a diverse mock community data set constructed by pooling DNA
107 from 64 species of bacteria and archaea and sequencing them with Illumina
108 HiSeq. The raw data set consisted of 109,629,496 reads from Illumina HiSeq
109 101 bp paired-end sequencing (2x101) with an untrimmed total length of
110 11.07 Gbp and an estimated fragment size of 380 bp [12].

111 The original reads are available through the NCBI Sequence Read Archive
112 at Accession SRX200676. We updated the 64 reference genomes sets from
113 NCBI GenBank using the latest available assemblies with plasmid content
114 (June 2017); the accession numbers are available as `accession-list-ref.txt`
115 in the Zenodo repository, DOI: 10.5281/zenodo.821919. For convenience, the
116 updated reference genome collection is available for download at the archival
117 URL <https://osf.io/vbhy5/>.

118 Methods

119 The analysis code and run scripts for this paper are written in Python and
120 bash, and are available at [https://github.com/dib-lab/2016-metagenome-](https://github.com/dib-lab/2016-metagenome-assembly-eval/)
121 `assembly-eval/` (archived at Zenodo DOI: 10.5281/zenodo.821919). The
122 scripts and overall pipeline were examined by the first and senior authors for
123 correctness. In addition, the bespoke reference-based analysis scripts were
124 tested by running them on a single-colony *E. coli* MG1655 data set with a
125 high quality reference genome [21].

126 **Quality Filtering**

127 We removed adapters with Trimmomatic v0.30 in paired-end mode with
128 the TruSeq adapters [22], using light quality score trimming (`LEADING:2`
129 `TRAILING:2 SLIDINGWINDOW:4:2 MINLEN:25`) as recommended in MacManes,
130 2014 [23].

131 **Reference Coverage Profile**

132 To evaluate how much of the reference metagenome was contained in the
133 read data, we used `bwa aln` (v0.7.7.r441) to map reads to the reference
134 genome [24]. We then calculated how many reference bases were covered by
135 mapped reads (custom script `coverage-profile.py`).

136 **Measuring k-mer inclusion and Jaccard similarity**

137 We used MinHashing as implemented in sourmash to estimate k-mer inclu-
138 sion and Jaccard similarity between data sets [25]. MinHash signatures were
139 prepared with `sourmash compute` using `--scaled 10000`. K-mer inclusion
140 was computed by taking the ratio of the number of intersecting hashes with
141 the query over the total number of hashes in the subject MinHash. Jac-
142 card similarity was computed as in [26] by taking the ratio of the number
143 of intersecting hashes between the query and subject over the number of
144 hashes in the union. K-mer sizes for comparison were chosen at 21, 31, or
145 51, depending on the level of taxonomic specificity desired - genus, species,
146 or strain, respectively, as described in [27].

147 Where specified, high-abundance k-mers were selected for counting by
148 using the script `trim-low-abund.py` script with `-C 5` from khmer v2 [28,
149 29].

150 **Assemblers**

151 We assembled the quality-filtered reads using three different assemblers:
152 IDBA-UD [18], MetaSPAdes [19], and MEGAHIT [20]. For IDBA-UD v1.1.3
153 [18], we used `--pre_correction` to perform pre-correction before assembly
154 and `-r` for the pe files. IDBA could not ingest orphan sequences so singleton
155 reads were omitted from this assembly.

156 For MetaSPAdes v3.10.1 [19], we used `--meta --pe1-12 --pe1-s` where
157 `--meta` is used for metagenomic data sets, `--pe1-12` specifies the interlaced

reads for the first paired-end library, and `--pe1-s` provides the orphan reads remaining from quality trimming.

For MEGAHIT v1.1.1-2-g02102e1 [20], we used `-l 101 -m 3e9 --cpu-only` where `-l` is for maximum read length, `-m` is for max memory in bytes to be used in constructing the graph, and `--cpu-only` uses only the CPU and no GPUs. We also used `--presets meta-large` for large and complex metagenomes, and `--12` and `-r` to specify the interleaved-paired-end and single-end files respectively. MEGAHIT allows the specification of a memory limit and we used `-M 1e+10` for 10 GB.

All three assemblies were executed on the same XSEDE Jetstream instance (S1.Xxlarge) at Indiana University, running Ubuntu 16.04 (install 6/21/17, Ubuntu 16.04 LTS Development + GUI support + Docker; based on Ubuntu cloud image for 16.04 LTS with basic dev tools, GUI/Xfce added). Assemblers were limited to 16 threads. We recorded RAM and CPU time for each assembly using `/usr/bin/time -v`. Install and execute details as well as output timings and logs are available in the `pipeline/runstats` directory of the Zenodo archive.

Unless otherwise mentioned, we eliminated all contigs less than 500 bp from each assembly prior to further analysis.

Mapping

We aligned all quality-filtered reads to the reference metagenome with `bwa aln` (v0.7.7.r441) [24]. We aligned paired-end and orphaned reads separately. We then used `samtools` (v0.1.19) [30] to convert SAM files to BAM files for both paired-end and orphaned reads. To count the unaligned reads, we included only those records with the “4” flag in the SAM files [30].

Assembly analysis using NUCmer

We used the NUCmer tool from MUMmer3.23 [31] to align assemblies to the reference genome with options `-coords -p`. Then we parsed the generated “coords” file using a custom script `analyze_assembly.py`, and calculated several analysis metrics across all three assemblies at a 99% alignment identity.

189 **Reference-based analysis of the assemblies**

190 We conducted reference-based analysis of the assemblies under two condi-
191 tions. “Loose” alignment conditions used all available alignments, including
192 redundant and overlapping alignments. “Strict” alignment conditions took
193 only the longest alignment for any given contig, eliminating all other align-
194 ments.

195 The script `summarize-coords2.py` was used to calculate aligned cov-
196 erage from the loose alignment conditions: each base in the reference was
197 marked as “covered” if it was included in at least one alignment. The script
198 `analyze_ng50.py` was used to calculate NGA 50 for each individual refer-
199 ence genome.

200 **Analysis of chimeric misassemblies**

201 We analyzed each assembly for chimeric misassemblies by counting the num-
202 ber of contigs that contained matches to two distinct reference genomes. In
203 order to remove secondary alignments from consideration, we included only
204 the longest non-overlapping NUCmer alignments for each contig at a mini-
205 mum alignment identity of 99%. We then used the script `analyze_chimeric2.py`
206 to find individual contigs that matched more than one distinct reference
207 genome. As a negative control on our analysis, we verified that this ap-
208 proach yielded no positive results when applied to the alignments of the
209 reference metagenome against itself.

210 **Analysis of unmapped reads**

211 We conducted assembly and analysis of unmapped reads with MEGAHIT,
212 NUCmer, and sourmash as above. The new GenBank genomes are listed in
213 the Zenodo archive at the file `accession-list-unmapped.txt` and for con-
214 venience are available for download at the archival URL <https://osf.io/34ef8/>.

215 **Results**

216 **The raw data is high quality.**

217 The reads contain 11,072,579,096 bp (11.07 Gbp) in 109,629,496 reads with
218 101.0 average length (2x101bp Illumina HiSeq).

219 Trimming removed 686,735 reads (0.63%). After trimming, we retained

Table 1: Jaccard containment of the reference in the reads

k-mer size	% reference in reads
21	96.8%
31	95.9%
41	94.9%
51	94.1%

220 108,422,358 paired reads containing 10.94 Gbp with an average length of
 221 100.9 bases. A total of 46.56 Mbp remained in 520,403 orphan reads with
 222 an average length of 89.5 bases. In total, the quality trimmed data contained
 223 10.98 Gbp in 108,942,761 reads. This quality trimmed (“QC”) data set was
 224 used as the basis for all further analyses.

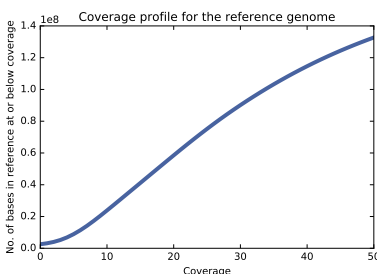


Figure 1: Cumulative coverage profile for the reference metagenome, based on read mapping.

225 **The reference metagenome is not completely present in the**
 226 **reads.**

227 We next evaluated the fraction of the reference genome covered by at least
 228 one read (see Methods for details). Quality filtered reads cover 203,058,414
 229 (98.76%) bases of the reference metagenome (205,603,715 bp total size). Fig-
 230 ure 1 shows the cumulative coverage profile of the reference metagenome,
 231 and the percentage of bases with that coverage. Most of the reference
 232 metagenome was covered at least minimally; only 3.33% of the reference
 233 metagenome had mapping coverage <5, and 1.24% of the bases in the ref-
 234 erence were not covered by any reads in the QC data set.

235 In order to evaluate reconstructability with De Bruijn graph assemblers,
 236 we next examined k-mer containment of the reference in the reads for k of

237 21, 31, 41, and 51 (Table 1). The k-mer overlap decreases from 96.8% to
 238 94.1% as the k-mer size increases. This could be caused by low coverage of
 239 some portions of the reference and/or variation between the reads and the
 240 reference.

241 **Some individual reference genomes are poorly represented in**
 242 **the reads.**

Table 2: Top uncovered genomes

Genome	Read coverage
<i>Desulfovibrio vulgaris</i> DP4	93.2%
<i>Thermus thermophilus</i> HB27	91.1%
<i>Enterococcus faecalis</i> V583	74.6%
<i>Fusobacterium nucleatum</i>	47.6%

243 To see if specific reference genomes exhibited low coverage, we analyzed
 244 read mapping coverage for individual genomes. Of the 64 reference genomes
 245 used in the metagenome, 60 had a per-base mapping coverage above 95%.
 246 The remaining four varied significantly (Table 2), with *F. nucleatum* the
 247 lowest – only 47.6% of the bases in the reference genome are covered by one
 248 or more mapped reads.

249 We next did a 51-mer containment analysis of each reference genome in
 250 the reads; k=51 was chosen so as to be specific to strain content [27]. 99%
 251 or more of the constituent 51-mers for 51 of the 64 reference genomes were
 252 present in the reads, suggesting that each of the 51 genomes was entirely
 253 present at some minimal coverage.

254 We excluded the remaining 13 genomes (see Table 3) from any fur-
 255 ther reference-based analysis because interpreting recovery and misassembly
 256 statistics for these genomes would be confounding; also see the discussion of
 257 strain variants, below.

258 **MEGAHIT is the fastest and lowest-memory assembler eval-**
 259 **uated**

260 We ran three commonly used metagenome assemblers on the QC data set:
 261 IDBA-UD, MetaSPAdes, and MEGAHIT. We recorded the time and mem-
 262 ory usage of each (Table 4). In computational requirements, MEGAHIT
 263 outperformed both MetaSPAdes and IDBA-UD, , producing an assembly in

Table 3: Genomes removed from reference for low 51-mer presence

51-mers in reads	Genome
98.7	<i>Leptothrix cholodnii</i>
98.7	<i>Haloferax volcanii</i> DS2
98.6	<i>Salinispora tropica</i> CNB-440
97.4	<i>Deinococcus radiodurans</i>
97.2	<i>Zymomonas mobilis</i>
97.1	<i>Ruegeria pomeroyi</i>
96.8	<i>Shewanella baltica</i> OS223
95.5	<i>B. bronchiseptica</i> D989
94.5	<i>Burkholderia xenovorans</i>
72.0	<i>Desulfovibrio vulgaris</i> DP4
65.0	<i>Thermus thermophilus</i> HB27
53.4	<i>Enterococcus faecalis</i>
4.7	<i>Fusobacterium nucleatum</i> ATCC 25586

Table 4: Running Time and Memory Utilization

Assembler	CPU time	Wall time	RAM (Max RSS)
MEGAHIT	1191m	1h 33m	10 GB
IDBA-UD	1904m	2h 27m	17 GB
MetaSPAdes	2554m	4h 7m	28 GB

1.5 hours (“wall time”) – 1.6 times faster than IDBA and 2.6 times faster than MetaSPAdes. MEGAHIT used only 10 GB of RAM as requested – about 60% of the memory used by IDBA and a third of the memory used by MetaSPAdes. CPU time measurements (which include processing on multiple CPU cores) show that all three assemblers use multiple cores effectively.

The assemblies contain most of the raw data

Table 5: Read and high-abundance (> 5) k-mer exclusion from assemblies

Assembly	Unmapped Reads	51-mers omitted
IDBA	3,328,674 (3.05%)	2.4%
MetaSPAdes	3,844,123 (3.52%)	3.2%
MEGAHIT	2,737,640 (2.51%)	2.8%

270 We assessed read inclusion in assemblies by mapping the QC reads to
 271 the length-filtered assemblies and counting the remaining unmapped reads.
 272 Depending on the assembly, between 2.7 million and 3.9 million reads (2.5-
 273 3.5%) did not map to the assemblies (Table 5). All of the assemblies included
 274 the large majority of high-abundance 51-mers (more than 96.8% in all cases).

275 **Much of the reference is covered by the assemblies.**

Table 6: Contig coverage of reference with loose alignment conditions.

Assembly	bases aligned	duplication	51-mers
MEGAHIT	94.8%	1.0%	96.7%
MetaSPAdes	93.1%	1.1%	96.2%
IDBA	93.6%	0.98%	97.2%

276 We next evaluated the extent to which the assembled contigs recovered
 277 the “known/true” metagenome sequence by aligning each assembly to the
 278 adjusted reference (Table 6). Each of the three assemblers generates contigs
 279 that cover more than 93.1% of the reference metagenome at high identity
 280 (99%) with little duplication (approximately 1%). All three assemblies con-
 281 tain between 96.2% and 97.2% of the 51-mers in the reference.

282 At 99% identity with the loose mapping approach, approximately 2.5% of
 283 the reference is missed by all three assemblers, while 1.7% is uniquely covered
 284 by MEGAHIT, 0.74% is uniquely covered by MetaSPAdes, and 0.64% is
 285 uniquely covered by IDBA.

286 **The generated contigs are broadly accurate.**

Table 7: Contig accuracy measured by reference coverage with strict alignment.

Assembly	% covered
MEGAHIT	89.3%
IDBA	87.7%
MetaSPAdes	83.4%

287 When counting only the best (longest) alignment per contig at a 99%
 288 identity threshold, each of the three assemblies recovers more than 87.3% of
 289 the reference, with MEGAHIT recovering the most – 89.3% of the reference
 290 (Table 7).

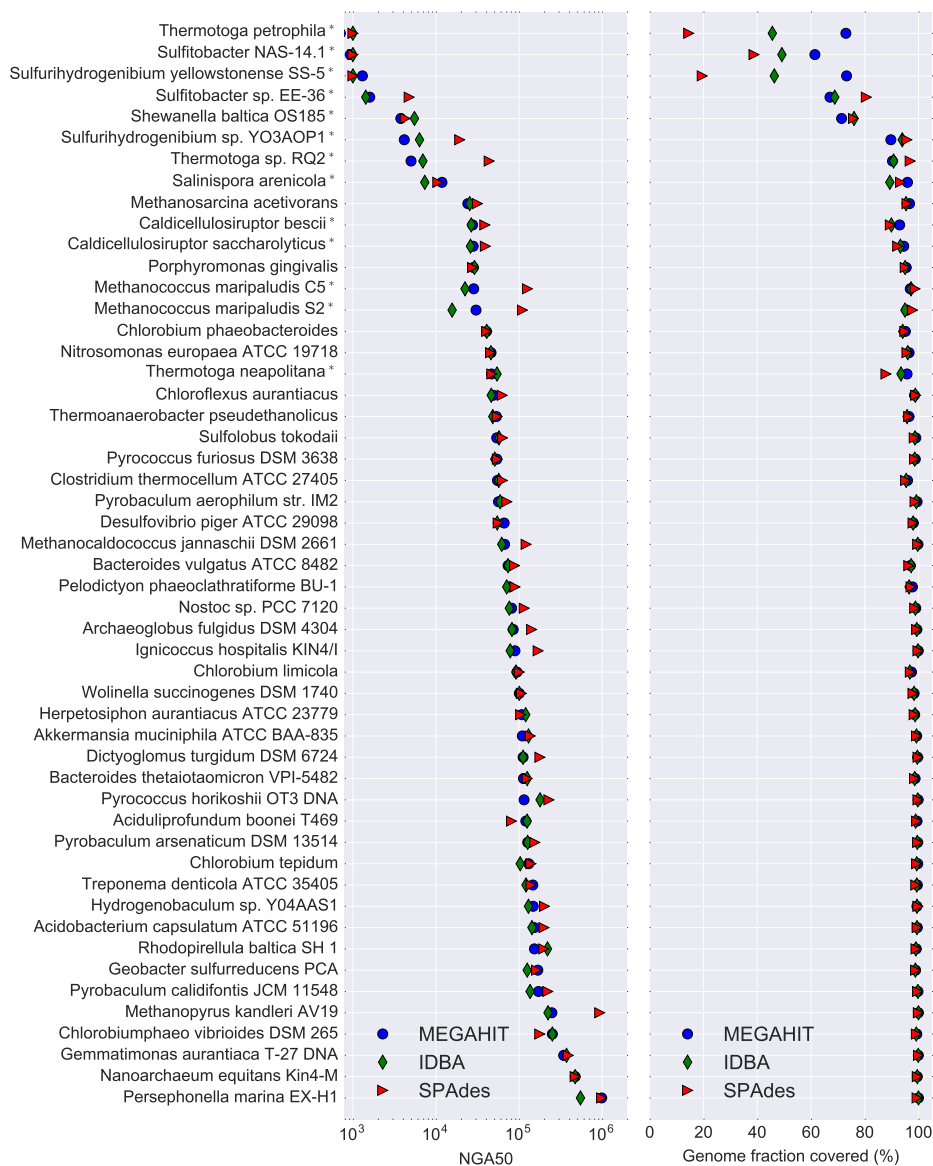


Figure 2: NGA50 and genome fraction covered, by genome and assembler. A '*' after the name indicates the presence of at least one other genome with > 2% Jaccard similarity at k=31 in the community. Where NGA50 cannot be calculated due to poor coverage, a marker is placed at 1kb.

291 **Individual genome statistics vary widely in the assemblies.**

292 We computed the NGA50 for each individual genome and assembly in order
 293 to compare assembler performance on genome recovery (see left panel of Fig-
 294 ure 2). The NGA50 statistics for individual genomes vary widely, but there
 295 are consistent assembler-specific trends: IDBA yields the lowest NGA50 for
 296 28 of the 51 genomes, while MetaSPAdes yields the highest NGA50 for 32
 297 of the 51 genomes.

298 We also evaluated aligned coverage per genome for each of the three
 299 assemblies (right panel, Figure 2). We found that 13 of the 51 genomes
 300 were missing 5% or more of bases in at least one assembly, despite all 51
 301 genomes having 99% or higher read- and 51-mer coverage. While some of
 302 these missing bases may be in the assembled contigs that are less than 500
 303 bp in length, contigs shorter than 500 bp are unlikely to contain more than
 304 half of a typical bacterial gene [32].

305 There are 12 genomes with k=31 Jaccard similarity greater than 2%
 306 to other genomes in the community, and these (denoted by '*' after the
 307 name) typically had lower NGA50 and aligned coverage numbers than other
 308 genomes. In particular, these constituted 12 of the 13 genomes missing 5%
 309 or more of their content, and the lowest eight NGA50 numbers.

310 **Longer contigs are less likely to be chimeric.**

Table 8: Chimeric contigs by contig length.

Assembly	> 50kb	> 5kb	> 500 bp
IDBA	0	1	7 (0.06%)
MEGAHIT	1	4	14 (0.13%)
MetaSPAdes	0	3	30 (0.48%)

311 Chimerism is the formation of contigs that include sequence from multi-
 312 ple genomes. We evaluated the rate of chimerism in contigs at three different
 313 contig length cutoffs: 500bp, 5kb, and 50kb (Table 8). We found that the
 314 percentage of contigs that match to the genomes of two or more different
 315 species drop as the minimum contig size increases, to the point where only
 316 the MEGAHIT assembly had a single chimeric contig longer than 50kb.
 317 Overall, chimeric misassemblies were rare, with no assembler generating
 318 more than 30 chimeric contigs out of thousands of total contigs.

Table 9: GenBank genomes detected in assembly of unmapped reads

match	GenBank genome
44.1%	<i>Fusobacterium</i> sp. OBRC1
23.0%	<i>P. ruminis</i> strain ML2
18.2%	<i>Thermus thermophilus</i> HB8
7.7%	<i>P. ruminis</i> strain CGMCC
8.2%	<i>Enterococcus faecalis</i> M7
7.3%	<i>F. nucleatum</i> 13_3C
3.7%	<i>F. nucleatum</i> subsp. <i>polymorphum</i>
2.9%	<i>Fusobacterium hwasookii</i>
1.0%	<i>E. coli</i> isolate YS
1.7%	<i>F. nucleatum</i> subsp. <i>polymorphum</i> , alt.
1.9%	<i>F. nucleatum</i> subsp. <i>vincentii</i>

319 The unmapped reads contain strain variants of reference genomes.

320 Approximately 4.8 million reads (4.4%) from the QC data set did not map
321 anywhere in the reference provided by the authors of [12]. We extracted
322 and assembled these reads in isolation using MEGAHIT, yielding 6.5 Mbp
323 of assembly in 1711 contigs > 500bp in length. We then did a k-mer in-
324 clusion analysis of this assembly against all of the GenBank genomes at
325 k=31, and estimated the fraction of the k-mers that belonged to different
326 species (Table 9). We find that 51.1% of the k-mer content of these contigs
327 positively match to a genome present in GenBank but not in the reference
328 metagenome.

329 To verify these assignments, we aligned the MEGAHIT assembly of un-
330 mapped reads to the GenBank genomes in Table 9 with NUCmer using
331 “loose” alignment criteria. We found that 1.78 Mbp of the contigs aligned
332 at 99% identity or better to these GenBank genomes. We also confirmed
333 that, as expected, there are no matches in this assembly to the full updated
334 reference metagenome.

335 We note that all but the two *P. ruminis* matches and the *E. coli* isolate
336 YS are strain variants of species that are part of the defined community
337 but are not completely present in the reads (see Table 2). For *Proteiniclas-*
338 *ticum ruminis*, there is no closely related species in the mock community
339 design, and very little of the MEGAHIT assembly aligns to known *P. ru-*
340 *minis* genomes at 99%. However, there are many alignments to *P. ruminis*
341 at 94% or higher, for approximately 2.73 Mbp total. This suggests that the

unmapped reads contain at least some data from a novel species of *Proteini-*
clasticum; this matches the observation in [12] of a contaminating genome
from an unknown *Clostridium* spp., as at the time there was no *P. ruminis*
genome.

Discussion

Assembly recovers basic content sensitively and accurately.

All three assemblers performed well in assembling contigs from the content that was fully present in reads and k-mers. After length filtering, all three assemblies contained more than 95% of the reference (Table 6); even with removal of secondary alignments, more than 87% was recovered by each assembler (Table 7). About half the constituent genomes had an NGA50 of 50kb or higher (Figure 2), which, while low for current Illumina single-genome sequencing, is sufficient to recover operon-level relationships for many genes.

The presence of multiple closely related genomes confounds assembly.

In agreement with CAMI, we also find that the presence of closely related genomes in the metagenome causes loss of assembly [3]. This is clearly shown by Figure 2, where 12 of the bottom 14 genomes by NGA50 (left panel) also exhibit poor genome recovery by assembly (right panel). Interestingly, different assemblers handle this quite differently, with e.g. MetaSPAdes failing to recover essentially any of *Thermotoga petrophila*, while MEGAHIT recovers 73%. The presence of nearby genomes is an almost perfect predictor that one or more assembler will fail to recover 5% or more - of the 13/51 genomes for which less than 95% is recovered, 12 of them have close genomes in the community. Interestingly, very little similarity is needed - all genomes with Jaccard similarity of 2% or higher at k=31 exhibit these problems.

The *Shewanella baltica* OS185 genome is a good example: there are two strain variants, OS185 and OS223, present in the defined community. Both are present at more than 99% in the reads, and more than 98% in 51-mers, but only 75% of *S. baltica* OS185 and 50% of *S. baltica* OS223 are recovered by assemblers. This is a clear case of “strain confusion” where the assemblers simply fail to output contigs for a substantial portion of the two genomes.

Another interest of this study was to examine cross-species chimeric as-

sembly, in which a single contig is formed from multiple genomes. In Table 8, we show that there is relatively little cross-species chimerism. Surprisingly, what little is present is length-dependent: longer contigs are less likely to be chimeric. This might well be due to the same “strain confusion” effect as above, where contigs that share paths in the assembly graphs are broken in twain.

MEGAHIT performs best by several metrics.

MEGAHIT is clearly the most efficient computationally, outperforming both MetaSPAdes and IDBA in memory and time (Table 4). The MEGAHIT assembly also included more of the reads than either IDBA or MetaSPAdes, and omitted only 0.4% more of the unique 51-mers from the reads than IDBA. MEGAHIT covered more of the reference genome with both loose and strict alignments (Table 6 and Table 7), with little duplication. This is clearly because of MEGAHIT’s generally superior performance in recovering the genomes of closely related strains (Figure 2, right panel). The sum “fraction of genome recovered” is arguably the most important measure of a metagenome assembler (see [5] in particular) and here MEGAHIT excels for individual genomes even in the presence of strain variation.

In general other studies have found that MEGAHIT excels in recovery of sequence through assembly [3, 17] and is considerably more computationally efficient than most other assemblers [3, 16]. However, studies have also shown that MEGAHIT produces more misassemblies than other assemblers [3] and performs poorly on high coverage portions of the data set [5]. Thus while we can recommend MEGAHIT as a good first assembler, we can also not unambiguously recommend it as the only assembler to use.

When comparing details of sequence recovery between the assemblers, the assembly content differs by only a small amount when loose alignments are allowed: all three assemblers miss more content (approximately 2.5% of the reference) than they generate uniquely (1.7% or less). In addition to preferring no one assembler over any other, this suggests that combining assemblies may have little value in terms of recovering additional metagenome content. The genome alignment statistics in Figure 2 suggest that much of this differential assembly content is due to the impact of strains.

409 **The missing reference may be present in strain variants of the**
410 **intended species.**

411 Several individual genomes are missing in measurable portion from the QC
412 reads (Table 2), and many QC reads (4.4% of 108m) did not map to the full
413 reference metagenome. These appear to be related issues: upon analysis of
414 the unmapped reads against GenBank, we find that many of the contigs as-
415 sembled from the unmapped reads can be assigned to strain variants of the
416 species in the mock community (Table 9) and align closely to the identified
417 genomes. This suggests that the constructors of the mock community may
418 have unintentionally included strain variants of *Fusobacterium nucleatum*,
419 *Thermus thermophilus* HB27, and *Enterococcus faecalis*; note that the mi-
420 crobes used were sourced from the community rather than the ATCC (M.
421 Podar, pers. communication). In addition, we detect what may be por-
422 tions of a novel member of the *Proteiniclasticum* genus in the assembly of
423 these reads - this is likely the *Clostridium* spp. detected through amplicon
424 sequencing in [12].

425 Without returning to the original DNA samples, it is impossible to con-
426 clusively confirm that unintended strains were used in the construction of the
427 mock community. In particular, our analysis is dependent on the genomes in
428 GenBank: the genomes we detect in the contigs are clearly closely related to
429 GenBank genomes not in the reference metagenome, based on k-mer anal-
430 ysis and contig alignment. However, GenBank is unlikely to contain the
431 exact genomes of the actually included strain variants, rendering conclusive
432 identification impossible.

433 Conclusions

434 Overall, assembly of this mock community works well, with good recovery
435 of known genomic sequence for the majority of genomes. All three assem-
436 blers that we evaluated recover similar amounts of most genomic sequence,
437 but (recapitulating several other studies [3, 5, 16]) MEGAHIT is compu-
438 tationally the most efficient of the three. We note that assembly resolves
439 substantial portions of several previously undetected strain variants, as well
440 as recovering a substantial portion of a novel *Proteiniclasticum* spp. that
441 was detected via amplicon analysis in [12], suggesting that assembly is a
442 useful complement to amplicon or reference-based analyses.

443 The presence of closely related strains is a major confounder of metagenome
444 assembly, and causes assemblers to drop considerable portions of genomes

445 that (based on read mapping and k-mer inclusion) are clearly present. In this
 446 relatively simple community, this strain confusion is present but does not
 447 dominate the assembly. However, real microbial communities are likely to
 448 have many closely related strains and any resulting loss of assembly would
 449 be hard to detect in the absence of good reference genomes. While high
 450 polymorphism rates in e.g. animal genomes are known to cause duplication
 451 or loss of assembly, some solutions have emerged that make use of assump-
 452 tions of uniform coverage and diploidy [33]. These solutions cannot however
 453 be transferred directly to metagenomes, which have unknown abundance
 454 distributions and strain content.

455 An additional concern is that metagenome assemblies are often per-
 456 formed after pooling data sets to increase coverage (e.g. [4, 34]); this pooled
 457 data is more likely to contain multiple strains, which would then in turn
 458 adversely affect assembly of strains. This may not be resolvable within the
 459 current paradigm of assembly, which focuses on outputting linear assem-
 460 blies that cannot properly represent strain variation. The human genomics
 461 community is moving towards using *reference graphs*, which can represent
 462 multiple incompatible variants in a single data structure [35]; this approach,
 463 however, requires high-quality isolate reference genomes, which are generally
 464 unavailable for environmental microbes.

465 Long read sequencing (and related technologies) will undoubtedly help
 466 resolve strain variation in the future, but even with highly accurate long-
 467 read sequencing, current sequencing depth is still too low to resolve deep
 468 environmental metagenomes [36, 37]. It is unclear how well long error-
 469 prone reads (such as those output by Pacific Biosciences SMRT [38] and
 470 Oxford Nanopore instruments [39]) will perform on complex metagenomes:
 471 with high error rates, deep coverage of each individual genome is required
 472 to achieve accurate assembly, and this may not be easily obtainable for
 473 complex communities. Single-molecule barcoding (e.g. 10X Genomics [40])
 474 and HiC approaches [41] show promise but these remain untested on well-
 475 defined complex communities and are still challenged by the complexity of
 476 complex environmental metagenomes; see [42, 43, 44].

477 Much of our analysis above depends on having a high-quality “mock”
 478 metagenome. While computationally constructed synthetic communities
 479 and computational “spike-ins” to real data sets can provide valuable controls
 480 (e.g. see [16] and [45]) we strongly believe that standardized communities
 481 constructed *in vitro* and sequenced with the latest technologies are critical
 482 to the evaluation of both canonical and emerging tools, e.g. efforts such as
 483 [46]. From the perspective of tool evaluation, we disagree somewhat with

484 Vollmers et al. [5]: good metagenome tool evaluation necessarily depends
485 on mock communities that are as realistic as we can make them. Likewise,
486 from the perspective of bench biologists, actually sequencing real DNA is
487 critical because it can evaluate confounding effects such as kit contamination
488 [47]. Large-scale studies of computational approaches systematically
489 applied to mock communities such as CAMI [3] can then provide fair comparisons
490 of entire toolchains (wet and dry combined) applied to these mock
491 communities.

492 We omitted two important questions in this study: binning and choice
493 of parameters. We chose not to evaluate genome binning because most binning
494 strategies either operate post-assembly (see e.g. [48]), in which case
495 the challenges with assembly discussed above will apply; or require multiple
496 samples (e.g. [49]), which we do not have. We also chose to use only
497 default parameters with all three assemblers, for two reasons. First, we
498 are not aware of any effective automated approaches for determining the
499 “best” set of parameters or evaluating the output for metagenome assemblers,
500 other than those integrated into the assemblers themselves (e.g. the choice
501 of k-mer sizes by MEGAHIT and MetaSPAdes), and absent such guidance we do not
502 feel comfortable blessing any particular set of parameters; here the choice of
503 default parameters is parsimonious (and also see [50] for the dangers of poorly
504 chosen objective functions). Second, any parameter exploration pipeline would
505 not only need to be automated but would need to run multiple assemblies, whose
506 time and resource usage should be measured; in this case, any comparison based
507 on runtime of the parameter choice pipeline should naturally favor MEGAHIT
508 because of its advantage in computational efficiency.

510 **Author contributions**

511 SA, LI and CTB developed, tested, and executed the analytical pipeline.
512 SA and CTB created the tables and figures and wrote the paper.

513 **Competing interests**

514 No competing interest to our knowledge.

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