

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 gbttools for binning [13] and benchmarking of the
64 MEGAHIT assembler [14].

65 More recently, several benchmark studies systematically evaluated metagenome
66 assembly of short reads. The Critical Assessment of Metagenome Interpre-
67 tation (CAMI) collaboration benchmarked a number of metagenome assem-
68 blers on several data sets of varying complexity, evaluating recovery of novel
69 genomes and multiple strain variants [3]. Notably, CAMI concluded that
70 “The resolution of strain-level diversity represents a substantial challenge
71 to all evaluated programs.” Another recent study evaluated eight assem-
72 blers on nine environmental metagenomes and three simulated data sets
73 and provided a workflow for choosing a metagenome assembler based on
74 the biological goal and computational resources available [15]. [5] explored
75 metagenome assembler performance on a pair of real data sets, again con-
76 cluding that the biological goal and computational resources defined the
77 choice of assembler. Also see [16] for an analysis of a previously generated
78 HMP benchmark data set; however, the Illumina reads used for this study
79 are much shorter than current sequencing and are arguably not relevant to
80 future studies.

81 In this study, we extend previous work by delving into questions of
82 chimeric misassembly and strain recovery in the Shakya et al. (2013) data
83 set. First, we update the list of reference genomes for Shakya et al. to in-
84 clude the latest GenBank assemblies along with plasmids. We then compare
85 IDBA [17], MetaSPAdes [18], and MEGAHIT [19] performance on assem-
86 bling this short-read data set, and explore concordance in recovery between
87 the three assemblers. We describe the effects of “strain confusion” between
88 multiple strains. We also detect and analyze several previously unreported
89 strains and genomes in the Shakya et al. data set. We find that in the ab-
90 sence of closely related genomes, all three metagenome assemblers recover
91 95% or more of known reference genomes. However, in the presence of
92 closely related genomes, these three metagenome assemblers vary widely in

93 their performance and, in extreme cases, can fail to recover the majority of
94 some genomes even when they are completely present in the reads. Our re-
95 port provides strong guidance on choice of assemblers and extends previous
96 analyses of this low-complexity metagenome benchmarking data set.

97 **Datasets**

98 We used a diverse mock community data set constructed by pooling DNA
99 from 64 species of bacteria and archaea and sequencing them with Illumina
100 HiSeq. The raw data set consisted of 109,629,496 reads from Illumina HiSeq
101 101 bp paired-end sequencing (2x101) with an untrimmed total length of
102 11.07 Gbp and an estimated fragment size of 380 bp [12].

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

110 **Methods**

111 The analysis code and run scripts for this paper are written in Python and
112 bash, and are available at [https://github.com/dib-lab/2016-metagenome-](https://github.com/dib-lab/2016-metagenome-assembly-eval/)
113 `assembly-eval/` (archived at Zenodo DOI: 10.5281/zenodo.821919). The
114 scripts and overall pipeline were examined by the first and senior authors for
115 correctness. In addition, the bespoke reference-based analysis scripts were
116 tested by running them on a single-colony *E. coli* MG1655 data set with a
117 high quality reference genome [20].

118 **Quality Filtering**

119 We removed adapters with Trimmomatic v0.30 in paired-end mode with
120 the TruSeq adapters [21], using light quality score trimming (`LEADING:2`
121 `TRAILING:2 SLIDINGWINDOW:4:2 MINLEN:25`) as recommended in MacManes,
122 2014 [22].

123 Reference Coverage Profile

124 To evaluate how much of the reference metagenome was contained in the
125 read data, we used `bwa aln` (v0.7.7.r441) to map reads to the reference
126 genome [23]. We then calculated how many reference bases were covered by
127 mapped reads (custom script `coverage-profile.py`).

128 Measuring k-mer inclusion and Jaccard similarity

129 We used MinHashing as implemented in `sourmash` to estimate k-mer inclu-
130 sion and Jaccard similarity between data sets [24]. MinHash signatures were
131 prepared with `sourmash compute` using `--scaled 10000`. K-mer inclusion
132 was computed by taking the ratio of the number of intersecting hashes with
133 the query over the total number of hashes in the subject MinHash. Jac-
134 card similarity was computed as in [25] by taking the ratio of the number
135 of intersecting hashes between the query and subject over the number of
136 hashes in the union. K-mer sizes for comparison were chosen at 21, 31, or
137 51, depending on the level of taxonomic specificity desired - genus, species,
138 or strain, respectively, as described in [26].

139 Where specified, high-abundance k-mers were selected for counting by
140 using the script `trim-low-abund.py` script with `-C 5` from `khmer` v2 [27,
141 28].

142 Assemblers

143 We assembled the quality-filtered reads using three different assemblers:
144 IDBA-UD [17], MetaSPAdes [18], and MEGAHIT [19]. For IDBA-UD v1.1.3
145 [17], we used `--pre_correction` to perform pre-correction before assembly
146 and `-r` for the pe files. IDBA could not ingest orphan sequences so singleton
147 reads were omitted from this assembly.

148 For MetaSPAdes v3.10.1 [18], we used `--meta --pe1-12 --pe1-s` where
149 `--meta` is used for metagenomic data sets, `--pe1-12` specifies the interlaced
150 reads for the first paired-end library, and `--pe1-s` provides the orphan reads
151 remaining from quality trimming.

152 For MEGAHIT v1.1.1-2-g02102e1 [19], we used `-l 101 -m 3e9 --cpu-only`
153 where `-l` is for maximum read length, `-m` is for max memory in bytes to
154 be used in constructing the graph, and `--cpu-only` uses only the CPU
155 and no GPUs. We also used `--presets meta-large` for large and complex
156 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) [23]. We aligned paired-end and orphaned reads separately. We then used `samtools` (v0.1.19) [29] 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 [29].

Assembly analysis using NUCmer

We used the NUCmer tool from MUMmer3.23 [30] 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.

Reference-based analysis of the assemblies

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

The script `summarize-coords2.py` was used to calculate aligned coverage from the loose alignment conditions: each base in the reference was marked as “covered” if it was included in at least one alignment. The script

190 `analyze_ng50.py` was used to calculate NGA 50 for each individual refer-
191 ence genome.

192 Analysis of chimeric misassemblies

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

202 Analysis of unmapped reads

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

207 Results

208 The raw data is high quality.

209 The reads contain 11,072,579,096 bp (11.07 Gbp) in 109,629,496 reads with
210 101.0 average length (2x101bp Illumina HiSeq).

211 Trimming removed 686,735 reads (0.63%). After trimming, we retained
212 108,422,358 paired reads containing 10.94 Gbp with an average length of
213 100.9 bases. A total of 46.56 Mbp remained in 520,403 orphan reads with
214 an average length of 89.5 bases. In total, the quality trimmed data contained
215 10.98 Gbp in 108,942,761 reads. This quality trimmed (“QC”) data set was
216 used as the basis for all further analyses.

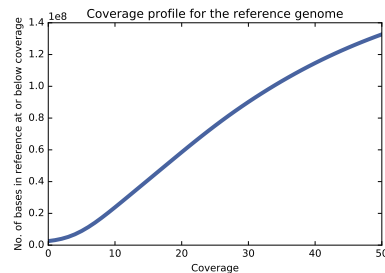


Figure 1: Cumulative coverage profile for the reference metagenome, based on read mapping.

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%

217 **The reference metagenome is not completely present in the**
218 **reads.**

219 We next evaluated the fraction of the reference genome covered by at least
220 one read (see Methods for details). Quality filtered reads cover 203,058,414
221 (98.76%) bases of the reference metagenome (205,603,715 bp total size). Fig-
222 ure 1 shows the cumulative coverage profile of the reference metagenome,
223 and the percentage of bases with that coverage. Most of the reference
224 metagenome was covered at least minimally; only 3.33% of the reference
225 metagenome had mapping coverage <5 , and 1.24% of the bases in the ref-
226 erence were not covered by any reads in the QC data set.

227 In order to evaluate reconstructability with De Bruijn graph assemblers,
228 we next examined k-mer containment of the reference in the reads for k of
229 21, 31, 41, and 51 (Table 1). The k-mer overlap decreases from 96.8% to
230 94.1% as the k-mer size increases. This could be caused by low coverage of
231 some portions of the reference and/or variation between the reads and the
232 reference.

233 **Some individual reference genomes are poorly represented in**
 234 **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%

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

241 We next did a 51-mer containment analysis of each reference genome in
 242 the reads; k=51 was chosen so as to be specific to strain content [26]. 99%
 243 or more of the constituent 51-mers for 51 of the 64 reference genomes were
 244 present in the reads, suggesting that each of the 51 genomes was entirely
 245 present at some minimal coverage.

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

250 **MEGAHIT is the fastest and lowest-memory assembler eval-** 251 **uated**

252 We ran three commonly used metagenome assemblers on the QC data set:
 253 IDBA-UD, MetaSPAdes, and MEGAHIT. We recorded the time and mem-
 254 ory usage of each (Table 4). In computational requirements, MEGAHIT
 255 outperformed both MetaSPAdes and IDBA-UD, , producing an assembly in
 256 1.5 hours (“wall time”) – 1.6 times faster than IDBA and 2.6 times faster
 257 than MetaSPAdes. MEGAHIT used only 10 GB of RAM as requested –
 258 about 60% of the memory used by IDBA and a third of the memory used by
 259 MetaSPAdes. CPU time measurements (which include processing on multi-
 260 ple CPU cores) show that all three assemblers use multiple cores effectively.

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

261 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%

262 We assessed read inclusion in assemblies by mapping the QC reads to
 263 the length-filtered assemblies and counting the remaining unmapped reads.
 264 Depending on the assembly, between 2.7 million and 3.9 million reads (2.5-
 265 3.5%) did not map to the assemblies (Table 5). All of the assemblies included
 266 the large majority of high-abundance 51-mers (more than 96.8% in all cases).

267 **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%

268 We next evaluated the extent to which the assembled contigs recovered
 269 the “known/true” metagenome sequence by aligning each assembly to the
 270 adjusted reference (Table 6). Each of the three assemblers generates contigs
 271 that cover more than 93.1% of the reference metagenome at high identity
 272 (99%) with little duplication (approximately 1%). All three assemblies con-
 273 tain between 96.2% and 97.2% of the 51-mers in the reference.

274 At 99% identity with the loose mapping approach, approximately 2.5% of
 275 the reference is missed by all three assemblers, while 1.7% is uniquely covered
 276 by MEGAHIT, 0.74% is uniquely covered by MetaSPAdes, and 0.64% is
 277 uniquely covered by IDBA.

278 **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%

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

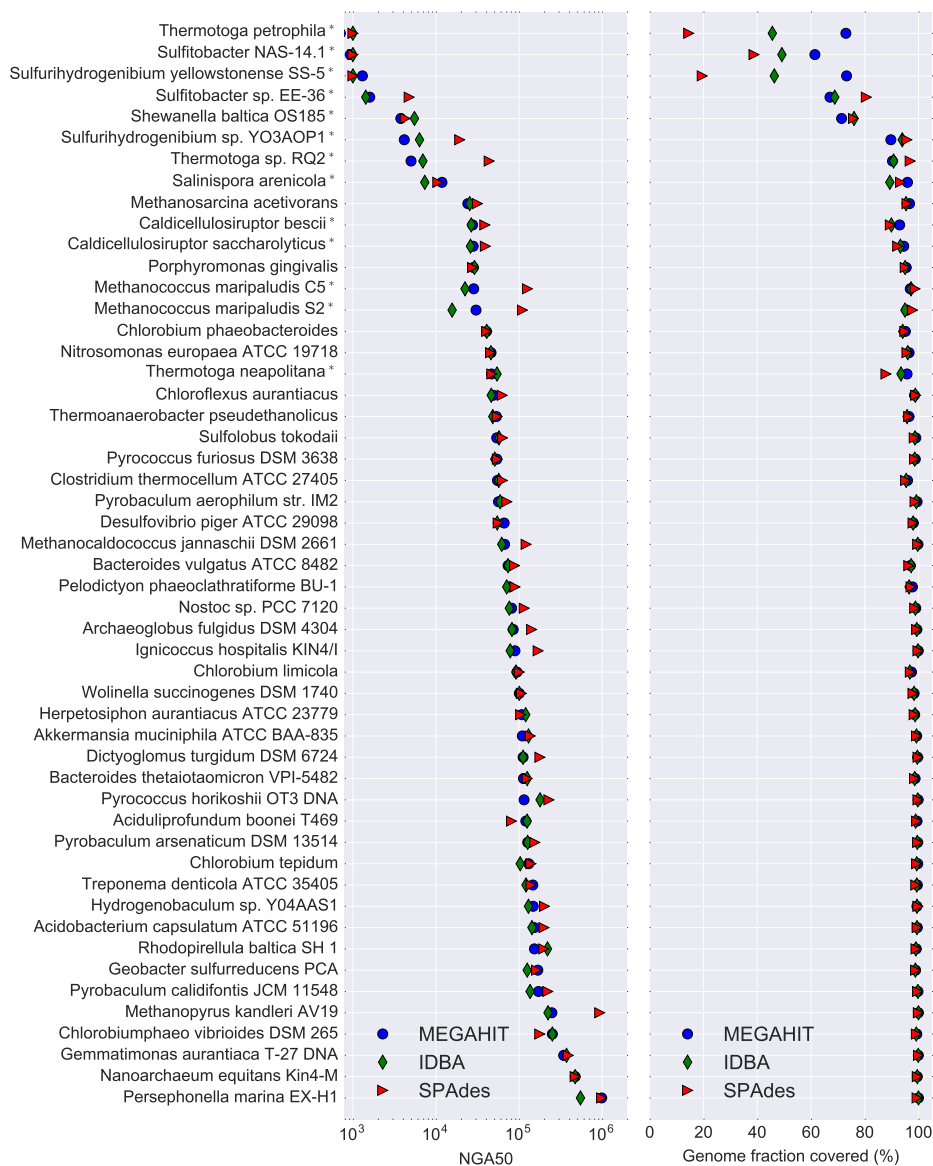


Figure 2: NGA50 and genome fraction covered, by genome and assembler. A '*' after the name indicates the presence of at least one other genome with > 2% Jaccard similarity at k=31 in the community. Where NGA50 cannot be calculated due to poor coverage, a marker is placed at 1kb.

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

284 We computed the NGA50 for each individual genome and assembly in order
 285 to compare assembler performance on genome recovery (see left panel of Fig-
 286 ure 2). The NGA50 statistics for individual genomes vary widely, but there
 287 are consistent assembler-specific trends: IDBA yields the lowest NGA50 for
 288 28 of the 51 genomes, while MetaSPAdes yields the highest NGA50 for 32
 289 of the 51 genomes.

290 We also evaluated aligned coverage per genome for each of the three
 291 assemblies (right panel, Figure 2). We found that 13 of the 51 genomes
 292 were missing 5% or more of bases in at least one assembly, despite all 51
 293 genomes having 99% or higher read- and 51-mer coverage. While some of
 294 these missing bases may be in the assembled contigs that are less than 500
 295 bp in length, contigs shorter than 500 bp are unlikely to contain more than
 296 half of a typical bacterial gene [31].

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

302 **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%)

303 Chimerism is the formation of contigs that include sequence from multi-
 304 ple genomes. We evaluated the rate of chimerism in contigs at three different
 305 contig length cutoffs: 500bp, 5kb, and 50kb (Table 8). We found that the
 306 percentage of contigs that match to the genomes of two or more different
 307 species drop as the minimum contig size increases, to the point where only
 308 the MEGAHIT assembly had a single chimeric contig longer than 50kb.
 309 Overall, chimeric misassemblies were rare, with no assembler generating
 310 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>

311 The unmapped reads contain strain variants of reference genomes.

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

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

327 We note that all but the two *P. ruminis* matches and the *E. coli* isolate
328 YS are strain variants of species that are part of the defined community
329 but are not completely present in the reads (see Table 2). For *Proteiniclas-*
330 *ticum ruminis*, there is no closely related species in the mock community
331 design, and very little of the MEGAHIT assembly aligns to known *P. ru-*
332 *minis* genomes at 99%. However, there are many alignments to *P. ruminis*
333 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, 16] and is considerably more computationally efficient than most other assemblers [3, 15]. 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.

401 **The missing reference may be present in strain variants of the**
402 **intended species.**

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

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

425 Conclusions

426 Overall, assembly of this mock community works well, with good recovery
427 of known genomic sequence for the majority of genomes. All three assem-
428 blers that we evaluated recover similar amounts of most genomic sequence,
429 but (recapitulating several other studies [3, 5, 15]) MEGAHIT is compu-
430 tationally the most efficient of the three. We note that assembly resolves
431 substantial portions of several previously undetected strain variants, as well
432 as recovering a substantial portion of a novel *Proteiniclasticum* spp. that
433 was detected via amplicon analysis in [12], suggesting that assembly is a
434 useful complement to amplicon or reference-based analyses.

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

437 that (based on read mapping and k-mer inclusion) are clearly present. In this
438 relatively simple community, this strain confusion is present but does not
439 dominate the assembly. However, real microbial communities are likely to
440 have many closely related strains and any resulting loss of assembly would
441 be hard to detect in the absence of good reference genomes. While high
442 polymorphism rates in e.g. animal genomes are known to cause duplication
443 or loss of assembly, some solutions have emerged that make use of assump-
444 tions of uniform coverage and diploidy [32]. These solutions cannot however
445 be transferred directly to metagenomes, which have unknown abundance
446 distributions and strain content.

447 An additional concern is that metagenome assemblies are often per-
448 formed after pooling data sets to increase coverage (e.g. [4, 33]); this pooled
449 data is more likely to contain multiple strains, which would then in turn
450 adversely affect assembly of strains. This may not be resolvable within the
451 current paradigm of assembly, which focuses on outputting linear assem-
452 blies that cannot properly represent strain variation. The human genomics
453 community is moving towards using *reference graphs*, which can represent
454 multiple incompatible variants in a single data structure [34]; this approach,
455 however, requires high-quality isolate reference genomes, which are generally
456 unavailable for environmental microbes.

457 Long read sequencing (and related technologies) will undoubtedly help
458 resolve strain variation in the future, but even with highly accurate long-
459 read sequencing, current sequencing depth is still too low to resolve deep
460 environmental metagenomes [35, 36]. It is unclear how well long error-
461 prone reads (such as those output by Pacific Biosciences SMRT [37] and
462 Oxford Nanopore instruments [38]) will perform on complex metagenomes:
463 with high error rates, deep coverage of each individual genome is required
464 to achieve accurate assembly, and this may not be easily obtainable for
465 complex communities. Single-molecule barcoding (e.g. 10X Genomics [39])
466 and HiC approaches [40] show promise but these remain untested on well-
467 defined complex communities and are still challenged by the complexity of
468 complex environmental metagenomes; see [41, 42, 43].

469 Much of our analysis above depends on having a high-quality “mock”
470 metagenome. While computationally constructed synthetic communities
471 and computational “spike-ins” to real data sets can provide valuable controls
472 (e.g. see [15] and [44]) we strongly believe that standardized communities
473 constructed *in vitro* and sequenced with the latest technologies are critical
474 to the evaluation of both canonical and emerging tools, e.g. efforts such as
475 [45]. From the perspective of tool evaluation, we disagree somewhat with

476 Vollmers et al. [5]: good metagenome tool evaluation necessarily depends
477 on mock communities that are as realistic as we can make them. Likewise,
478 from the perspective of bench biologists, actually sequencing real DNA is
479 critical because it can evaluate confounding effects such as kit contamination [46]. Large-scale studies of computational approaches systematically
480 applied to mock communities such as CAMI [3] can then provide fair com-
481 parisons of entire toolchains (wet and dry combined) applied to these mock
482 communities.
483

484 We omitted two important questions in this study: binning and choice
485 of parameters. We chose not to evaluate genome binning because most bin-
486 ning strategies either operate post-assembly (see e.g. [47]), in which case
487 the challenges with assembly discussed above will apply; or require multi-
488 ple samples (e.g. [48]), which we do not have. We also chose to use only
489 default parameters with all three assemblers, for two reasons. First, we
490 are not aware of any effective automated approaches for determining the
491 “best” set of parameters or evaluating the output for metagenome assem-
492 blers, other than those integrated into the assemblers themselves (e.g. the
493 choice of k-mer sizes by MEGAHIT and MetaSPAdes), and absent such
494 guidance we do not feel comfortable blessing any particular set of param-
495 eters; here the choice of default parameters is parsimonious (and also see [49]
496 for the dangers of poorly chosen objective functions). Second, any param-
497 eter exploration pipeline would not only need to be automated but would
498 need to run multiple assemblies, whose time and resource usage should be
499 measured; in this case, any comparison based on runtime of the parameter
500 choice pipeline should naturally favor MEGAHIT because of its advantage
501 in computational efficiency.

502 **Author contributions**

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

505 **Competing interests**

506 No competing interest to our knowledge.

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