

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

157 single-end files respectively. MEGAHIT allows the specification of a memory
158 limit and we used `-M 1e+10` for 10 GB.

159 All three assemblies were executed on the same XSEDE Jetstream in-
160 stance (S1.Xxlarge) at Indiana University, running Ubuntu 16.04 (install
161 6/21/17, Ubuntu 16.04 LTS Development + GUI support + Docker; based
162 on Ubuntu cloud image for 16.04 LTS with basic dev tools, GUI/Xfce
163 added). Assemblers were limited to 16 threads. We recorded RAM and CPU
164 time for each assembly using `/usr/bin/time -v`. Install and execute details
165 as well as output timings and logs are available in the `pipeline/runstats`
166 directory of the Zenodo archive.

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

169 Mapping

170 We aligned all quality-filtered reads to the reference metagenome with `bwa`
171 `aln` (v0.7.7.r441) [23]. We aligned paired-end and orphaned reads separately.
172 We then used `samtools` (v0.1.19) [29] to convert SAM files to BAM files for
173 both paired-end and orphaned reads. To count the unaligned reads, we
174 included only those records with the “4” flag in the SAM files [29].

175 Assembly analysis using NUCmer

176 We used the NUCmer tool from MUMmer3.23 [30] to align assemblies to the
177 reference genome with options `-coords -p`. Then we parsed the generated
178 “.coords” file using a custom script `analyze_assembly.py`, and calculated
179 several analysis metrics across all three assemblies at a 99% alignment iden-
180 tity.

181 Reference-based analysis of the assemblies

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

187 The script `summarize-coords2.py` was used to calculate aligned cov-
188 erage from the loose alignment conditions: each base in the reference was
189 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.

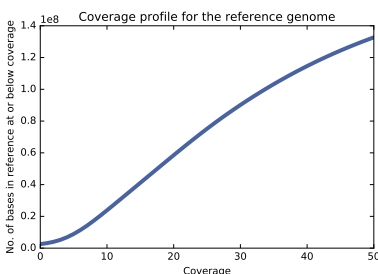


Figure 1: Cumulative coverage profile for the reference metagenome, based on read mapping.

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 refer-
226 ence 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).

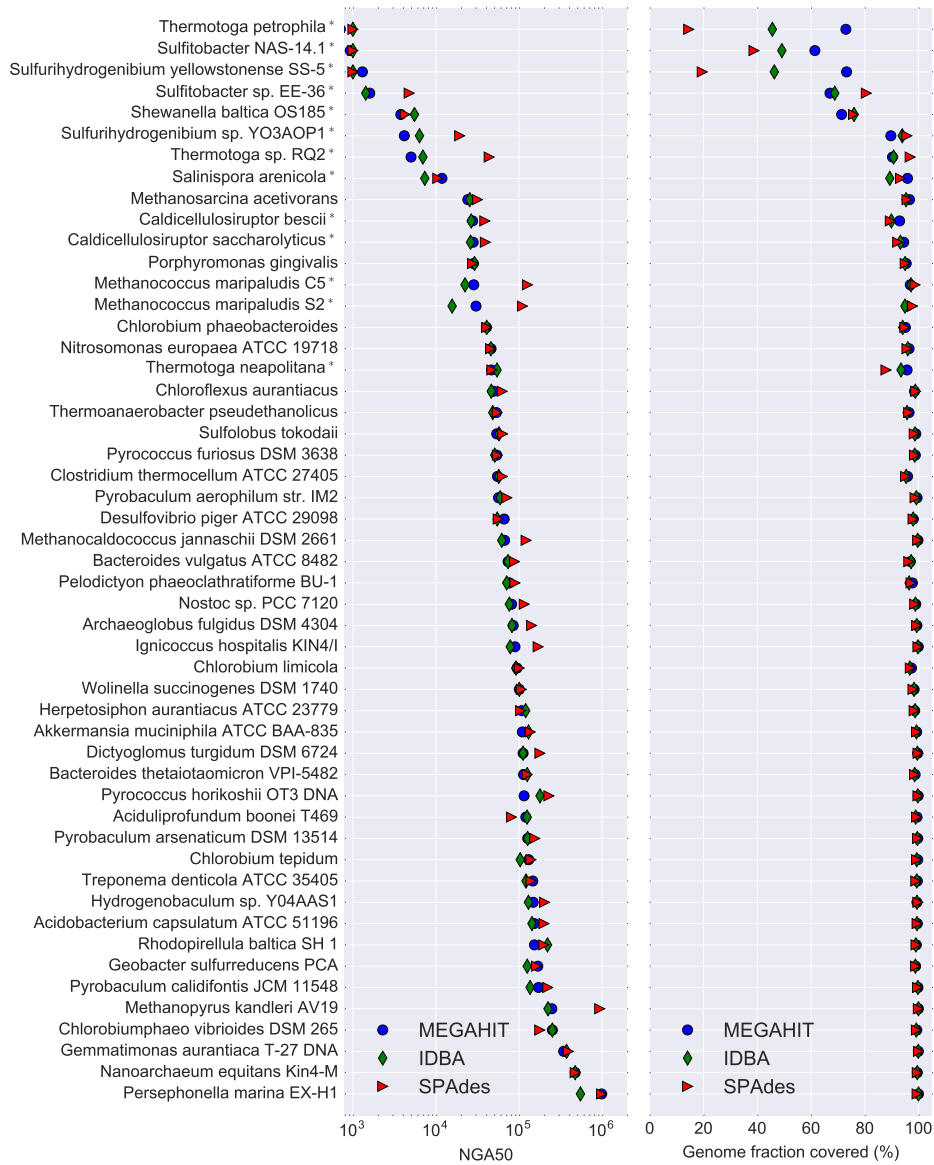


Figure 2: NGA50 and genome fraction covered, by genome and assembler. A '*' after the name indicates the presence of at least one other genome with > 2% Jaccard similarity at k=31 in the community. Where NGA50 cannot be calculated due to poor coverage, a marker is placed at 1kb.

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 were
292 missing 5% or more of bases in at least one assembly, despite all 51 genomes
293 having 99% or higher read- and 51-mer coverage.

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

299 **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%)

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

308 **The unmapped reads contain strain variants of reference genomes.**

309 Approximately 4.8 million reads (4.4%) from the QC data set did not map
310 anywhere in the reference provided by the authors of [12]. We extracted

Table 9: GenBank genomes detected in assembly of unmapped reads

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

311 and assembled these reads in isolation using MEGAHIT, yielding 6.5 Mbp
312 of assembly in 1711 contigs > 500bp in length. We then did a k-mer in-
313 clusion analysis of this assembly against all of the GenBank genomes at
314 k=31, and estimated the fraction of the k-mers that belonged to different
315 species (Table 9). We find that 51.1% of the k-mer content of these contigs
316 positively match to a genome present in GenBank but not in the reference
317 metagenome.

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

324 We note that all but the two *P. ruminis* matches and the *E. coli* isolate
325 YS are strain variants of species that are part of the defined community
326 but are not completely present in the reads (see Table 2). For *Proteini-
327 clasticum ruminis*, there is no closely related species in the mock community
328 design, and very little of the MEGAHIT assembly aligns to known *P. ru-
329 minis* genomes at 99%. However, there are many alignments to *P. ruminis*
330 at 94% or higher, for approximately 2.73 Mbp total. This suggests that the
331 unmapped reads contain at least some data from a novel species of *Proteini-
332 clasticum*; this matches the observation in [12] of a contaminating genome
333 from an unknown *Clostridium* spp., as at the time there was no *P. ruminis*
334 genome.

335 Discussion

336 Assembly recovers basic content sensitively and accurately.

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

345 The presence of multiple closely related genomes confounds 346 assembly.

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

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

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

371 **MEGAHIT performs best by several metrics.**

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

383 In general other studies have found that MEGAHIT excels in recovery of
384 sequence through assembly [3, 16] and is considerably more computationally
385 efficient than most other assemblers [3, 15]. However, studies have also
386 shown that MEGAHIT produces more misassemblies than other assemblers
387 [3] and performs poorly on high coverage portions of the data set [5] Thus
388 while we can recommend MEGAHIT as a good first assembler, we can also
389 not unambiguously recommend it as the only assembler to use.

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

398 **The missing reference may be present in strain variants of the**
399 **intended species.**

400 Several individual genomes are missing in measurable portion from the QC
401 reads (Table 2), and many QC reads (4.4% of 108m) did not map to the full
402 reference metagenome. These appear to be related issues: upon analysis of
403 the unmapped reads against GenBank, we find that many of the contigs as-
404 sembled from the unmapped reads can be assigned to strain variants of the
405 species in the mock community (Table 9) and align closely to the identified
406 genomes. This suggests that the constructors of the mock community may
407 have unintentionally included strain variants of *Fusobacterium nucleatum*,

408 *Thermus thermophilus* HB27, and *Enterococcus faecalis*; note that the mi-
409 crobes used were sourced from the community rather than the ATCC (M.
410 Podar, pers. communication). In addition, we detect what may be por-
411 tions of a novel member of the *Proteiniclasticum* genus in the assembly of
412 these reads - this is likely the *Clostridium* spp. detected through amplicon
413 sequencing in [12].

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

422 Conclusions

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

432 The presence of closely related strains is a major confounder of metagenome
433 assembly, and causes assemblers to drop considerable portions of genomes
434 that (based on read mapping and k-mer inclusion) are clearly present. In this
435 relatively simple community, this strain confusion is present but does not
436 dominate the assembly. However, real microbial communities are likely to
437 have many closely related strains and any resulting loss of assembly would
438 be hard to detect in the absence of good reference genomes. While high
439 polymorphism rates in e.g. animal genomes are known to cause duplication
440 or loss of assembly, some solutions have emerged that make use of assump-
441 tions of uniform coverage and diploidy [31]. These solutions cannot however
442 be transferred directly to metagenomes, which have unknown abundance
443 distributions and strain content.

444 An additional concern is that metagenome assemblies are often per-
445 formed after pooling data sets to increase coverage (e.g. [4, 32]); this pooled
446 data is more likely to contain multiple strains, which would then in turn
447 adversely affect assembly of strains. This may not be resolvable within the
448 current paradigm of assembly, which focuses on outputting linear assem-
449 blies that cannot properly represent strain variation. The human genomics
450 community is moving towards using *reference graphs*, which can represent
451 multiple incompatible variants in a single data structure [33]; this approach,
452 however, requires high-quality isolate reference genomes, which are generally
453 unavailable for environmental microbes.

454 Long read sequencing (and related technologies) will undoubtedly help
455 resolve strain variation in the future, but even with highly accurate long-
456 read sequencing, current sequencing depth is still too low to resolve deep
457 environmental metagenomes [34, 35]. It is unclear how well long error-
458 prone reads (such as those output by Pacific Biosciences SMRT [36] and
459 Oxford Nanopore instruments [37]) will perform on complex metagenomes:
460 with high error rates, deep coverage of each individual genome is required
461 to achieve accurate assembly, and this may not be easily obtainable for
462 complex communities. Single-molecule barcoding (e.g. 10X Genomics [38])
463 and HiC approaches [39] show promise but these remain untested on well-
464 defined complex communities and are still challenged by the complexity of
465 complex environmental metagenomes; see [40, 41, 42].

466 Much of our analysis above depends on having a high-quality “mock”
467 metagenome. While computationally constructed synthetic communities
468 and computational “spike-ins” to real data sets can provide valuable controls
469 (e.g. see [15] and [43]) we strongly believe that standardized communities
470 constructed *in vitro* and sequenced with the latest technologies are critical
471 to the evaluation of both canonical and emerging tools, e.g. efforts such as
472 [44]. From the perspective of tool evaluation, we disagree somewhat with
473 Vollmers et al. [5]: good metagenome tool evaluation necessarily depends
474 on mock communities that are as realistic as we can make them. Likewise,
475 from the perspective of bench biologists, actually sequencing real DNA is
476 critical because it can evaluate confounding effects such as kit contamina-
477 tion [45]. Large-scale studies of computational approaches systematically
478 applied to mock communities such as CAMI [3] can then provide fair com-
479 parisons of entire toolchains (wet and dry combined) applied to these mock
480 communities.

481 We omitted two important questions in this study: binning and choice
482 of parameters. We chose not to evaluate genome binning because most bin-

483 ning strategies either operate post-assembly (see e.g. [46]), in which case
484 the challenges with assembly discussed above will apply; or require multi-
485 ple samples (e.g. [47]), which we do not have. We also chose to use only
486 default parameters with all three assemblers, for two reasons. First, we
487 are not aware of any effective automated approaches for determining the
488 “best” set of parameters or evaluating the output for metagenome assem-
489 blers, other than those integrated into the assemblers themselves (e.g. the
490 choice of k-mer sizes by MEGAHIT and MetaSPAdes), and absent such
491 guidance we do not feel comfortable blessing any particular set of param-
492 eters; here the choice of default parameters is parsimonious (and also see [48]
493 for the dangers of poorly chosen objective functions). Second, any param-
494 eter exploration pipeline would not only need to be automated but would
495 need to run multiple assemblies, whose time and resource usage should be
496 measured; in this case, any comparison based on runtime of the parameter
497 choice pipeline should naturally favor MEGAHIT because of its advantage
498 in computational efficiency.

499 **Author contributions**

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

502 **Competing interests**

503 No competing interest to our knowledge.

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