

1 **Oxford Nanopore R10.4 long-read sequencing enables near-perfect**  
2 **bacterial genomes from pure cultures and metagenomes without**  
3 **short-read or reference polishing**

4 Mantas Sereika<sup>a\*</sup>, Rasmus Hansen Kirkegaard<sup>a,b\*</sup>, Søren Michael Karst<sup>a</sup>, Thomas Yssing  
5 Michaelsen<sup>a</sup>, Emil Aarre Sørensen<sup>a</sup>, Rasmus Dam Wollenberg<sup>c</sup> and Mads Albertsen<sup>a\*\*</sup>

6 <sup>a</sup>Center for microbial communities, Aalborg University, Denmark

7 <sup>b</sup>Joint Microbiome Facility, University of Vienna, Austria

8 <sup>c</sup>DNASense ApS, Denmark

9 \*These authors contributed equally to the paper

10 \*\*Corresponding author [ma@bio.aau.dk](mailto:ma@bio.aau.dk)

11

12 **ABSTRACT**

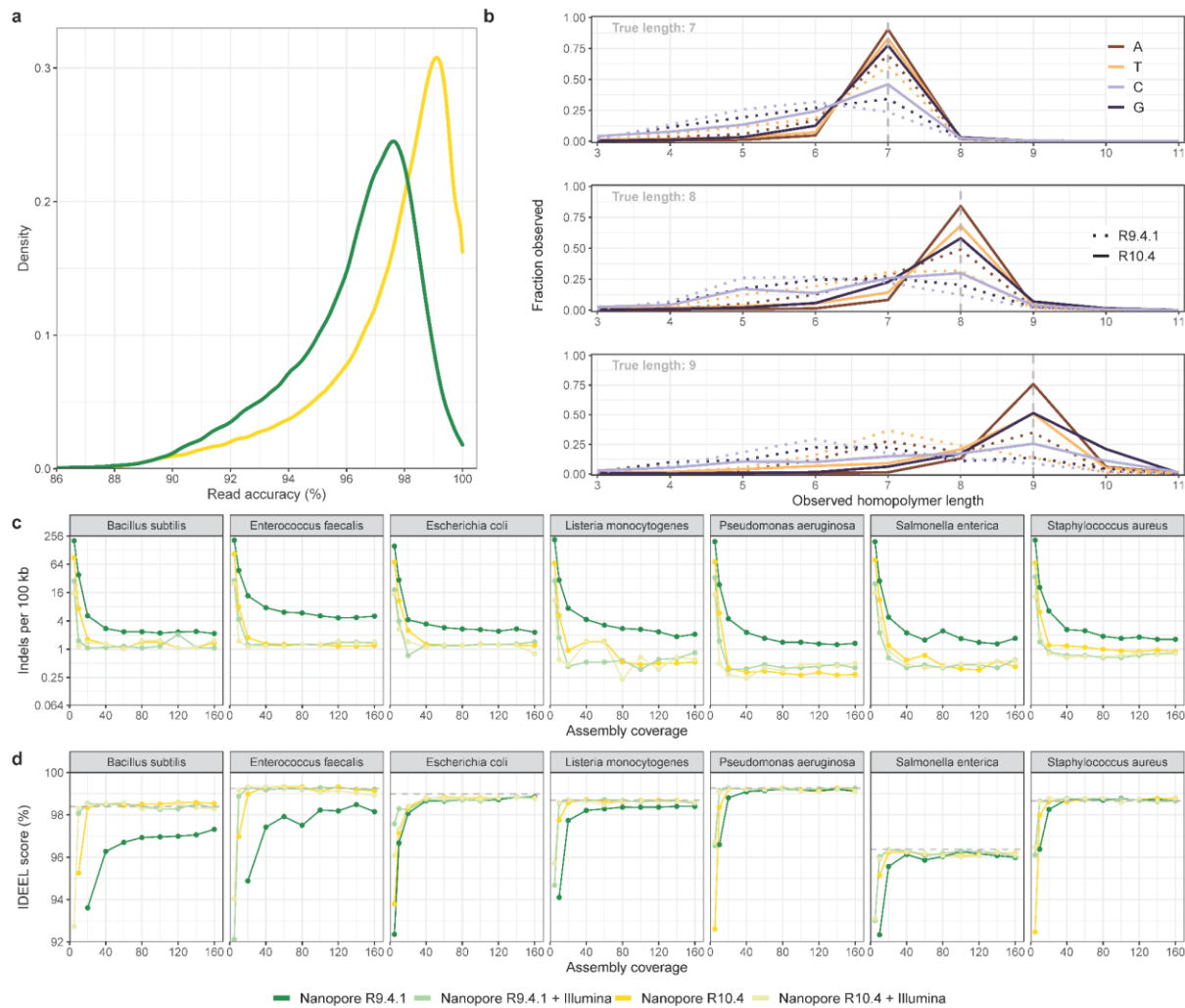
13 **Long-read Oxford Nanopore sequencing has democratized microbial genome**  
14 **sequencing and enables the recovery of highly contiguous microbial genomes from**  
15 **isolates or metagenomes. However, to obtain near-perfect genomes it has been**  
16 **necessary to include short-read polishing to correct insertions and deletions derived**  
17 **from homopolymer regions. Here, we show that Oxford Nanopore R10.4 can be used to**  
18 **generate near-perfect microbial genomes from isolates or metagenomes without short-**  
19 **read or reference polishing.**

20 **MAIN TEXT**

21 Bacteria live in almost every environment on Earth and the global microbial diversity is  
22 estimated to entail more than  $10^{12}$  species<sup>1</sup>. To obtain representative genomes, sequencing  
23 of pure cultures or genome recovery directly from metagenomes are often employed<sup>2-4</sup>. High-  
24 throughput short-read sequencing has for many years been the method of choice<sup>5,6</sup> but fails  
25 to resolve repeat regions larger than the insert size of the library<sup>7</sup>. This is especially  
26 problematic in metagenome samples where related species or strains often contain long  
27 sequences of near-identical DNA. More recently, long-read sequencing has emerged as the  
28 method of choice for both pure culture genomes<sup>8,9</sup> and metagenomes<sup>10-12</sup>. PacBio HiFi reads

29 combine low error rates with relatively long reads and generate near-perfect microbial  
30 genomes from pure cultures or metagenomes<sup>13–15</sup>. Despite very high-quality raw data, the  
31 relatively high cost pr. base remains an economic hindrance for many research projects. A  
32 widely used alternative is Oxford Nanopore sequencing which offers low-cost long-read data.  
33 However, numerous studies have shown that despite vast improvements in raw error rates,  
34 assembly consensus sequences still suffer from insertion and deletions in homopolymers that  
35 often cause frameshift errors during gene calling<sup>16–18</sup>. A commonly adopted solution has been  
36 to include short-read data for post-assembly error correction<sup>12,19</sup>, although it increases the cost  
37 and complexity overhead. Another solution has been to apply reference-based polishing to  
38 correct frameshift errors<sup>20–22</sup>, but while it provides a practical solution, which allows gene  
39 calling, it does not provide true near-perfect genomes.

40 We first evaluated the ability for Oxford Nanopore R9.4.1 and R10.4 data to obtain near-  
41 perfect microbial genomes through sequencing of the ZymoBIOMICS HMW DNA Standard  
42 #D6322 (Zymo mock) consisting of 7 bacterial species and 1 fungus. A single PromethION  
43 R10.4 flowcell generated 52.3 gbp of data with a modal read accuracy of 99 % (**Figure 1A**,  
44 **Table S1**). In contrast to R9.4.1 data, we do not see any significant improvement in assembly  
45 quality for R10.4 by the addition of Illumina polishing (**Figure 1C**, **Figure S1**). This indicates  
46 that near-perfect microbial reference genomes can be obtained from R10.4 data alone at a  
47 coverage of approximately 40x. The improvement in assembly accuracy from R9.4.1 to R10.4  
48 is largely due to an improved ability to call homopolymers, as R10.4 is able to correctly call  
49 the length of the majority of homopolymers up to a length of 10 (**Figure 1B**, **Figure S2-3**). In  
50 general, a homopolymer length of more than 10 is very rare in bacteria, with an estimate of  
51 less than 10 per species on average<sup>18</sup>.



52

53 **Figure 1:** Sequencing and assembly statistics for the Zymo mock. **A)** Observed raw read  
 54 accuracies measured through read-mapping. **B)** Observed homopolymer length of raw reads  
 55 compared to the reference genomes (see **Figure S2-3** for a complete overview). **C)** Observed  
 56 indels of de novo assemblies per 100 kbp at different coverage levels, with and without Illumina  
 57 polishing. Note that the reference genomes available for the Zymo mock are not identical to  
 58 the sequenced strains (**Table S3**). **D)** IDEEL<sup>23</sup> score calculated as the proportion of predicted  
 59 proteins which are  $\geq 95\%$  the length of their best-matching known protein in a database<sup>16</sup>. The  
 60 dotted line represents the IDEEL score for the reference genome.

61 To assess the performance of state-of-the-art sequencing technologies in recovering near-  
 62 perfect microbial genomes from metagenomes we sequenced activated sludge from an  
 63 anaerobic digester using single runs of Illumina MiSeq 2x300 bp, PacBio HiFi, and Oxford  
 64 Nanopore R9.4.1 and R10.4. Despite being the same sample, direct comparisons are difficult  
 65 as the additional size selection of the PacBio CCS dataset both increased the read length  
 66 (**Figure S4**) and altered the relative abundances of the species in the sample (**Figure S5**).  
 67 Furthermore, Nanopore R9.4.1 produced more than twice the amount of data compared to the

68 other datasets, while the Illumina data featured variations in relative abundances presumably  
 69 due to GC bias (**Figure S5**). To assist automated contig binning, we performed Illumina  
 70 sequencing of 9 additional samples from the same anaerobic digester spread over 9 years  
 71 (**Table S2**) and used the coverage profiles as input for binning using multiple different  
 72 approaches. Furthermore, to evaluate the impact of micro-diversity on MAG quality, we  
 73 calculated the polymorphic site rates for each MAG as a simple proxy for the presence of  
 74 micro-diversity<sup>6</sup>.

75 After performing automated contig binning it is evident that micro-diversity has a  
 76 large impact on MAG fragmentation, but that long-read sequencing data results in much less  
 77 fragmentation of bins at higher amounts of micro-diversity (**Figure S6**). Despite large  
 78 differences in read length for Nanopore and PacBio CCS data (N50 read length 6 kbp vs. 15  
 79 kbp), only small differences in bin fragmentation were observed, as compared to the  
 80 Illumina-based results (**Table 1, Figures S6**).

81

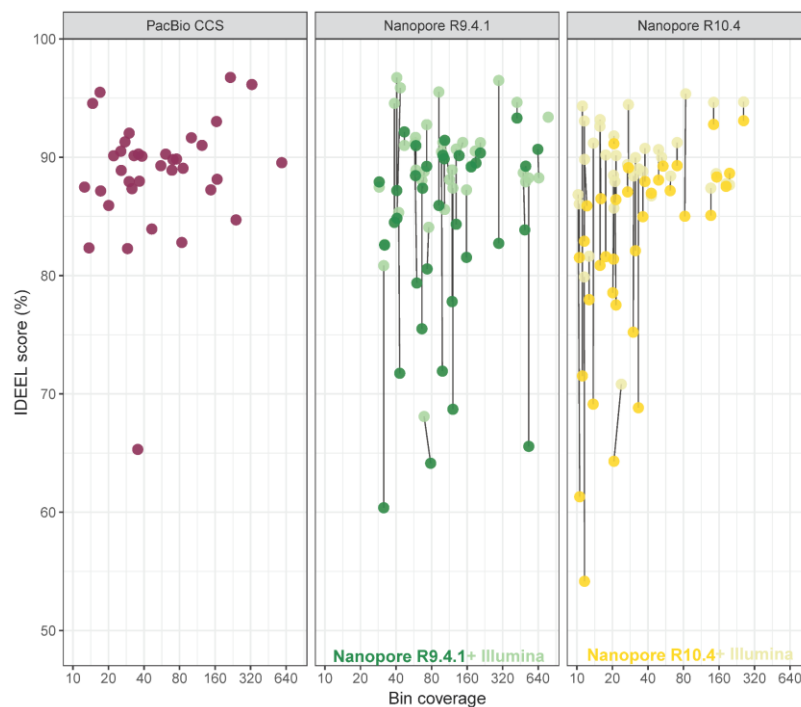
82 **Table 1:** Sequencing and assembly statistics for the anaerobic digester sample using  
 83 different technologies and approaches. \*Costs refer to the expenses encountered at the time  
 84 of conducting the experiments and may differ for other research groups.

	Illumina MiSeq	R9.4.1 / +Illumina	R10.4 / +Illumina	PacBio HiFi
<b>Total Yield (Gbp)</b>	13	35	14	15
<b>Read N50 (kbp)</b>	0.3	5.9	5.6	15.4
<b>Observed modal read accuracy (%)</b>	100	96.76	98.21	99.86
<b>Assembly size (Mbp)</b>	409	754	379	606
<b>Contigs (&gt; 1kbp)</b>	145,976	24,680	21,585	8,989
<b>Circular contigs (&gt; 0.5 Mbp)</b>	0	7	3	9
<b>Contig N50 (kbp)</b>	3.5	79.9	40.1	172.5
<b>Reads mapped to contigs (%)</b>	88.1	93.5	95.4	95.2
<b>HQ MAGs</b>	8	64/86	34/36	74
<b>MQ MAGs</b>	83	114/95	65/67	72
<b>Contigs pr. HQ MAG (median)</b>	184	15/16	21/21	9
<b>Mapped reads in HQ MAGs (%)</b>	16	46/49	39/40	48
<b>Costs (\$)*</b>	1,200	811/2,011	811/2,011	4,420
<b>Cost per HQ MAG (\$)</b>	150	13/23	24/56	60

85

86 All long-read methods produce high numbers of high-quality (HQ) MAGs, which capture 39-  
87 49% of all reads (**Table 1**). Nanopore R9.4.1 is able to produce HQ MAGs as a standalone  
88 technology, but Illumina polishing increases the number of HQ MAGs from 64 to 86. For  
89 Nanopore R10.4, Illumina polishing increases the number of HQ MAGs from 34 to 36. Using  
90 the IDEEL test (**Figure 2**), it can be seen that Illumina polishing results in minor  
91 improvements for Nanopore R10.4 above a coverage of 40, and that the Nanopore R10.4 is  
92 in the same IDEEL range as PacBio HiFi MAGs. As with sequencing of the Zymo mock, the  
93 difference from R9.4.1 to R10.4 is largely due to significantly better accuracy in  
94 homopolymers for lengths up to 10 (**Figure S7**).

95  
96



97  
98 **Figure 2:** IDEEL score vs. coverage for metagenome bins from the anaerobic digester  
99 sample. The Nanopore bins are shown with and without Illumina polishing connected by a  
100 line.

101

102 Since its introduction as an early access program in 2014 Oxford Nanopore  
103 sequencing technology has democratized sequencing and enabled every laboratory and  
104 classroom to engage in microbial genome sequencing. However, for the generation of high-  
105 quality genomes, additional short-read polishing has been essential, as indels in  
106 homopolymer regions cause fragmented gene calls. The additional sequencing requirements  
107 have been one of the barriers to widespread uptake. Here we show that Oxford Nanopore  
108 R10.4 enables the generation of near-perfect microbial genomes from pure cultures or

109 metagenomes at coverages of 40x without short-read polishing. While homopolymers of 10  
110 or more bases will likely still be problematic, they constitute a minor part of microbial  
111 genomes.

112 For genome-recovery from metagenomes, low-coverage bins (<40X) do need  
113 Illumina polishing to attain quality comparable to PacBio HiFi. Hence, in some cases, the  
114 most economic option could be Nanopore R9.4.1 supplemented with short-read sequencing,  
115 as the throughput is currently at least 2 times higher on R9.4.1 compared to R10.4 and no  
116 difference is seen between the methods after Illumina short-read polishing.

117

#### 118 **Data availability**

119 Anaerobic digester sequencing data are available at the ENA with bio project ID  
120 PRJEB48021, while the Zymo mock community sequencing data is available at  
121 PRJEB48692. The code and datasets used to generate the figures and supplementary  
122 material are available at <https://github.com/Serka-M/Digester-MultiSequencing>.

123

#### 124 **Acknowledgments**

125 We would like to acknowledge the plant operators at Fredericia wastewater treatment plant  
126 for supplying the sample material. The study was funded by research grants from VILLUM  
127 FONDEN (15510) and the Poul Due Jensen Foundation (Microflora Danica).

128

#### 129 **Author contributions**

130 MS and RHK performed DNA extraction, and sequencing of the anaerobic digester and  
131 selected Zymo mock samples. RWO prepared and sequenced the Zymo mock using R9.4.1  
132 and Illumina. MS, RHK, and MA wrote the first draft of the manuscript. SMK, TYM, RWO,  
133 and EAS contributed to experiment design, result interpretation, and writing of the  
134 manuscript. All authors reviewed the manuscript.

135

#### 136 **Conflict of interest**

137 EAS, SMK, MA, RHK, and RWO are employed at DNASense ApS that consults and  
138 performs sequencing. The remaining authors declare no conflict of interest.

139

140

141

142 **Materials and methods**

143

144 **Sampling**

145 Sludge biomass was sampled from the anaerobic digester at Fredericia wastewater  
146 treatment plant (Latitude 55.552219, Longitude 9.722003) at multiple time points and stored  
147 as frozen 2 mL aliquots at -20°C. For the Zymo sample, the ZymoBIOMICS HMW DNA  
148 Standard #D6322 (Zymo Research, USA) was used.

149

150 **DNA extraction**

151 DNA was extracted from the anaerobic digester sludge using DNeasy PowerSoil Kit  
152 (QIAGEN, Germany) following the manufacturer's protocol. The extracted DNA was then  
153 size selected using the SRE XS (Circulomics, USA), according to the manufacturer's  
154 instructions.

155

156 **DNA QC**

157 DNA concentrations were determined using Qubit dsDNA HS kit and measured with a Qubit  
158 3.0 fluorimeter (Thermo Fisher, USA). DNA size distribution was determined using an Agilent  
159 2200 TapeStation system with genomic screentapes (Agilent Technologies, USA). DNA  
160 purity was determined using a NanoDrop One Spectrophotometer (Thermo Fisher, USA).

161

162 **Oxford Nanopore DNA sequencing**

163 Library preparation was carried out using the ligation sequencing kits (Oxford Nanopore  
164 Technologies, UK) SQK-LSK109 and SQK-LSK112 for sequencing on R.9.4.1 and the  
165 R.10.4 flowcells, respectively. Anaerobic digester and Zymo R.9.4.1 datasets were  
166 generated on a MinION Mk1B (Oxford Nanopore Technologies, UK) device, while Zymo  
167 R10.4 dataset was produced on a PromethION and digester R10.4 read sequences were  
168 generated on a GridION.

169

170 **Illumina DNA sequencing**

171 The anaerobic digester Illumina libraries were prepared using the Nextera DNA library  
172 preparation kit (Illumina, USA), while the Zymo Mock sample was prepared with NEB Next  
173 Ultra II DNA library prep kit for Illumina (New England Biolabs, USA) following the  
174 manufacturer's protocols and sequenced using the Illumina MiSeq platform.

175

176

177 **PacBio HiFi**



178 A size-selected DNA sample was sent to the DNA Sequencing Center at Brigham Young  
179 University, USA. The DNA sample was fragmented with Megaruptor (Diagenode, Belgium)  
180 to 15 kb and size-selected using the Blue Pippin (Sage Science, USA) and prepared for  
181 sequencing using SMRTbell Express Template Preparation Kit 1.0 (PacBio, USA) according  
182 to manufacturers' instructions. Sequencing was performed on the Sequel II system (PacBio,  
183 USA) using the Sequel II Sequencing Kit 1.0 (PacBio, USA) with the Sequel II SMRT Cell  
184 8M (PacBio, USA) for a 30 hour data collection time.

185

### 186 **Read processing**

187 Illumina reads were trimmed for adapters using Cutadapt v. 1.16<sup>24</sup>. The generated raw  
188 Nanopore data was basecalled in super-accurate mode with using Guppy v. 5.0.16  
189 (<https://community.nanoporetech.com/downloads>) with dna\_r9.4.1\_450bps\_sup.cfg model  
190 for R9.4.1 and dna\_r10.4\_e8.1\_sup.cfg model for R10.4 chemistry. Concatemers in R10.4  
191 data were split by using "split\_on\_adapter" command (5 iterations) of duplex-tools v. 0.2.5  
192 (<https://github.com/nanoporetech/duplex-tools>). Adapters for Nanopore reads were removed  
193 using Porechop v. 0.2.3<sup>25</sup> and reads with Phred quality scores below 7 and 10 for R9.4.1 and  
194 R10.4 reads, respectively, were removed using NanoFilt v. 2.6.0<sup>26</sup>. The CCS tool v. 6.0.0  
195 (<https://ccs.how/>) was utilized with the sub-read data from PacBio CCS to produce HiFi  
196 reads. Read statistics were acquired via NanoPlot v. 1.24.0<sup>26</sup>. Zymo read datasets were  
197 subsampled to custom coverage profiles using Rasusa v. 0.3.0  
198 (<https://github.com/mbhall88/rasusa>). Counterr v. 0.1 (<https://github.com/dayzerodx/counterr>)  
199 was used to assess homopolymer calling in reads.

200

### 201 **Read assembly and binning**

202 Long reads were assembled using Flye v. 2.9-b1768<sup>13,27</sup> with the "--meta" setting enabled  
203 and the "--nano-hq" option for assembling Nanopore reads, whereas "--pacbio-hifi" and "--  
204 min-overlap 7500 --read-error 0.01" options were used for assembling PacBio CCS reads,  
205 as it resulted in more HQ MAGs than using the default settings. Polishing tools for  
206 Nanopore-based assemblies: Minimap2 v. 2.17<sup>28</sup>, Racon v. 1.3.3 (used thrice)<sup>29</sup>, and  
207 Medaka v. 1.4.4 (used twice, <https://github.com/nanoporetech/medaka>). The trimmed  
208 Illumina reads were assembled using Megahit v. 1.1.4<sup>30</sup>.

209

210 Automated binning was carried out using MetaBAT2 v. 2.12.1<sup>31</sup>, with "-s 500000" settings,  
211 MaxBin2 v. 2.2.7<sup>32</sup> and Vamb v. 3.0.2<sup>33</sup> with "-o C --minfasta 500000" settings. Contig  
212 coverage profiles from different sequencer data as well as 9 additional time-series Illumina  
213 datasets of the same anaerobic digester were used for generating the bins. The binning  
214 output of different tools was then integrated and refined using DAS Tool v. 1.1.2<sup>34</sup>. CoverM



215 v. 0.6.1 (<https://github.com/wwood/CoverM>) was applied to calculate the bin coverage (“-m  
216 mean” settings) and relative abundance (“-m relative\_abundance”) values.

217

## 218 **Assembly processing**

219 The completeness and contamination of the genome bins were estimated using CheckM v.  
220 1.1.2<sup>35</sup>. The bins were classified using GDTB-Tk v. 1.5.0<sup>36</sup>, R202 database. Protein  
221 sequences were predicted using Prodigal v. 2.6.3<sup>37</sup> with “p meta” setting, while rRNA genes  
222 were predicted using Barrnap v. 0.9 (<https://github.com/tseemann/barrnap>) and tRNAscan-  
223 SE v. 2.0.5<sup>38</sup> was used for tRNA predictions. Bin quality was determined following the  
224 Genomic Standards Consortium guidelines, wherein a MAG of high quality featured genome  
225 completeness of more than 90 %, less than 5 % contamination, at least 18 distinct tRNA  
226 genes and the 5S, 16S, 23S rRNA genes occurring at least once<sup>39</sup>. MAGS with  
227 completeness above 50 % and contamination below 10 % were classified as medium  
228 quality, while low quality MAGs featured completeness below 50 % and contamination below  
229 10 %. MAGs with contamination estimates higher than 10 % were classified as  
230 contaminated.

231

232 Illumina reads were mapped to the assemblies using Bowtie2 v. 2.4.2<sup>40</sup> with the “--very-  
233 sensitive-local” setting. The mapping was converted to BAM and sorted using SAMtools v.  
234 1.9<sup>41</sup>. Single nucleotide polymorphism rate was then calculated using CMseq v. 1.0.3<sup>6</sup> from  
235 the mapping using poly.py script with “--mincov 10 --minqual 30” settings.

236

237 Bins were clustered using dRep v. 2.6.2<sup>42</sup> with “-comp 50 -con 10 -sa 0.95” settings. Only the  
238 bins that featured higher coverage than 10 in their respective sequencing platform and a  
239 higher Illumina read coverage than 5 for bins from the hybrid approach were included in  
240 downstream analysis. For IDEEL test<sup>17,23</sup>, the predicted protein sequences from clustered  
241 bins and Zymo assemblies were searched against the UniProt TrEMBL<sup>43</sup> database (release  
242 2021\_01) using Diamond v. 2.0.6<sup>44</sup>. Query matches, which were not present in all datasets,  
243 were omitted to reduce noise. The IDEEL scores were assigned as described by<sup>16</sup>.

244

245 QUAST v. 4.6.3<sup>45</sup> was applied on the Zymo assemblies and the clustered bins with less than  
246 0.5 % SNP rate to acquire mismatch and indels metrics. Cases with Quast parameters  
247 “Genome Fraction” of less than 75 % and “Unaligned length” of more than 250 kb were  
248 omitted to reduce noise. For homopolymer analysis, the clustered bins were mapped to each  
249 other using “asm5” mode of Minimap2 and Counterr was used on the mapping files to get  
250 homopolymer calling errors. For QUAST and Counterr, PacBio CCS bins were used as  
251 reference sequences. FastANI v. 1.33<sup>46</sup> was used to calculate identity scores between Zymo

252 assemblies and the Zymo reference sequences. The Zymo mock reference genome  
253 sequences were obtained from a link in the accompanying instruction manual to the  
254 ZymoBIOMICS HMW DNA Standard Catalog No. D6332 at  
255 <https://s3.amazonaws.com/zymo-files/BioPool/D6322.refseq.zip>.

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## Supplementary information for

# Oxford Nanopore R10.4 long-read sequencing enables near-perfect bacterial genomes from pure cultures and metagenomes without short-read or reference polishing

Mantas Sereika<sup>a\*</sup>, Rasmus Hansen Kirkegaard<sup>a,b\*</sup>, Søren Michael Karst<sup>a</sup>, Thomas Yssing Michaelsen<sup>a</sup>, Emil Aarre Sørensen<sup>a</sup>, Rasmus Dam Wollenberg<sup>c</sup> and Mads Albertsen<sup>a\*\*</sup>

<sup>a</sup>Center for microbial communities, Aalborg University, Denmark

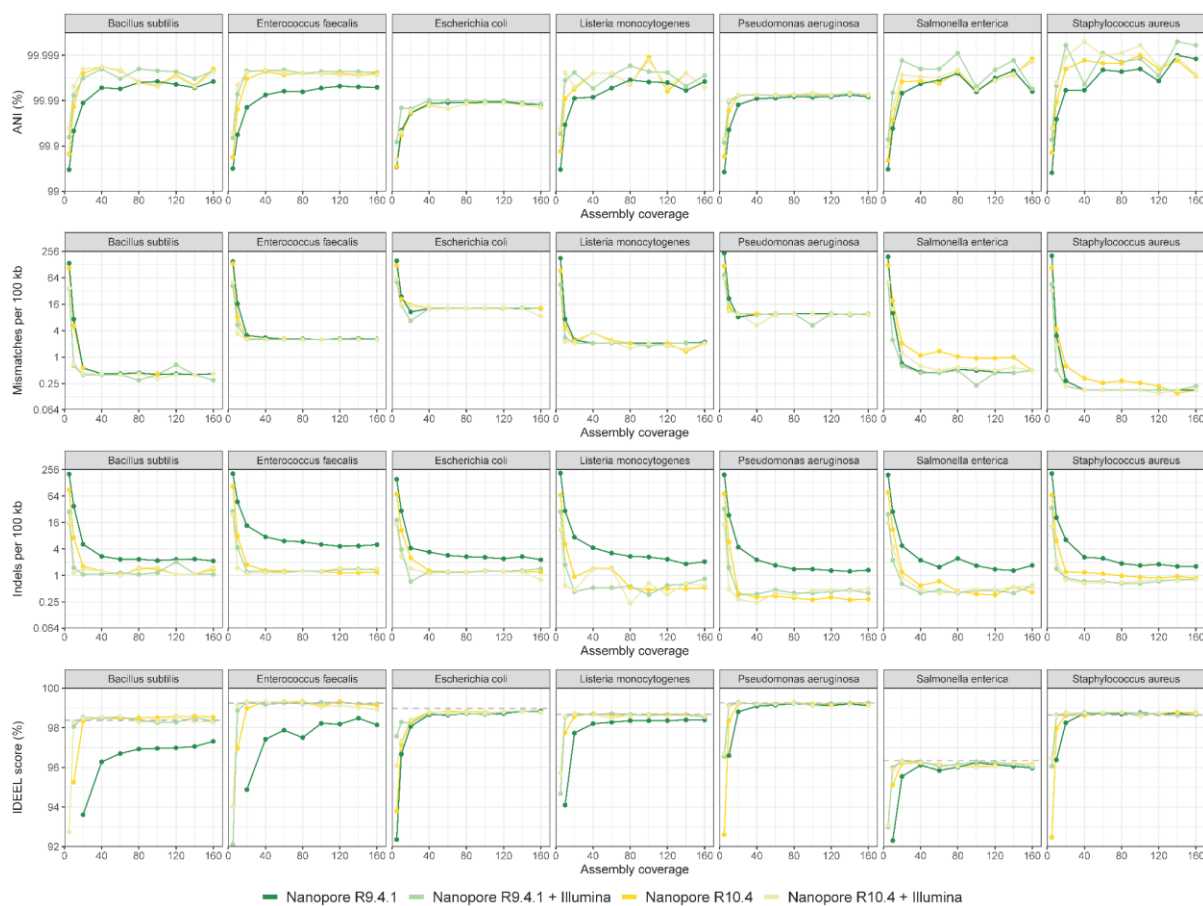
<sup>b</sup>Joint Microbiome Facility, University of Vienna, Austria

<sup>c</sup>DNASense ApS, Denmark

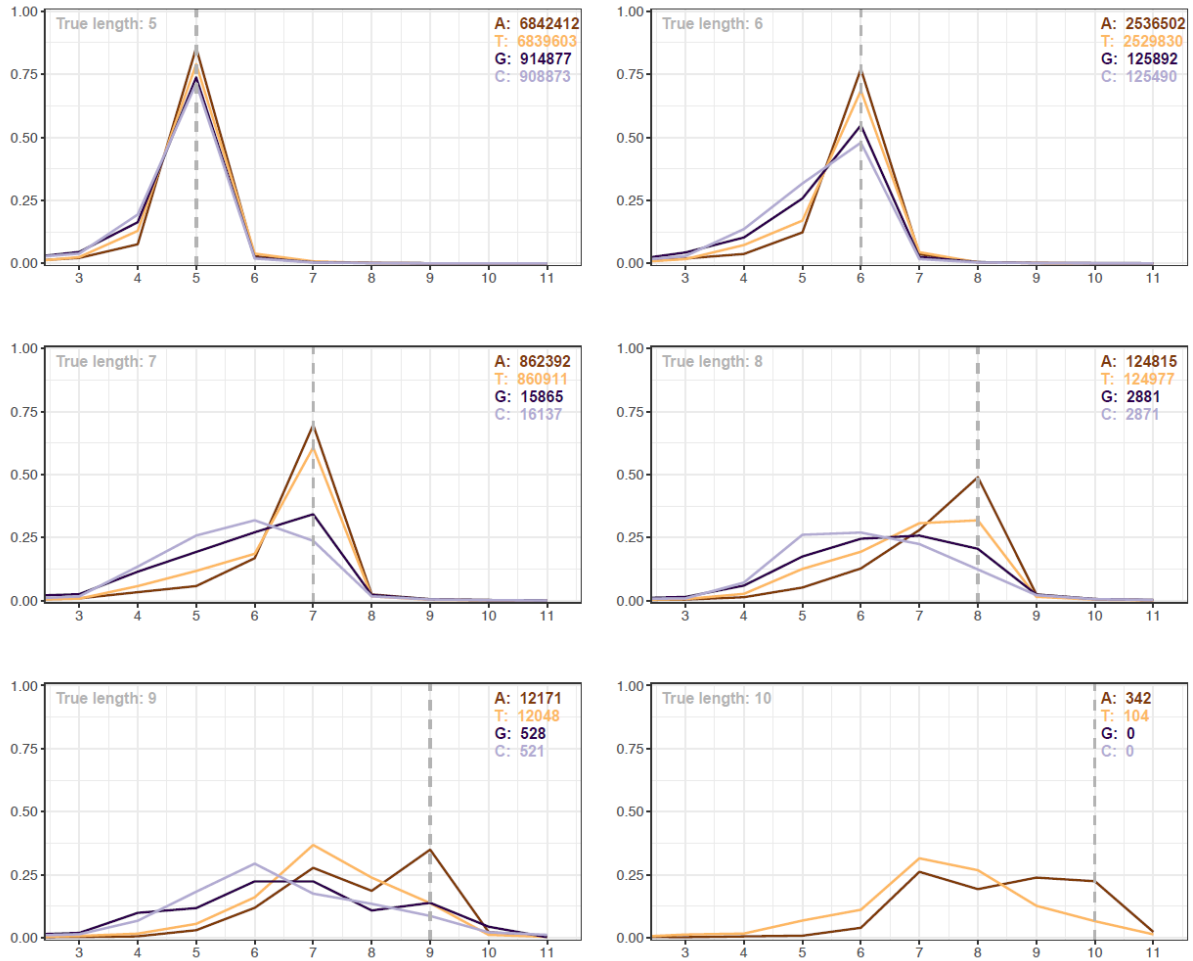
\*These authors contributed equally to the paper

\*\*Corresponding author [ma@bio.aau.dk](mailto:ma@bio.aau.dk)

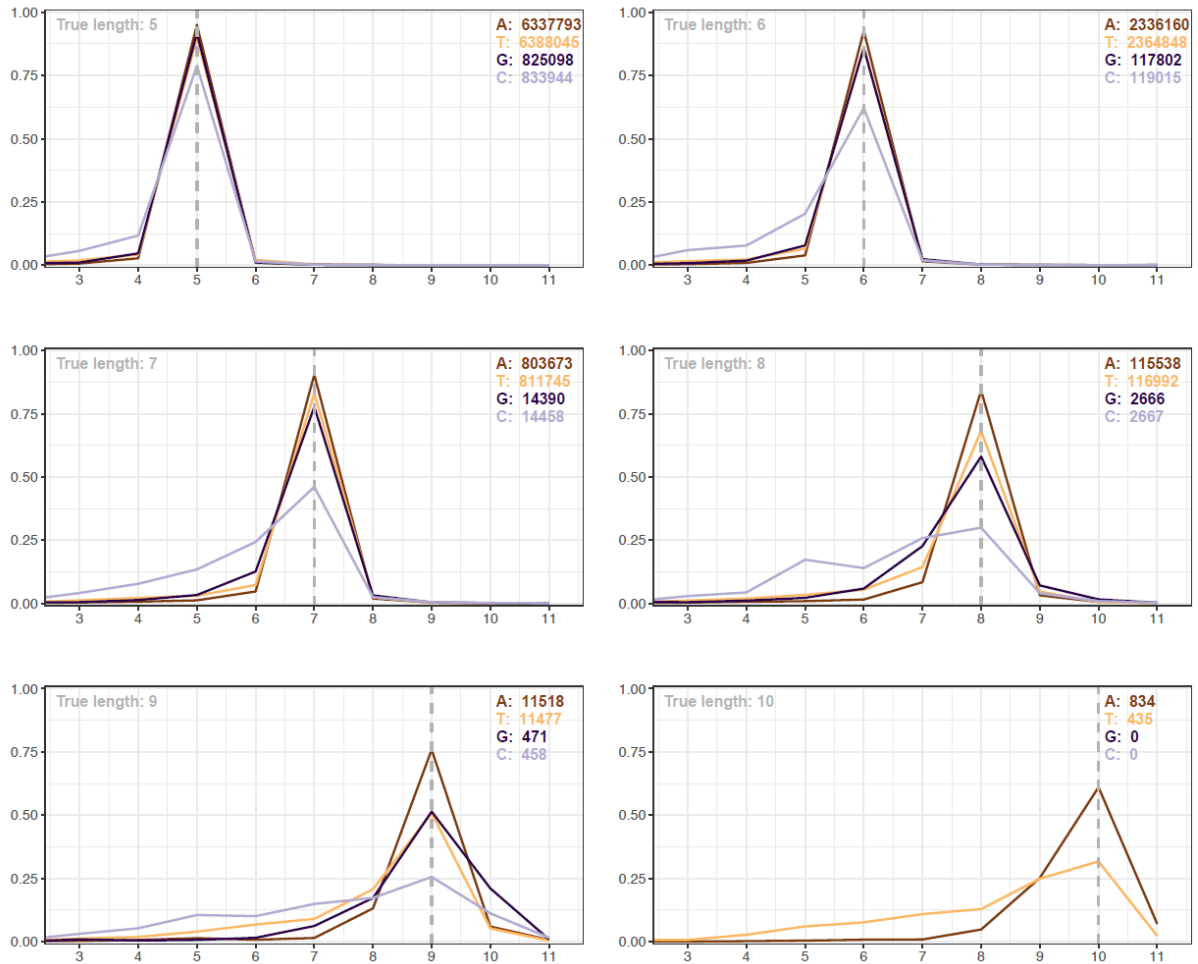




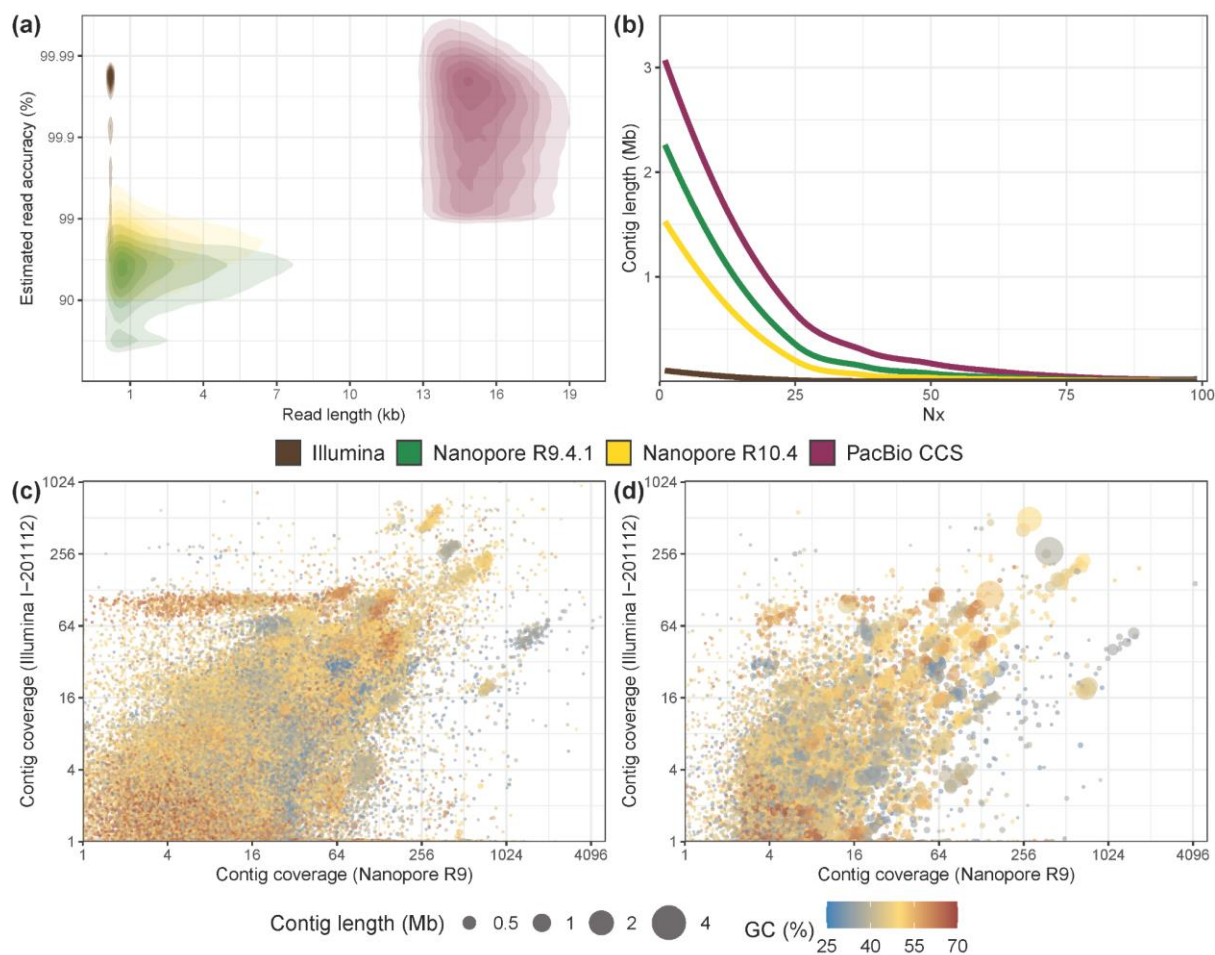
**Figure S1:** Assembly metrics for the ZYMO Mock HMW DNA.



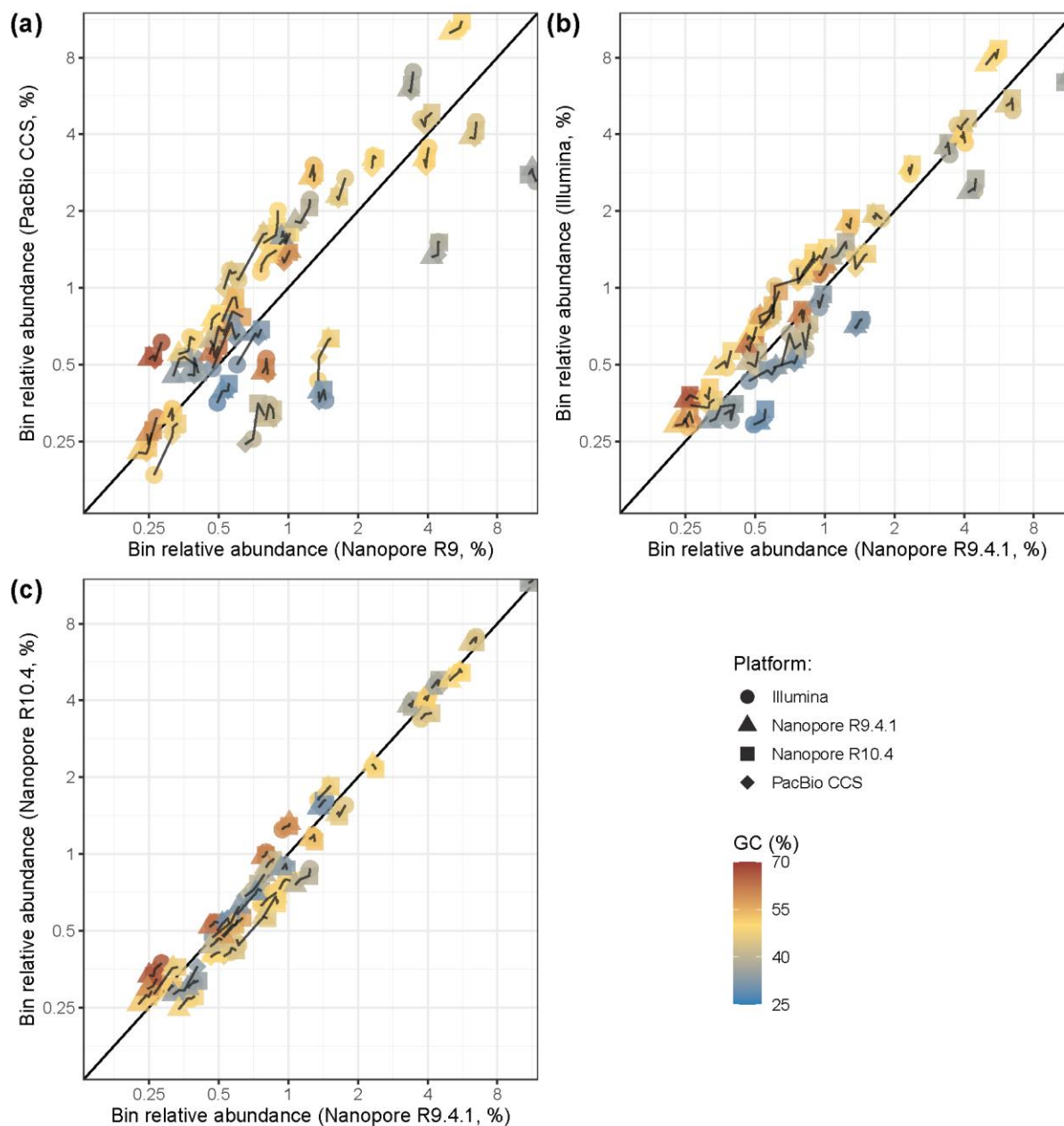
**Figure S2:** Counter homopolymer plot for Nanopore R9.4.1 read data of the Zymo mock. Reads for each Zymo mock species, subsetted to a coverage of 160 were used for the analysis.



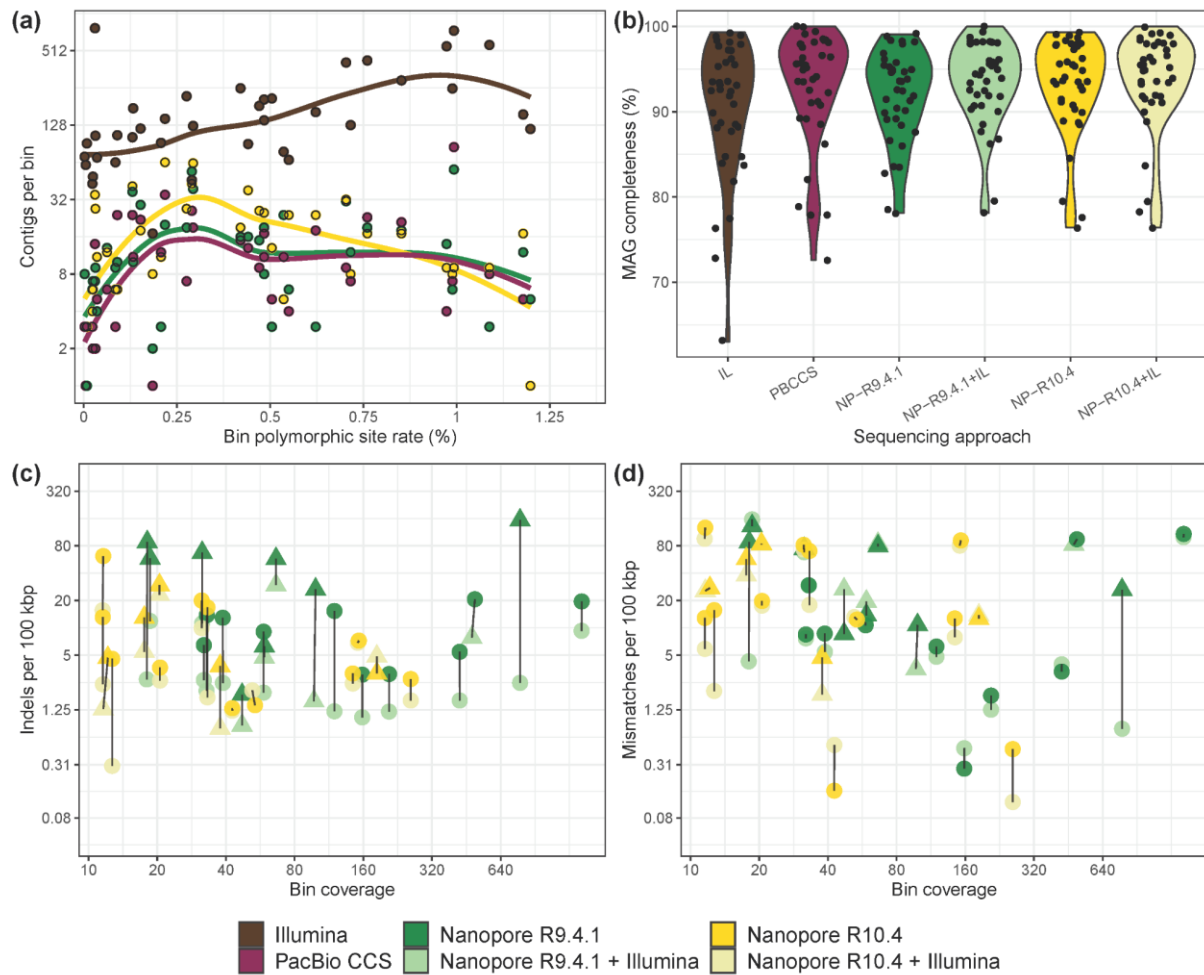
**Figure S3:** Counter homopolymer plot for Nanopore R10.4 read data of the Zymo mock. Reads for each Zymo mock species, subsetted to a coverage of 160 were used for the analysis.



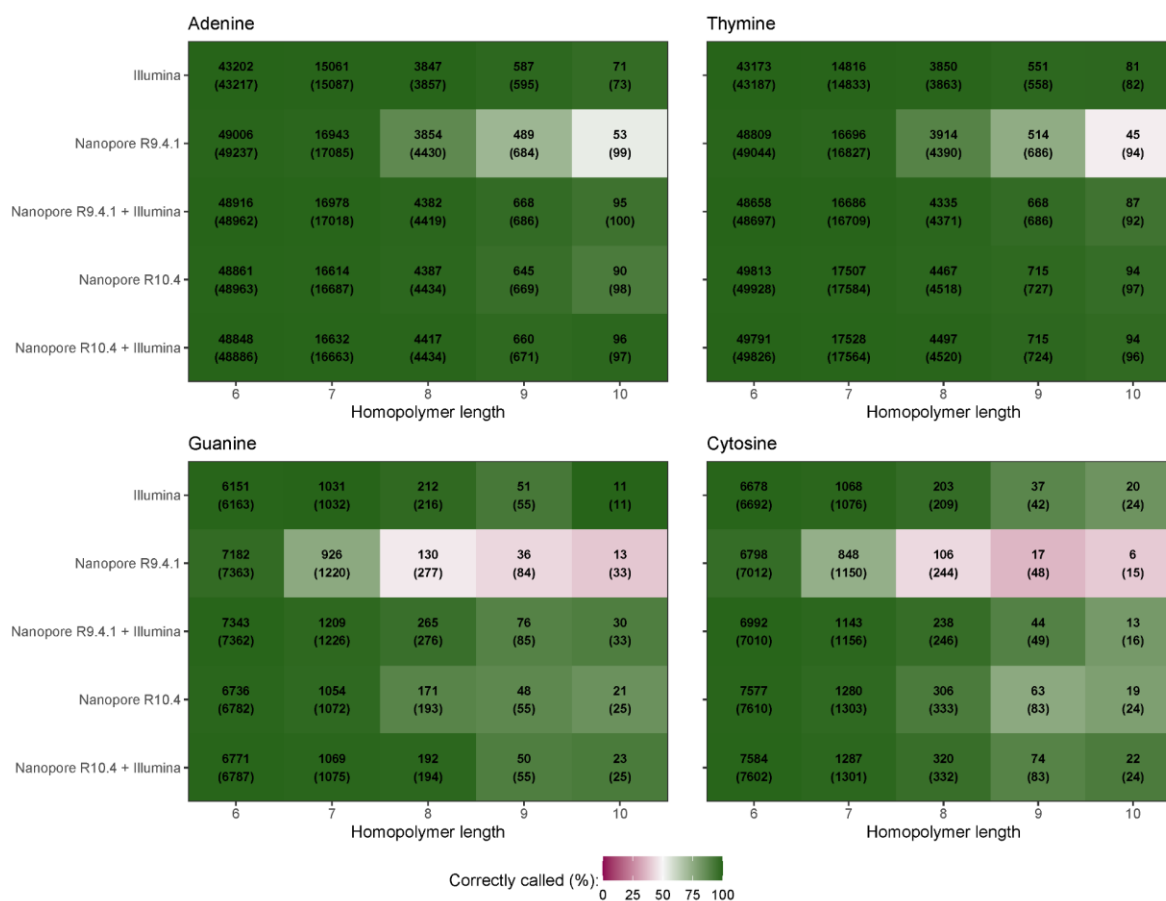
**Figure S4:** Sequencing and assembly overview for the anaerobic digester sample. **A)** Estimated read accuracy (from Q-scores) versus read length. Note that the PacBio HiFi sample underwent additional size selection prior to sequencing. **B)** Nx plot of the assemblies produced from different sequencing technologies. **C)** Differential coverage plot of the Illumina assembly. **D)** Differential coverage plot of the Nanopore R9.4.1 assembly.



**Figure S5:** Comparison of bin relative abundances between different sequencing platforms. Relative abundance values (log-scaled) are presented between the Nanopore R9 data and **a)** PacBio CCS, **b)** Illumina, **c)** Nanopore R10. Only the bins that were clustered together between different platforms are presented in the plots and are interlinked.



**Figure S6:** Comparison of bins from different sequencing approaches. **a)** MAG fragmentation (log-scaled) at different bin SNP rates in PacBio CCS MAGs. **b)** Genome bin completeness estimates for different sequencing platforms. IL — Illumina, NP — Nanopore, PBCCS — PacBio CCS. Bin **c)** indel and **d)** mismatch rates (log-scaled) for MAGs from Nanopore sequencing with and without Illumina read polishing, compared to MAGs from PacBio CCS. The presented bin coverage on the x axis (log-scaled) is for the corresponding Nanopore chemistry type. HQ MAGs are represented by circle, while triangles denote MQ MAGs. For all figures, only the bins that were clustered together between all the different sequencing platforms (see Materials and methods) are presented.



**Figure S7:** Homopolymer calling estimates in metagenomes (consensus sequences) from different sequencing platforms. Values in the heatmap show observed homopolymer counts estimated to be called correctly at a given sequence length. The total count of homopolymers (called correctly and incorrectly) are in brackets. Only the contigs for bins that were clustered together between different platforms were used to generate values for the plot.



**Table S1:** Sequence statistics for the Zymo HMW Mock using different sequencing platforms. Estimated modal read accuracy is measured using the reported Q-score for each read type. Observed modal read accuracy was measured by read-mapping to the reference genomes.

	<b>Illumina</b>	<b>Nanopore R9.4.1</b>	<b>Nanopore R10.4</b>
<b>Total read count</b>	48,123,500	8,846,993	22,452,567
<b>Total yield (Gbp)</b>	7,2	31,6	52,3
<b>N50 (bp)</b>	151	14,018	5,992
<b>Estimated modal read accuracy (%)</b>	99.99	96.89	98.22
<b>Observed modal read accuracy (%)</b>	99.98	97.59	99.07

**Table S2:** Overview of read datasets used in the study.

Read dataset	Instrument	Yield (Gb)	Read N50 (kb)	Read count	ENA sample ID	LOT#
IL-201104	Illumina HiSeq	6.2	0.15	42,727,130	ERS7673063	
IL-201112	Illumina HiSeq	11.4	0.15	79,619,634	ERS7673064	
IL-201301	Illumina HiSeq	7.5	0.25	31,702,618	ERS7673065	
IL-201308	Illumina HiSeq	6.7	0.25	28,067,586	ERS7673066	
IL-201502	Illumina HiSeq	5.3	0.25	22,351,578	ERS7673067	
IL-201702	Illumina HiSeq	15.9	0.25	66,225,442	ERS7673068	
IL-201705	Illumina HiSeq	4.9	0.25	20,492,240	ERS7673069	
IL-201707	Illumina HiSeq	5.5	0.25	23,663,146	ERS7673070	
IL-201804	Illumina MiSeq	3.2	0.3	11,981,252	ERS7673071	
IL-202001	Illumina MiSeq	13.3	0.3	47,091,904	ERS7673072	
PB-202001	PacBio Sequel II	15.3	15.4	992,914	ERS7673073	
R9-202001	MinION Mk1B	35.2	5.9	10,266,261	ERS7673074	
R10-202001	MinION Mk1B	13.0	6.4	3,646,771	ERS7673075	
R104-202001	GridION	14.0	7.5	3,514,955	ERS7672969	
IL-ZYMO	Illumina MiSeq	7.5	0.15	49,774,986	ERS8296812	ZRC195845
R941-ZYMO	MinION Mk1B	32.0	1.8	8,851,918	ERS8296813	ZRC195845
R104-ZYMO	PromethION	5.2	7.5	18,831,686	ERS8296814	

**Table S3:** CMSeq SNP calling statistics for the Zymo mock reference sequences.

	<b>Covered bases (Mb)</b>	<b>Polymorphic bases (bp)</b>	<b>Polymorphic rate</b>
<b>Bacillus subtilis</b>	4.0	10	2.5e-06
<b>Enterococcus faecalis</b>	2.8	113	4.0e-05
<b>Escherichia coli</b>	4.8	1156	2.4e-04
<b>Listeria monocytogenes</b>	3.0	80	2.7e-05
<b>Pseudomonas aeruginosa</b>	6.8	1222	1.8e-04
<b>Salmonella enterica</b>	4.8	41	8.6e-06
<b>Staphylococcus aureus</b>	2.7	18	6.6e-06