

# Genome Enrichment of Rare, Unknown Species from Complicated Microbiome by Nanopore Selective Sequencing

Yuhong Sun <sup>a, #</sup>, Xiang Li <sup>a, b, c, #</sup>, Qing Yang <sup>a</sup>, Bixi Zhao <sup>a</sup>, Ziqi Wu <sup>a</sup>, Yu Xia <sup>a, b, c \*</sup>

<sup>a</sup> School of Environmental Science and Engineering, College of Engineering, Southern University of Science and Technology, Shenzhen 518055, China

<sup>b</sup> State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China

<sup>c</sup> Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen, 518055, China

## Keywords:

Nanopore sequencing; selective sequencing; Read Until; Readfish; rare species; thermophilic anaerobic digestion

# These authors contributed equally to this work

\*Corresponding author:

Yu Xia

Address: School of Environmental Science and Engineering, College of Engineering, Southern University of Science and Technology, Shenzhen 518055, China

E-mail: [xiay@sustech.edu.cn](mailto:xiay@sustech.edu.cn)

## 1 **Abstract**

2 Rare species are vital members of a microbial community, but retrieving their genomes  
3 is difficult due to their low abundance. The ReadUntil (RU) approach allows nanopore  
4 devices to sequence specific DNA molecules selectively in real-time, which provides an  
5 opportunity for enriching rare species. However, there is still a gap in RU-based  
6 enriching of rare and unknown species in environmental samples whose community  
7 composition is unclear, and many species lack corresponding reference in public  
8 databases. Here we present metaRU<sub>pore</sub> to overcome this challenge. We applied  
9 metaRU<sub>pore</sub> to a thermophilic anaerobic digester (TAD) community, it successfully  
10 redirected the sequencing throughput from high-abundance populations to rare species  
11 while facilitating the recovery of 41 high-quality metagenome-assembled genomes  
12 (MAGs) at low sequencing effort. The simplicity and robustness of the approach make  
13 it accessible for labs with moderate computational resources and hold the potential to  
14 become the standard practice in future metagenomic sequencing of complicated  
15 microbiomes.

## 16 **1 Introduction**

17 Microbial communities are composed of a high number of rare species<sup>1</sup>. Rare species  
18 play a vital role in ecosystem health and stability<sup>2</sup>. For example, the slow-growing  
19 autotrophic microbes of ammonia-oxidizing bacteria or archaea (AOB/AOA) and  
20 anammox enable the rate-limiting step for natural nitrogen turnover<sup>3,4</sup>. Therefore,  
21 identifying the functional capacities of these rare species is essential to understanding  
22 the community dynamics and ecological function of a natural microbiome<sup>2,3</sup>.

23

24 The recovery of draft genomes (referred to as metagenome-assembled genomes, MAGs)

25 from high-throughput metagenomic whole-genome sequencing (thereafter short as  
26 metagenomic) datasets ushered in a new era for understanding the ecological and  
27 evolutionary traits of the unculturable majority of natural microbiomes. However, high-  
28 quality (HQ, usually defined as >90% completeness with <5% contamination and the  
29 intact rRNA operon<sup>44</sup>) MAGs recovery for low abundant species is always difficult. In  
30 metagenomic sequencing, the low-abundance microorganisms are often missed or  
31 simply neglected due to low sequencing coverage. To get sufficient genome coverage of  
32 low-abundance species, extremely deep sequencing will be required. It would be a great  
33 waste if the study aims were to focus on rare species. Things can become more  
34 intractable during the data analyses that recovering the unknown genomes from  
35 hundreds of gigabytes to terabytes of data is a massive computational challenge<sup>4</sup>.

36

37 To raise coverage of rare taxa from a high-abundance background, molecular biology-  
38 based methods including hybrid capture or CRISPR-Cas9 enrichment are adapted in  
39 library preparation to enrich target<sup>5,6</sup>. On the other hand, depletion of high abundance  
40 species may serve the same purpose. Saponin-based host DNA depletion in human  
41 metagenomic communities is used for rapid clinical diagnosis of relatively low  
42 abundance pathogenic bacteria<sup>7</sup>. What is evident, however, is that these approaches  
43 require the use of extra reagents and preparatory procedures. This is compounded by the  
44 fact that they require known information about the enrichment or depletion targets in  
45 order to design the experiment, which does not appear to work for enriching low  
46 abundance species in metagenomic communities with unknown compositions.

47

48 Unlike the endeavors made prior to sequencing, Nanopore sequencing (Oxford  
49 Nanopore Technology, ONT) users can program their system to reverse the voltage  
50 polarity of the sequencing pore to eject reads identified as not of interest, which provides  
51 a potential solution to enrich for rare species in metagenomic samples. This ‘selective  
52 sequencing’ or Read Until (RU) strategy was first implemented by Loose and colleagues  
53 in 2016<sup>8</sup>. The earliest adopted dynamic time warp (DTW) algorithm-based approach  
54 could not scale to references larger than millions of bases, which limits its widespread

55 usage<sup>8</sup>. With the similar goal of mapping streaming raw signal to DNA reference,  
56 UNCALLED has a lighter computational footprint than DTW<sup>9</sup>. Still, it requires abundant  
57 computational resources. The newly designed Readfish toolkit eliminates the need for  
58 complex signal mapping algorithms, and exploits existing ONT tools to provide a robust  
59 toolkit for designing and controlling selective sequencing experiments<sup>10</sup>. Until now, the  
60 application of RU is principally limited to the elimination of known host species<sup>9, 10, 11</sup>  
61 or the enrichment of known targets such as mitogenomes of blood-feeding insects<sup>12, 13</sup>.

62  
63 By ejecting dominant species while accepting low-abundance species, selective  
64 sequencing provides a potential solution to enrich rare species in metagenomic samples.  
65 Nonetheless, enrichment for low abundance species in real metagenomic samples by  
66 selective sequencing remains challenging because the community composition is never  
67 known, and a large proportion of the species lacks a corresponding reference in public  
68 databases. To specifically address such metagenomic-issue and to realize effective  
69 targeted enrichment of rare species within a complicated environment microbiome, here  
70 we introduced metaRU<sub>pore</sub>, a protocol consisting of know-how for configuring selective  
71 nanopore sequencing and necessary bioinformatic scripts to achieve efficient enrichment  
72 of rare species within a complicated environment microbiome. We initially assessed the  
73 efficacy of enriching low abundance species in a mock community. Based on this  
74 evaluation, we elaborated the principles and processes of metaRU<sub>pore</sub> and applied it to  
75 a thermophilic anaerobic digester (TAD) community that was treating waste sludge of a  
76 domestic wastewater treatment plant (WWTP). Meanwhile, we demonstrate a robust and  
77 effective procedure for assembling and binning HQ-MAGs from RU-based nanopore  
78 datasets. And an archaeal HQ-MAG retrieved from the TAD community revealed a giant  
79 (112Kbp) function-related genomic island, extending the evolutionary traits of the  
80 important *Bathyarchaeota* phylum.

## 81 **2 Results**

### 82 ***H. mediterrane* enrichment in a mock community**

83 To evaluate nanopore performance on enriching low abundance species with RU, we  
84 firstly constructed a mock community. The *Haloferax mediterranei* strain which  
85 accounts for 1% of the mock community, was the target of our enrichment, while the  
86 other seven bacteria species were targets to be depleted during the RU run. In the mock  
87 run, a MinION flow cell was configured into two parts, where the first half of the  
88 channels did selective sequencing, and the other half did normal sequencing as a control.  
89 In the RU channels, the reads were basecalled and then mapped to a 33-M reference  
90 which contained all these eight microorganisms when they are being sequenced. A DNA  
91 molecule would be firstly sequenced for 0.4s before the obtained sequence was aligned  
92 to decide it should be sequenced continually or ejected. The average length of rejected  
93 reads was 537 bases, it demonstrated that the entire process of basecalling, mapping, and  
94 rejection decision could be completed in about 1.3s, based on the average nanopore  
95 sequencing speed of 400bp/s with R9.4.1 chemistry<sup>10</sup>. In the RU-delivered dataset, >99.9%  
96 of archaeal reads were kept while >99% of bacterial reads were ejected. *H. mediterranei*  
97 got enriched to the absolute dominant population within the community with a relative  
98 abundance of 62% in kept reads (Fig. 1a) with the coverage increased twice to 21.19×  
99 in RU data (Fig. 1b).

100

101 Despite the high rejection precision and fairly ideal enrichment result, it must be noted  
102 that the total yield of selective sequencing was approximately 60% lower than that of  
103 normal sequencing (Fig. 1c). This reduction in throughput can be partly attributed to the  
104 increased idle time of each nanopore caused by a large number of ejections<sup>9</sup>. At an  
105 enriched target prevalence of 1% within a community, each nanopore ejected an average  
106 of 2,430 short fragments while 267 continuous long fragments were sequenced in a 7-  
107 hour run. In addition, a rapid drop in active channels happened after 1-hour sequencing in  
108 RU channels (Fig. 1 d and Supplementary Fig. 1) and the effective pore got depleted

109 after 6-hour runtime which was 4 times shorter than normal run whose pores could  
110 normally last for 24 hours (Fig. 1d). Consequently, it's critical to establish an appropriate  
111 target proportion for selective sequencing to achieve the best tradeoff between  
112 enrichment effectiveness and throughput loss. Fortunately, increasing sequencing effort  
113 could easily compensate for the RU-induced per flow cell throughput loss.

## 114 **In situ Metagenomic selective sequencing protocol and** 115 **performance**

116 We introduced a pipeline, MetaRUpore (<https://github.com/sustc-xylab/metaRUpore>),  
117 to selectively sequence rare populations in complex microbiome samples. The protocol  
118 consists of three consecutive steps (Fig. 2a): (1) 1h normal sequencing to obtain an  
119 overall picture of the community structure and the genomic profile of the dominant  
120 populations, (2) bioinformatics analysis to determine the reference and target dataset for  
121 optimized RU configuration, and (3) finally a 40h selective sequencing for enriching  
122 rare populations in the sample. The pore control of the nanopore device was  
123 implemented by Readfish<sup>10</sup> which combines Guppy with minimap2<sup>14</sup> to determine the  
124 eject/keep action for a pore.

125

126 Here we show our results in applying the metaRUpore protocol to facilitate the genome  
127 recovery of rare populations within the TAD community, which consists of 2,977 OTUs  
128 with a Shannon index of 8.74, representing a typical diversity level of bioreactor systems  
129 (Supplementary Fig. 2). Rarefaction analysis demonstrated that the reads sequenced in  
130 the first 1 h normal sequencing already cover 90% of the overall diversity in the TAD  
131 community (Supplementary Fig. 5). Among the 125,606 reads sequenced, 66% of them  
132 could be assigned to a known reference by Centrifuge<sup>15</sup>. All of these classified reads  
133 obtained in the first 1 h run were set as the target for ejection in subsequent RU run as it  
134 mostly consisted of the known and abundant populations within the community. Notably,  
135 using whole-genome sequences from close species (same family or genus) as the  
136 reference for RU run will result in poor performance in ejecting the dominant

137 populations because environmental microbiomes typically contain a high proportion of  
138 genetic fragments that are distinct from all the sequences deposited in whole-genome  
139 collections. In fact, even with the entire bacterial whole genome collection set as the  
140 ejection target, only an ejection efficiency of 22% was achieved in RU sequencing of  
141 the TAD community, leaving the community profile largely unchanged after selective  
142 sequencing. Another thing to note is that the classified reads obtained in the firstly 1h  
143 normal sequencing, inevitably contain genomic fragments from the rare and unknown  
144 populations we intend to enrich, which will result in incomplete genome coverage of  
145 rare populations in the sequences obtained in the RU channels. Therefore, a small  
146 fraction of the channels still needed to be set to normal sequencing in the subsequent 40h  
147 RU run and the delivered dataset needs to be assembled together with the RU-derived  
148 datasets. For our RU-sequencing of the TAD community, we set 1/8 channels to normal  
149 sequencing (--channels 1 448) (Fig. 2b). Our subsequent data analysis revealed that 29  
150 HQ-MAGs would be missed if reads derived from selective sequencing were assembled  
151 alone. To further manipulate the selection, the users can manually select which taxa to  
152 keep during subsequent RU run; reads belonging to these taxa will be subtracted from  
153 the target dataset based on their taxonomic affiliations determined by ARGpore2<sup>16</sup>. For  
154 example, in our TAD community, we intended to keep all the archaea reads, so we  
155 eliminated them from the ejection target datasets. The entire aforementioned  
156 bioinformatic analysis can be completed in less than 30 min, such short suspension will  
157 not affect the flow cell chemistry and the subsequent RU run may directly start without  
158 refreshing the sequencing library.

159

160 The 40h RU run on one flow cell delivered 6.84 Gbp of effective long reads with an  
161 average read length of 3.46 kbp, while the normal sequencing channels produced 1.71  
162 Gbp reads with an average read length of 3.60 kbp (Supplementary Fig. 3). To ensure  
163 adequate genome coverage, we sequenced the TAD community following metaRU-pore  
164 protocol using three flow cells one by one on GridION X5. Given the concern to exhaust  
165 computation capacity on GridION X5, we did not test RU run with multiple flow cells  
166 sequenced simultaneously. RU sequencing using metaRU-pore protocol resulted in a

167 marked change in the community structure. As shown in the 3D density plot of  
168 phylogeny distribution of the overall TAD community (Fig. 3a), several density peaks  
169 of the original TAD community were depleted in the RU-run delivered datasets,  
170 indicating DNA of the high abundance populations of the TAD community was  
171 effectively ejected during RU-sequencing and the community got homogeneous with  
172 coverage of different populations become much more unified. Such unified coverage of  
173 different populations will help to minimize the disparity of kmer frequency in the dataset,  
174 preventing kmers of the rare species from being filtered out as error-containing kmers  
175 due to coverage drop during the kmer-counting step of a *de novo* assembly algorithm<sup>17</sup>,  
176 <sup>18</sup>.

## 177 **Bioinformatics pipeline for *de novo* metagenomic assembly** 178 **and genome recovery**

179 As illustrated in the assembly pipeline (Fig. 2c), the 31G data consisting of RU and  
180 normal sequencing were assembled together respectively using three different  
181 assemblers, namely Canu<sup>19</sup>, Unicycler<sup>20</sup>, and metaFlye<sup>21</sup>. The basic statistics of  
182 assembled contigs were summarized in Supplementary Table 1. To improve the  
183 robustness of the binning, 139 > 1Mbp contigs were firstly picked, as the candidate of  
184 HQ genome<sup>22</sup>. The rest shorter contigs derived by the three assemblers were respectively  
185 binned by MetaBAT2<sup>23</sup>. Only contigs longer than 100 kbp were kept for subsequent  
186 binning. The MAGs retrieved above were subject to consensus correction by Medaka  
187 with nanopore data and polished by Pilon<sup>24</sup> with Illumina short reads (SRs). Next,  
188 polished MAGs were further corrected for frame-shift errors using MEGAN-LR<sup>22</sup> based  
189 on DIAMOND alignment against the *nr* database. Finally, MAGs obtained by the  
190 different assemblers were de-duplicated using dRep<sup>25</sup> with a relatedness threshold of  
191 ANI > 0.95 to obtain species-level representative MAGs. Totally, we obtained 46 draft-  
192 quality MAGs after dereplication. Among them, 41 MAGs including 6 complete circular  
193 genomes were high-quality (HQ) (Supplementary Fig. 8 and Supplementary Table 2).  
194 32 of these HQ MAGs were firstly picked single >1Mbp contigs, while the remaining



195 15 HQ MAGs were obtained by binning. All of these MAGs contained less than 13  
196 contigs with an average N50 > 2 Mbp, demonstrating that they are highly continuous. In  
197 comparison, the normal nanopore sequencing dataset yielded 29 draft-quality MAGs,  
198 including 16 HQ MAGs. 15 of them were included in the 41 HQ MAGs retrieved by  
199 metaRU-pore strategy (Supplementary Fig. 8). Worth noting is that the 26 HQ MAGs  
200 that are additionally obtained by RU-based selective sequencing were mainly from the  
201 rare populations of the TAD community (Fig. 3b). Additionally, evident coverage  
202 reduction was observed in the dominant populations that the coverage of MAG17,  
203 MAG4, and MAG30, which together accounted for 21% of the TAD community,  
204 dramatically reduced by 78% after RU-based selective sequencing (Fig. 3b and  
205 Supplementary Table 3), demonstrating the effectiveness of metaRU-pore protocol in  
206 eliminating dominant populations during sequencing. Despite the lowered overall  
207 throughput, coverage of the rare species MAG33, MAG35, MAG57, and MAG56 was  
208 doubled at the current sequencing effort and the application of the metaRU-pore protocol  
209 has reduced the abundance limit for HQ-MAG recovery in the TAD community to 0.7%.  
210 It could be expected that by using additional flow cells, HQ-MAGs could be obtained  
211 for populations with even lower prevalence.

212

### 213 **3 Discussion**

#### 214 **Complete genomes recovered from TAD community**

215 The 41 HQ MAGs introduce 5 new phyla, namely *WOR-3*, *OLB16*, *Omnitrophota*,  
216 *Gemmatimonadota*, and *Deferribacterota*, into the global HQ genome collection of AD  
217 microbiome<sup>26</sup> (Fig. 4). Furthermore, our MAGs show much better integrity and  
218 continuity than those in the previous collection assembled with SRs in terms of N50,  
219 number of contigs as well as intact rRNA operon. Additionally, evolutionary traits  
220 analysis reveals a much more conservative scale of gene flow based on HQ genomes we  
221 assembled than that based on fragmented MAGs<sup>27</sup> (Supplementary Fig. 9).

222

## 223 **Versatile metabolic capacities of *Bathyarchaeota* phylum in** 224 **TAD community**

225 *Bathyarchaeota* was recently recognized as a methanogenesis contributor<sup>28</sup> that may  
226 play active roles in global biogeochemical cycles<sup>31</sup>. However, the absence of pure  
227 cultures of the phyla has hampered our understanding of their ecological functions and  
228 evolutionary positions from a genome-centric perspective<sup>29,30</sup>. Genomes reported for this  
229 phylum so far are highly fragmented (Fig 5a). In this work, MetaRU pore has boosted  
230 the abundance of *Bathyarchaeota* in the TAD community from 0.19% to 0.32%,  
231 facilitating its genome recovery as MAG56, which to the best of our knowledge, is the  
232 first complete genome for this phylum. MAG56 represented a novel *Bathyarchaeota*  
233 lineage with the closest neighbor being Bathy-5 (Fig 5b). The genome size of MAG56  
234 is 1.9Mbp, notably larger than the average size of previously assembled genomes of  
235 *Bathyarchaeota* phylum (1.23Mbp)<sup>29,30,31</sup>. *Bathyarchaeota* was previously proposed to  
236 have methyl-dependent hydrogenotrophic methanogenic potential<sup>28,32</sup> as MAGs  
237 recovered from deep aquifers<sup>34</sup> possess an MCR-like complex. However, no MCR  
238 homology could be detected in MAG56. Given the complete nature of the genome  
239 obtained in this study, a functioning methanogenic pathway in the TAD community  
240 lineage of *Bathyarchaeota* seemed implausible.

241

242 Remarkably, we found three genomic islands (GIs) (Fig 5a) in MAG56 with the largest  
243 being 36 kbp in length. These GIs were always missing in previously genomes  
244 assembled by short reads due to the defective resolving of repetitive fragments flanking  
245 the exogenous genetic island<sup>33,34</sup>. In the largest GIs of 36 Kbp, we identified six copies  
246 of Tyrosine recombinase (*xerA*, *xerC*, or *xerD*), which had previously been reported to  
247 facilitate the insertion of gene islands into the host chromosome by catalyzing site-  
248 specific, energy-independent DNA recombination<sup>34,36</sup>. Additionally, we identified a heat  
249 shock protein, *HtpX*, that may contribute to the heat shock response facilitating the cell's

250 survival in a thermophilic environment. Collectively, this GI represents a highly mobile  
251 fitness island<sup>33</sup> that offers selective advantages for the archaeal population within the  
252 thermophilic digester community. And the recovery of complete MAGs by metaRU pore  
253 undoubtedly enabled the discovery of the role of large GIs in shaping *Bathyarchaeota*'s  
254 evolution.

255

256 Overall, we proposed metaRU pore, a method for enriching low-abundance and  
257 undiscovered microorganisms in complex microbial communities based on nanopore  
258 selective sequencing. The heuristic ejecting targets determined through initial short-term  
259 *de novo* sequencing of the dominant populations, overcome the constraints imposed by  
260 the absence of reference genomes for selective sequencing of complex communities.  
261 metaRU pore unifies the sequenced community structure and increases the genome  
262 coverage of low-abundance species, facilitating the assembly of additional HQ genomes  
263 of rare species within the microbiota. HQ MAGs retrieved from the TAD community by  
264 metaRU pore contribute to the building of a more comprehensive database of AD-  
265 associated microbes, which will ultimately allow for an in-depth understanding of their  
266 biological characteristics. More importantly, metaRU pore protocol is robust and requires  
267 minimal modification to the experimental procedure of nanopore library construction  
268 and sequencing, making it easily applicable to metagenomic investigations of other  
269 environmental microbiomes. Even though selective sequencing for the rare sphere is  
270 inevitably associated with a reduction in per-flow cell data yield. Future implementation  
271 of the RU API on PromethION will easily provide a throughput boost, overcoming the  
272 coverage barrier and enabling complete genome recovery of rare species with even lower  
273 abundance from complex microbiomes using the metaRU pore protocol.

## 274 **4 Methods**

### 275 **Sampling and DNA extraction**

276 Genomic DNA of the eight microorganisms of the mock community was extracted by QIAamp DNA  
277 Micro Kit (50). Samples for TAD community were taken when the methanogenic bacteria were at  
278 their highest activity. Genomic DNA of the TAD community samples was extracted by QIAGEN  
279 DNeasyR PowerSoilR Kit (100). DNA concentration was determined using the Life Technologies

280 Qubit high sensitivity assay kits. The quality of the DNA was measured by Thermo Scientific™  
281 NanoDrop™ to assure that it all met the requirements for library construction.

282

### 283 **Construction of the synthetic mocks**

284 We synthesized a mock community of eight microorganisms, of which Archaea accounted for 1%  
285 and the other seven bacteria species shared the rest equally based on DNA concentration determined  
286 from qubit average measurements. The archaeal species is *Haloferax mediterranei* and these seven  
287 bacteria are *Acinetobacter baumannii*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella*  
288 *pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Staphylococcus aureus*.

289

### 290 **Library construction and Sequencing**

291 All sequencing libraries were constructed using the ONT Ligation Sequencing Kit (no. SQK-LSK109)  
292 according to the manufacturer's instructions. When preparing the reactor sample libraries, in order to  
293 remove as many very short DNA fragments as possible, 0.4X beads was used for each step of the  
294 cleanup, and therefore the initial amount of genomic DNA was increased to 2ug to ensure a sufficient  
295 amount of DNA of the final library. ONT MinION flowcells v.R9.4.1 were used for all sequencing  
296 on an ONT GridION.

297

### 298 **Selective sequencing via metaRUopore**

299 The execution of metaRUopore to enrich for unknown low abundance taxa is divided into the  
300 following three steps: firstly, a period (in this case 60 min) of normal sequencing is performed to  
301 generate reference file for selective sequencing using Readfish<sup>10</sup> which should contain the vast  
302 majority of taxa in the community. Next, the sequenced data is fed into metaRUopore to obtain the  
303 reference and target needed to configure Readfish TOML for selective sequencing. During this time,  
304 it is advisable to keep the MinION flowcell with the DNA library in a 4°C refrigerator to avoid the  
305 loss of activity of the nanopores affecting the subsequent sequencing. We put the reference and target  
306 paths into the TOML file and set `config_name = "dna_r9.4.1_450bps_fast"`, `single_on = unblock`,  
307 `multi_on = unblock`, `single_off = stop_receiving`, `multi_off = stop_receiving`, `no_seq = proceed`,  
308 `no_map = proceed`. As recommended by the author of Readfish, we deactivated adapter scaling by  
309 editing the config files (`dna_r9.4.1_450bps_fast.cfg`) in the guppy data directory. Next, selective  
310 sequencing was started. the configuration on MinKNOW was the same as for normal sequencing.  
311 Readfish runs at the same time as the sequencing starts.

312

### 313 **Analysis of long-read sequence data**

314 Sequencing-derived fastq reads were performed adaptor trimming using Porechop ([GitHub -](#)  
315 [rrwick/Porechop](#)) (version 0.2.2) with default settings. These reads were subsequently assembled by  
316 the three tools: Canu<sup>19</sup> (version 2.2, default setting except `-nanopore`, `genomeSize=3m`,  
317 `maxInputCoverage=10000`, `corOutCoverage=10000`, `corMhapSensitivity=high`, `corMinCoverage=0`,  
318 `redMemory=32`, `oeaMemory=32`, `batMemory=200` `useGrid=false`), Unicycler<sup>20</sup> (version 0.4.9b,  
319 default setting except `-t 40`, `--keep 3`) and Flye<sup>17</sup> (version 2.8.3, default setting except `-nano-raw`, `--`  
320 `threads 50`, `--plasmids`, `--meta`, `--debug`). Generated contigs that was at least 1Mbp in length were  
321 regarded as potential whole-chromosome sequence. Among the remaining contigs that are less than  
322 1Mbp, we did metagenomic binning for the contigs that are greater than 100kbp in length. Metabat2<sup>21</sup>

323 (version 2.12.1 with default setting) is used to respectively binning the contigs assembled by above  
324 three assemblers.

325 Next, we took multiple steps to correct the >1Mbp potential chromosome and bins we obtained.  
326 Firstly, we used nanopore data to perform consensus correction on them using Medaka ([GitHub -  
327 nanoporetech/medaka](#)) (version 1.4.3, default setting except -t 20, -m r941\_min\_high\_g360). They  
328 were then further corrected with the short reads data using Pilon<sup>23</sup> (version 1.24 with default setting  
329 except --fix all, --vcf). We used DIAMOND<sup>35</sup> (version 0.9.24) to align the Pilon polished potential  
330 chromosome (with default settings except -f 100 -p 40 -v --log --long-reads -c1 -b12) against the  
331 NCBI-NR database<sup>38</sup> (July 2021). We used daa-meganizer in MEGAN Community Edition suite<sup>39</sup>  
332 (version 6.21.7, run with default settings except --longReads, --lcaAlgorithm longReads, --  
333 lcaCoveragePercent 51, --readAssignmentMode alignedBases) to format the .daa output file and  
334 receive frame-shift corrected sequence with 'Export Frame-Shift Corrected Reads' option.  
335 We checked the completeness and contamination of these potential genomes with CheckM<sup>40</sup> (version  
336 v1.0.12, run with default setting except lineage\_wf, -t 20). All the putative genomes were de-  
337 replicated using the dRep<sup>25</sup> (version 3.2.2, run with default setting except -p 40 -sa 0.95 --genomeInfo)  
338 to get species-level unique MAGs. Next, gene annotations were obtained using Prokka<sup>41</sup> (version  
339 1.13). Microbial taxonomic classifications were assigned using GTDB-Tk<sup>42</sup> (version 1.3.0, GTDB-  
340 Tk reference data version r89).

341

#### 342 **Calculation of the abundance and assessment of the quality of MAG**

343 Abundance was calculated from both selective sequencing data and normal sequencing data, by  
344 mapping these data to the MAGs using minimap2<sup>14</sup> (version 2.17) separately using the following  
345 flags -ax map-ont -t 40. We used samtools<sup>43</sup> (version 1.11) to extract .sam file that matched each  
346 MAG individually. The abundance of each MAG is calculated by dividing the number of bases in all  
347 reads in this .sam file by the total number of bases selectively sequenced or normally sequenced.  
348 Analogously, sorted .bam files were used in the calculation of coverage of the MAGs.

349 We defined high-quality (HQ) MAGs as encoding multiple rRNA genes (23S/16S/5S), SCG-  
350 completeness > 90% and contamination < 5%<sup>44</sup>. Draft-quality (DQ) MAGs means MAGs having >  
351 70% SCG-completeness, < 10% contamination, and the presence of 16S rRNA. While if a MAG  
352 meets all of the DQ criteria but misses 16S rRNA were regarded as low-quality (LQ) genomes.

353

354

#### 355 **Code availability**

356 The metaRU pore workflow is available on the GitHub page: [https://github.com/sustc-  
357 xyllab/metaRU pore](https://github.com/sustc-xyllab/metaRU pore).

358

#### 359 **Availability of data and materials**

360 The raw nucleotide sequence data (both Illumina and Nanopore) used in the present study has  
361 been deposited in the NCBI database under project ID PRJNA794848.

362

#### 363 **Acknowledgements**

364 This work was supported by the National Key Research and Development Program of China  
365 (Grant No. 2021YFA1202500), the National Natural Science Foundation of China (Grant No.  
366 42007216) and Shenzhen Science and Technology Innovation Committee (Grant No.

367 JCYJ20210324104412033). Also, we want to thank the Center for Computational Science and  
368 Engineering at Southern University of Science and Technology (SUSTech) and core research  
369 facilities at SUSTech to provide quality resources and services.  
370

### 371 **Conflict of interests**

372 The authors claim no conflict of interests.  
373

## 374 **Reference**

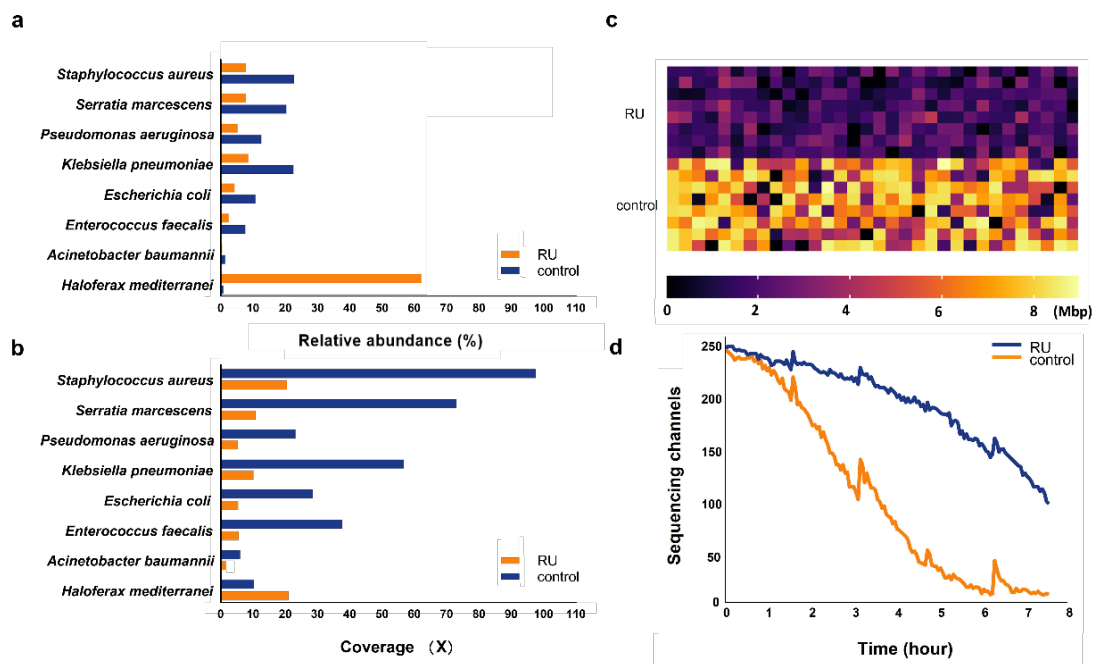
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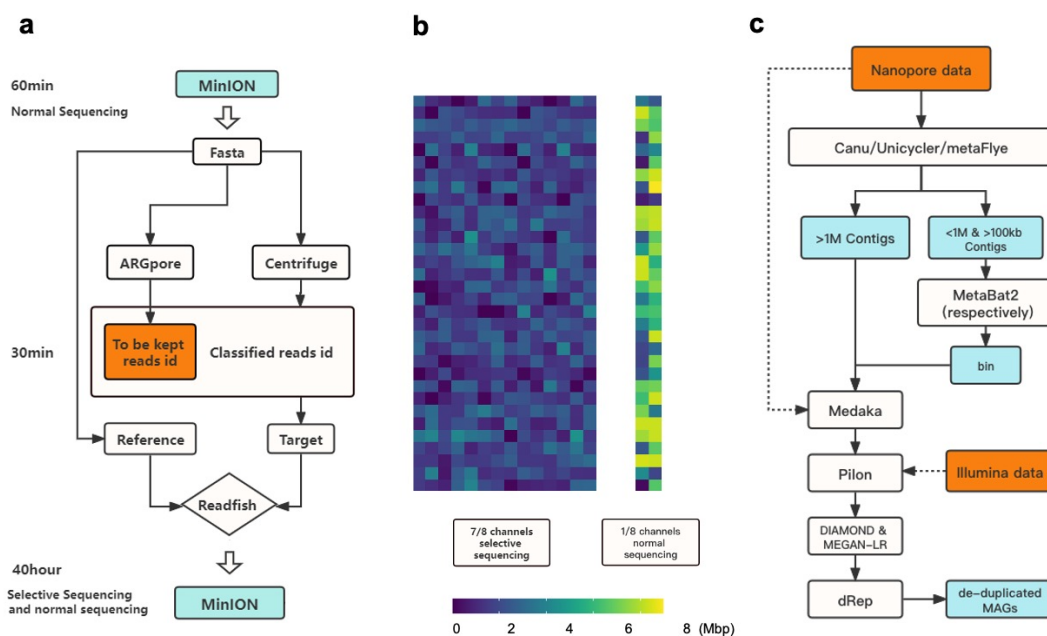
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484

485 **Fig. 1 Enriching low abundance species in mock community with RU.** **a**, Bar plot of the  
 486 abundance of the seven microbial species in RU and control runs. **b**, Bar plot of the coverage of the  
 487 seven microbial species' genome in RU and control runs. **c**, heatmap of data yield per channel in RU  
 488 and control runs, and **d**, plot of the number of sequencing channels over the course of the sequencing  
 489 run.



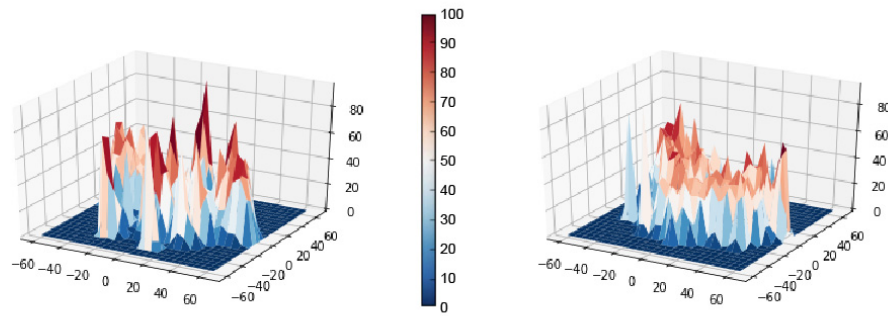
490

491 **Fig. 2 a**, The workflow of metaRUopore. **b**, A MinION flow cell in metaRUopore is  
 492 configured into two parts, 1/8th of the channels for normal sequencing and the remaining  
 493 channels for selective sequencing. **c**, The bioinformatic workflow for HQ-MAGs  
 494 retrieval based on datasets derived from nanopore selective sequencing and Illumina  
 495 sequencing.

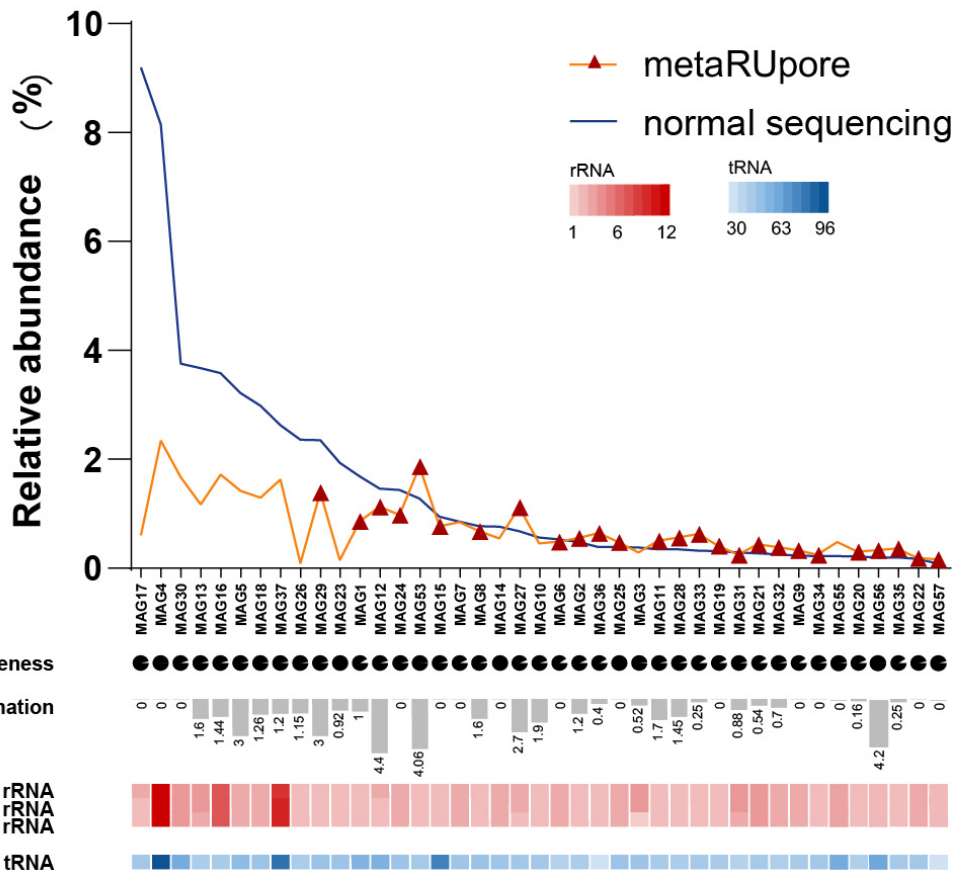
a

normal sequencing

metaRU pore



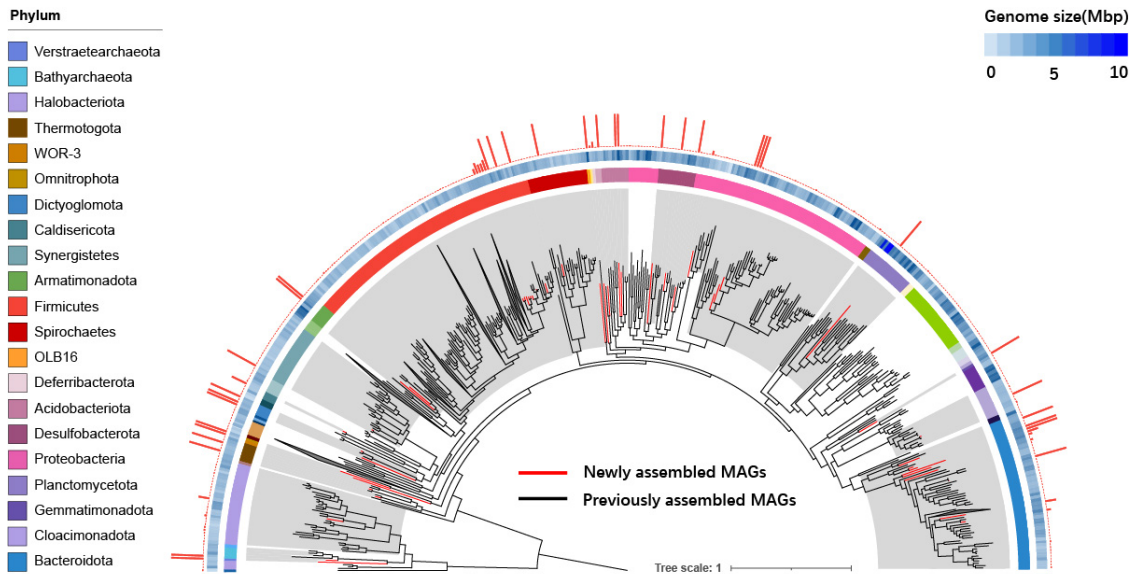
b



496

497 **Fig. 3 Performance of metaRU pore on recovery of high-quality MAGs in TAD community.** a, 3D  
 498 density plots of t-SNE downscaling results for normal sequencing datasets and selective sequencing  
 499 datasets by metaRU pore at four base frequencies, showing that metaRU pore renders the community  
 500 structure homogenous. b, The distribution of 41 retrieved HQ MAGs in normal and RU sequencing  
 501 dataset. The red triangles indicate MAGs that were could only be assembled in the metaRU pore dataset.  
 502 The pie chart and bar chart represent the level of genomic completeness and contamination by CheckM.  
 503 The copy number of 16S rRNA, 23S rRNA, and 5S rRNA is represented by the red heatmap, while the  
 504 copy number of tRNA is represented by the blue heatmap.

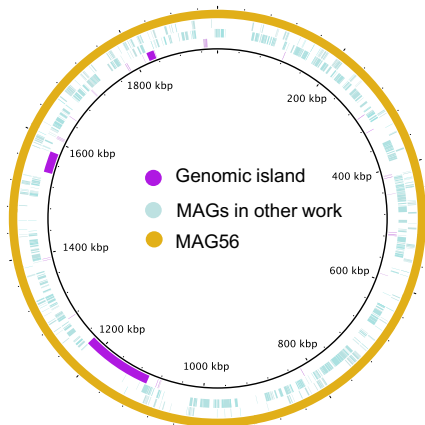
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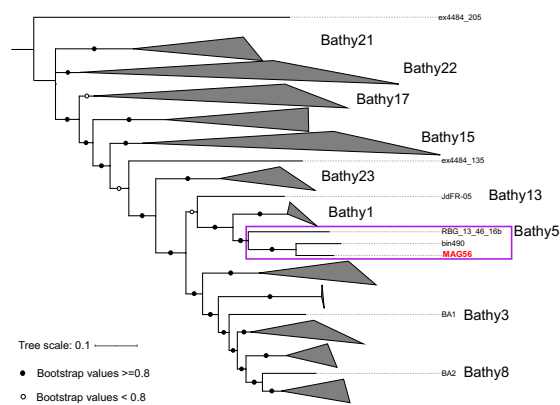
506

507 **Fig. 4 Phylogenomics of MAGs in anaerobic reactor.** A phylogenetic tree was constructed from 41  
 508 HQ-MAGs derived by metaRU-pore (red branches) and 1,108 HQ-MAGs collection derived from  
 509 other AD systems (black branches). External circles represent, respectively: (1) taxonomic  
 510 assignment at phylum level, (2) genome size (heatmap), (3) bar plot representing the genome  
 511 continuity, which is calculated as the reciprocal of the number of contigs. The grey shaded areas  
 512 indicate phyla with near-complete genomes obtained by metaRU-pore, and the name of each phylum  
 513 is in the legend on the left.

a



b



514

515 **Fig. 5 a,** Genomes comparison of MAG56 and other MAGs of Bathyarchaeota from prior research.  
 516 The outermost ring stands for the circular genome of MAG56 reconstructed by metaRU-pore. The  
 517 second to third circles from the outside represent the MAGs of phylum Bathyarchaeota  
 518 reconstructed by short reads-only assembly method (MAGs covered by purple boxes in Figure 5c).  
 519 The innermost purple circle represents the genomic island. b, A Maximum Likelihood Tree  
 520 showing the phylogeny of Bathyarchaeota based on the MAGs from the current study (MAG56)  
 521 and prior research<sup>29</sup>. Bootstrap values for these phylogenies are shown with open (< 80%) and  
 522 filled ( $\geq 80\%$ ) circles.

523