Untangling Microbiota Diversity and Assembly Patterns in the World's Largest Water Diversion Canal

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28 **Abstract**

29 Large water diversion projects are important constructions for reallocation of human-essential
30 water resources. Deciphering microbiota dynamics and assembly mechanisms underlying
31 canal water ecosystem services especially during long-distance diversion is the prerequisite
32 for water quality monitoring, biohazard warning and sustainable management. Using a
33 1432-km canal of the South-to-North Water Diversion Projects as a model system, we answer
34 three central questions: how bacterial and micro-eukaryotic communities spatio-temporally
35 develop, how much ecological stochasticity contributes to microbiota assembly, and which
36 immigrating populations better survive and navigate across the canal. We applied quantitative
37 ribosomal RNA gene sequence analyses to investigate canal water microbial communities
38 sampled over a year, as well as null model- and neutral model-based approaches to
39 disentangle the microbiota assembly processes. Our results showed clear microbiota dynamics
40 in community composition driven by seasonality more than geographic location, and
41 seasonally dependent influence of environmental parameters. Overall, bacterial community
42 was largely shaped by deterministic processes, whereas stochasticity dominated
43 micro-eukaryotic community assembly. We defined a local growth factor (LGF) and
44 demonstrated its innovative use to quantitatively infer microbial proliferation, unraveling
45 taxonomically dependent population response to local environmental selection across canal
46 sections. Using LGF as a quantitative indicator of immigrating capacities, we also found that
47 most micro-eukaryotic populations (82%) from the source lake water sustained growth in the
48 canal and better acclimated to the hydrodynamical water environment than bacteria (67%).

49 Taxa inferred to largely propagate include *Limnohabitans* sp. and *Cryptophyceae*, potentially
 50 contributing to water auto-purification. Combined, our work poses first and unique insights
 51 into the microbiota assembly patterns and dynamics in the world's largest water diversion
 52 canal, providing important ecological knowledge for long-term sustainable water quality
 53 maintenance in such a giant engineered system.

54 Key words: Engineered system; Bacterial community; Micro-eukaryote; Deterministic
 55 assembly; Stochastic assembly; Population growth

56

57 **Introduction**

58 Microorganisms are fundamental components of ecosystems and play vital roles in global
59 biogeochemical cycling (Arrigo, 2005; Battin et al., 2016; Du et al., 2021). Knowledge about
60 composition and dynamics of microbial communities across space and time is a prerequisite
61 for predicting and manipulating the microorganisms in both natural and engineered systems
62 (Bell et al., 2005; Bru et al., 2011; Ju and Zhang, 2015). Due to artificial system design and
63 control, microbial communities in engineered ecosystems have received increasing attention
64 to unravel the community dynamics underlying their eco-environmental or biotechnological
65 services (Briones and Raskin, 2003; Ju et al., 2017; Ju et al., 2014; Vanwonterghem et al.,
66 2014; Wu et al., 2019). Specifically, microorganisms in drinking water systems are closely
67 linked with water auto-purification and eventually to public health (Berry et al., 2006). Thus,
68 comprehensive microbiome monitoring is needed to differentiate baseline and disturbed
69 microbial communities in engineered water systems to facilitate system management (Hull et
70 al., 2019; Sagova-Mareckova et al., 2021).

71 The South-to-North Water Diversion (SNWD) Projects were built to aid in water supply
72 in the drought-stricken north China, which represent the world's largest water diversion
73 projects. The projects consist of the Eastern Route Project, the Middle Route Project (MRP)
74 and the Western Route Project. The 1432-km MRP went into service in 2014 and has drawn
75 40 billion m³ of water from the Danjiangkou Reservoir to He'nan and Hebei Provinces, and
76 Beijing and Tianjin Municipalities, benefiting tens of millions of people. Despite their

77 important roles in controlling geochemical processes and indicating water quality (Chen et al.,
78 2018; Dang et al., 2018), little has been known about the microbial constituents throughout
79 the system (Luo et al., 2019), neither for other engineered long-distance water diversion
80 canals. Especially, the MRP is a closed engineered system where microorganisms are subject
81 to potentially less complex biotic and abiotic interactions, while the numerous hydraulic
82 structures and engineered perturbations (e.g., inverted siphon, aqueduct and tunnels) along the
83 main canal add to challenges to model microbial community dynamics, calling for
84 comprehensive investigation of microbial community patterns in the MRP canal, to assist in
85 establishing integrative assessment of water quality with microbial community monitoring
86 (Sagova-Mareckova et al., 2021).

87 Understanding processes and mechanisms driving the community patterns underlying
88 ecosystem services is one of the key issues in microbial ecology (Zhou et al., 2014).
89 Microbial community composition in aquatic ecosystems can be affected deterministically by
90 both abiotic or environmental factors, e.g., water temperature, pH, dissolved oxygen, and
91 nutrient availability (Hu et al., 2017; Liu et al., 2020a; Zhang et al., 2019b), and biotic factors,
92 such as competition and predation. In addition to deterministic mechanisms, stochastic
93 processes, for instance, dispersal and ecological drift, also contribute to microbial community
94 assembly. Efforts have been made to disentangle the relative importance of deterministic vs.
95 stochastic processes based on a variety of null and neutral modeling methods (Ning et al.,
96 2019; Sloan et al., 2006; Stegen et al., 2013). Depending on which ecosystems or organism
97 types these methods are applied to, the relative importance of deterministic vs. stochastic

98 processes may greatly vary (Chen et al., 2019; Liu et al., 2018b; Wang et al., 2020; Yan et al.,
99 2020; Niederdorfer et al., 2021). For instance, bacterial communities in lake water were
100 revealed to be predominantly structured by determinism, whereas micro-eukaryotic
101 communities largely by stochasticity (Logares et al., 2018). For the MRP, the closed channel,
102 the environmental gradients and the variety of hydraulic constructions along the canal make it
103 obscure to what extent ecological stochasticity determines the assembly of microbiota
104 community, which is essential for an improved understanding of the ecosystem processes and
105 microbial dynamics prediction.

106 Other emerging questions in such large water-diversion canal and the comparable
107 systems, like river continuum (Gweon et al., 2021), include which immigrating
108 microorganisms are adapted to local niches in the receiving water and how they functionally
109 contribute to the ecological service. Especially in the MRP, how many of the microbial
110 populations originated from a natural reservoir may survive in the contrastingly engineered
111 canal remains an open question. Although population growth associated with microbial
112 immigration has been quantified in other engineered water systems (Mei and Liu, 2019), such
113 as activated sludge reactors (Mei et al., 2019), efforts in estimating microbial growth in
114 long-distance and evolving systems are still lacking.

115 In the present study, we followed the spatio-temporal dynamics of prokaryotic and
116 micro-eukaryotic community along the main canal of MRP of the SNWD project over a year
117 to address: 1) How does microbial community develop with the water flow? 2) How is the

relative importance of deterministic and stochastic processes on shaping the community assembly in this closed turbulent engineered system? 3) How do the immigrating bacteria and micro-eukaryotes from water source survive, grow, or die off in the canal? Based on high-throughput marker gene (i.e., 16S and 18S) amplicon and metagenomic sequencing, bioinformatics, and statistical modeling of water microbiota, we elucidated the microbial diversity and population dynamics along the canal over four seasons and uncovered the key patterns and processes driving community assembly. By overcoming the limitations of widely used relative abundance measures with estimated absolute abundance (Barlow et al., 2020; Tkacz et al., 2018), we demonstrated the first use of local growth factor to predict growth capacity of the immigrating microbial populations along the water canal and revealed the divergent microbial fates and population lifestyles throughout the world's largest water diversion canal.

Materials and Methods

A full version of the Materials and methods are available in the Supplementary Information.

Study sites, sampling and physiochemical monitoring

The Middle Route Project (MRP) of the South-to-North Water Diversion (SNWD) Projects started from Danjiangkou reservoir and diverted water northwards until Beijing and Tianjin, which is an artificial system without direct connection with surface waters in the area. Fifty samples were collected from 19 sites (Fig. 1), including one at the Danjiangkou reservoir (S01) and 18 along the main canal of the MRP (S02-S18). The 18 sampling sites along the

138 canal were selected to capture the spatial dynamics of microbial communities from canal head
139 to end across the subtropical and warm-temperate climate zones. The sites were grouped to
140 three sections: S02-S08 (Section1), S09p-S14 (Section2) and S15-S18 (Section3). Canal
141 water was sampled and filtered through 0.22- μ m cellulose nitrate filters (Sartorius, Germany).
142 Physiochemical properties, including water temperature (T), pH, dissolved oxygen (DO), total
143 nitrogen (TN) and fluoride (F⁻), were measured according to the environmental quality
144 standard for surface water of China (GB3838-2002). Details about the sampling sites and
145 physiochemical measurements are in the Supplementary Method S1.

146 **DNA extraction, PCR amplification and sequencing**

147 Genomic DNA was extracted from filters using FastDNA Spin Kit for Soil (MP Biomedicals,
148 USA). Bacterial 16S rRNA gene V3-V4 region and eukaryotic 18S rRNA gene V4 region
149 were amplified and sequenced on the Illumina platforms producing 250 bp paired-end reads at
150 the Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China). Real-time
151 quantitative PCR (qPCR) was additionally performed to quantify the 16S rRNA gene copies
152 (as a proxy for bacterial biomass) in each genomic DNA sample, as described in Ju et al.
153 (2019). Detailed methodologies are provided in the Supplementary Method S2.

154 Each genomic DNA was also used for shot-gun metagenomic sequencing on the
155 Illumina's Novaseq platform using a 150 bp paired-end sequencing strategy at the Novogene
156 (Beijing, China). The sequencing data generated in this study have been deposited into CNGB
157 Sequence Archive (CNSA) (Guo et al., 2020) of China National GeneBank DataBase

158 (CNCBdb) (Chen et al., 2020) with accession number CNP0001593.

159 **Bioinformatics and statistical analyses of microbial diversity**

160 The 16S and 18S rRNA gene amplicon sequence data were processed using Qiime2 pipeline
 161 (Bolyen et al., 2019) and DADA2 algorithm (Callahan et al., 2016) as described in the
 162 Supplementary Method S3, generating final abundance tables of amplicon sequence variants
 163 (ASVs) consisting of 6705 bacterial and 3217 eukaryotic ASVs, respectively. The
 164 alpha-diversity metrics of bacterial and micro-eukaryotic communities were further computed
 165 in Qiime2. All the univariate and multivariate statistics were performed in R (R Core Team,
 166 2020). A set of spatial factors was generated for the sampling sites based on the geographic
 167 coordinates using the principal coordinates of neighboring matrices (PCNM) analysis
 168 (Borcard and Legendre, 2002).

169 The metagenomic data were pretreated and searched against the SILVA SSU132 database
 170 to identify rRNA gene reads. This information was further used to estimate the 16S rRNA and
 171 18S rRNA gene copies detected in each metagenome based on the sum for the read coverage
 172 over the full length of each matched reference sequence in the database. Equation and details
 173 are available in the Supplementary Method S3. The copies of 18S rRNA genes in each water
 174 sample were estimated as 16S rRNA gene copies (determined by the aforementioned qPCR
 175 analysis) divided by ratios of 16S rRNA to 18S rRNA gene copy (Table S1) as sequenced and
 176 estimated in the metagenomic data.

177 **Neutral and null model analyses of microbial community patterns**

178 To disentangle the relative contribution of stochastic and deterministic processes to microbial
 179 community assembly, Sloan neutral model (Sloan et al., 2006) and null model-based NST
 180 approach (Ning et al., 2019) were applied to the 16S and 18S community data. Fitting of the
 181 model were performed in R according to Burns et al. (2016). Accordingly, ASVs were
 182 classified into three groups depending on whether their observed occurrence falling higher
 183 than (“above prediction”), within (“as predicted”) or lower than (“below prediction”) the 95%
 184 confidence interval of the neutral model predictions. Normalized stochasticity ratio (NST)
 185 was used to quantify ecological stochasticity in communities within canal sections (Ning et al.,
 186 2019). NST analysis was performed in R using the NST package (Ning et al., 2019). More
 187 details about the methodology are in the Supplementary Method S4.

188 **Population lifestyle and local growth factor analyses**

189 The absolute abundance of bacterial and micro-eukaryotic ASVs was calculated by
 190 multiplying relative abundance of each ASV computed from amplicon sequence data by the
 191 overall 16S and 18S rRNA gene copies detected in a given metagenome for all the Oct
 192 samples (see the Supplementary Method S5 for more details).

193 To characterize fate of microorganisms originated from natural reservoir after transported
 194 into the closed canal, we defined a local growth factor (LGF) to depict whether the
 195 populations grew or restrained, by comparing their mean absolute abundance in each canal

196 section to the abundance at the canal head, as follows:

$$197 \quad LGF_x = \log_{10} \frac{AA_{x,N} + 1}{AA_{x,OctS02} + 1} \quad (1)$$

198 where $AA_{x,N}$ is the average absolute abundance of ASV x in Section N of sampling sites and
 199 $AA_{x,OctS02}$ is the absolute abundance of ASV x at OctS02, the canal head. OctS02 was
 200 excluded from Section1 for the LGF calculation. An average LGF > 0 across the three
 201 sections was considered to indicate growth in the canal. The potentially pathogenic bacterial
 202 ASVs were identified referring to a list of potentially pathogenic genera (Table S2)
 203 summarized from Woolhouse et al. (2015), and LGFs of these ASVs were examined.

204 **Results**

205 **Microbiota biomass and diversity shifted with seasonality**

206 Microbial communities showed clear seasonal shifts (Fig. 2), whether for their biomass (A
 207 and B) or alpha (C-F) and beta diversity (Fig. 2G, 2H and Fig. S1). Both the bacterial and
 208 micro-eukaryotic biomass, as measured by 16S rRNA (A) and 18S rRNA (B) gene copies,
 209 were the highest in August and the lowest in October (Fig. 2). PERMANOVA showed that
 210 seasonality significantly ($P < 0.001$) influenced both the bacterial ($F = 22.78$) and
 211 micro-eukaryotic ($F = 25.57$) community composition. Moreover, the seasonal impacts were
 212 much more pronounced than the spatial effects along the canal, whether for bacteria ($R^2 =$
 213 0.52 vs. 0.14) or micro-eukaryotes ($R^2 = 0.55$ vs. 0.14). Accordingly, Bray Curtis-based
 214 NMDS analyses of all canal water samples ($n = 52$) revealed strong clustering of bacterial

(Fig. 2G) and micro-eukaryotic (Fig. 2H) communities in relation to seasons, of which May and August had witnessed greater intra-season community variations along the canal. Seasonal variation was also observed for specific bacterial and micro-eukaryotic lineages (Fig. S2 and S3).

Microbiota composition and diversity changed along the canal

Both the source water from Danjiangkou reservoir (S01) and the canal water were dominated by phyla *Actinobacteria* (39.7 - 65.0%, mainly hgcI clade and CL500-29 marine group), *Proteobacteria* (17.9 - 38.3%), *Bacteroidetes* (0.9 - 29.5%), *Verrucomicrobia* (1.2 - 9.2%) and *Cyanobacteria* (0.2 - 8.6%, Fig. 3A). Overall, bacterial communities at the canal head (S02) were most structurally similar to the source water (Fig. 3A and Fig. S1A). However, compared with the source water, relative abundance of *Proteobacteria* at the canal head (i.e., S02) decreased whereas *Actinobacteria* and *Bacteroidetes* increased. Within the canal, both *Cyanobacteria* (mostly *Cyanobium_PCC-6307*, $F = 16.77$, $P < 0.001$) and *Bacteroidetes* ($F = 6.06$, $P < 0.05$) showed significant decrease in relative abundance with water flow (Fig. 3A). In contrast, *Limnohabitans* ($F = 19.87$) and *Candidatus Methylopusillus* ($F = 58.69$) within *Proteobacteria* became increasingly abundant along the canal ($P < 0.001$). Likewise, both the overall bacterial biomass (Fig. S4A) and Chao1 richness (Fig. S5A) exhibited highly dynamic variation patterns along the canal. The variation trend of Shannon diversity index largely agreed with (though relatively weaker than) that of Chao1 richness (Fig. S5B).

Unlike bacterial communities, micro-eukaryotic communities appeared more dynamic

235 along the canal water flow (Fig. 3B). The relative abundance of *Metazoa*, mostly *Calanoida*,
 236 was as high as 24.0% in Danjiangkou Reservoir. These organisms were drastically enriched
 237 by 2.3 times upon entering the canal head site, but largely declined in the downstream sites
 238 (S03 to S18). Two predominant lineages of *Cryptophyceae*, i.e., *Cryptomonas* ($F = 4.11$, $P =$
 239 0.06) and *Teleaulax* ($F = 9.50$, $P < 0.01$), were more abundant in the source water and canal
 240 upstream (S02-S08) than downstream sites (S09p-S14), accompanied by their gradual
 241 replacement by two uncultured *Cryptophyceae* lineages ($F = 30.53$, $P < 0.001$; $F = 24.60$, $P <$
 242 0.001 , respectively), especially close to the canal end (S15-S18). The canal head communities
 243 were distinct from water source and the downstream communities (Fig. 3B and Fig. S1B).
 244 This special pattern was manifested by the dominance of *Calanoida* (72.0%) at the canal head
 245 site where micro-eukaryotic biomass was much higher (3.2×10^9 vs. average 5.9×10^8 18S
 246 rRNA gene copies L^{-1} , Fig. S4B) whereas alpha diversity metrics much lower compared with
 247 the other sites (Fig. S6), suggesting intriguing local micro-eukaryotic selection in the canal
 248 head.

249 **Environmental factors influenced canal microbiota diversity and biomass**

250 The influence of five environmental parameters, namely water temperature, pH, dissolved
 251 oxygen (DO), total nitrogen (TN) and fluoride (Table S3), and spatial factors (i.e., ten PCNM
 252 variables representing the quantitative spatial patterns, Table S4) on the microbial
 253 communities was tested by redundancy analysis. All the above-mentioned environmental
 254 variables and PCNM1, 2 and 4 were identified to exert significant impacts on bacterial

community composition ($P < 0.05$, Fig. 4A). These variables except for PCNM4 also represented the significant influential factors for micro-eukaryotic communities ($P < 0.05$; Fig. 4B). Seasonal variation of environmental factors appeared based on the RDA plots (Fig. 4A and 4B), for instance, fluoride concentration was especially higher in March, which was supported by the significant seasonal difference in the environmental variables by Kruskal-Wallis one-way ANOVA (Table S5). The abundant actinobacterial hgcI clade ($r_s = 0.63$, $P < 0.01$) and CL500-29 ($r_s = -0.68$, $P < 0.01$) marine group were among the bacterial lineages positively and negatively related to fluoride concentration, respectively (Table S6). For micro-eukaryotes, for instance, an ASV affiliated to *Mediophyceae* ($r_s = 0.66$, $P < 0.01$) was positively correlated with fluoride (Table S7).

The identified significant variables were employed in further variation partitioning analysis (VPA). The result showed clearly stronger influence of seasonality than that of spatial and examined environmental factors for both bacterial and eukaryotic communities (Figure 4C and 4D). Seasonality alone could explain 19% and 17% of the bacterial and micro-eukaryotic community variance, respectively, while only 6 - 9% of the variance could be explained exclusively by environmental or spatial factors. Notably high proportions of the community variance could be attributed to the co-effects of seasonality and environmental factors (29% for bacteria and 23% for micro-eukaryotes), implying their strong interplay in shaping microbial communities. Besides, temperature, DO and TN were found to be significantly correlated with bacterial biomass, while only DO for micro-eukaryotic biomass (as measured by 16S rRNA and 18S rRNA gene copies, Table S8).

276 **Bacterial and micro-eukaryotic communities exhibited distinct and spatio-temporally**
 277 **dynamic stochasticity**

278 The apparent dispersal of microorganisms in the canal water and engineered hydraulic
 279 manipulations (i.e., inverted siphon, aqueduct, and tunnels) along the main canal drove us to
 280 question on the relative contribution of stochastic vs. deterministic processes to the canal
 281 bacterial and micro-eukaryotic community assembly, which was first quantitatively assessed
 282 with a recently proposed metric, normalized stochasticity ratio (NST) (Ning et al., 2019). This
 283 new metric quantifies the relative importance of stochasticity in governing community
 284 assembly ranging from 0 to 1, with a value > 0.5 indicating more stochastic assembly and <
 285 0.5 more deterministic assembly. NST analysis revealed contrasting trends between bacteria
 286 and micro-eukaryotes. In the upstream section, a highest NST of 0.78 was estimated for
 287 bacteria (Fig. 5A). This value decreased to 0.46 in the middle section, and to as low as 0.32
 288 close to the canal end. On the contrary, NST was generally above 0.5 for micro-eukaryotes
 289 throughout the canal, with the highest value of 0.75 in the middle section (Fig. 5A).

290 While NST provides rigorous quantification of relative importance of stochasticity, the
 291 Sloan neutral model analysis (Sloan et al., 2006) allows for assessing how well the observed
 292 community patterns could be explained by neutral processes (e.g., birth, death, and
 293 immigration) and attributing the observed community-level assembly pattern to the divergent
 294 population behavior. According to seasonal comparison of the model fitness, neutral model
 295 could explain much higher proportions of community patterns in October ($R^2 = 0.73$ and 0.67

for bacteria and micro-eukaryotes, respectively) and March ($R^2 = 0.77$ and 0.72), but lowest in May ($R^2 = 0.42$ and 0.13 , Fig. S7).

To link community patterns to the underlying population dynamics, bacterial and micro-eukaryotic ASVs were classified into three groups based on the neutral model analysis of the October samples. Depending on whether they empirically occurred more frequently than, as frequently as or less than predicted by the neutral model, ASVs were assigned to “above prediction”, “as predicted” and “below prediction” groups that were different in abundance and composition (Fig. 5B-D). We found that 7.8% of the bacterial community richness (i.e., number of ASVs) accounted by above-prediction group corresponded to 67.9% of the total number of sequences, which was dominated by *Actinobacteria* and *Proteobacteria*, whereas the neutrally distributed group contributed 86.8% of the richness but only 23.9% of the abundance (Fig. 5B and Fig. S8). Similar trend was detected for micro-eukaryotes (Fig. 5B). The three partitioning groups of ASVs also showed distinct taxonomic composition (Fig. 5C, 5D and Fig. S8). *Cryptomonas* constituted 13.7% of the above-prediction group in abundance, but they were almost absent in the neutral and below-prediction groups. *Metazoa*, in contrast, accounted for only 0.6% of the above-prediction group, but as high as 32.9% and 62.3% of the abundance of the neutral and below-prediction groups (Fig. 5D).

Selective growth of immigrating bacterial and micro-eukaryotic populations along the canal

To resolve which populations of the water communities died off, sustained, or proliferated

during dispersal over 1432-km canal, we proposed local growth factor (LGF) (see equation 1 in Methods) as a quantitative measure of variation in population abundance in each canal section compared to the abundance at the canal head, with an average LGF > 0 across the three sections indicating growth in the canal. LGF analysis was applied with the Oct sample dataset composed of 1389 bacterial and 1229 eukaryotic ASVs, using absolute abundance of ASVs estimated by multiplying relative abundance of ASVs at each canal site with the total copies of bacterial 16S rRNA (7.89×10^8 to 2.93×10^9 copies L⁻¹) or micro-eukaryotic 18S rRNA (3.62×10^8 to 3.19×10^9 copies L⁻¹) genes therein. Micro-eukaryotes showed higher overall occurrence (0.313 ± 0.008 vs. 0.216 ± 0.007 ; $P < 0.001$) while lower variability (2.879 ± 0.033 vs. 3.055 ± 0.032 ; $P < 0.001$) in absolute abundance compared to bacteria.

In total, 67.1% of the bacterial ASVs (Table S9) and 82.3% of the micro-eukaryotic ASVs (Table S10) displayed positive average LGFs. The ASVs were further observed to form clearly three clusters based on their LGFs in each section: LGF > 3, $-3 < \text{LGF} < 3$ and LGF < -3, which could indicate substantial growth, survival, and die-off of the microbial populations (Fig. 6). The survival group represented the largest proportion of the ASVs (45.6 - 60.2%), while only 4.1 - 21.7% of the ASVs went extinction in the canal. We also found that both the contrastingly high and low LGFs tended to be attributed to high-abundance microbial lineages (Fig. 6A and B). For example, the abundant *Luteolibacter* sp. (ASV106) and *Limnohabitans* sp. (ASV27) exhibited the highest LGF values in Section 2 and Section 3, respectively (Table S9). In stark contrast, ASV138 assigned to actinobacterial CL500-29 marine group and two *Cyanobium* PCC-6307 lineages (ASV144 and ASV250) were especially restrained in growth

close to the canal end (Table S9). A total of 53 bacterial ASVs in the canal were related to 16 genera included in a published reference pathogen list (Table S2, summarized from Woolhouse et al., 2015). While LGF of most (39 out of 53) of these potentially pathogenic ASVs fell between -3 and 3, three *Acinetobacter*-associated ASVs (ASV794, ASV1108 and ASV 1261) showed LGFs ranging from -5.9 to -6.1, indicating notable depletion along the canal. For micro-eukaryotes, two uncultured *Cryptophyceae* lineages (ASV54 and ASV80) as well as two ASVs related to *Acineta flava* (ASV224) and *Spumella* sp. (ASV59) were elevated throughout the canal (Table S10).

Discussion

Diversion canals widely built for reallocation of water resources represent an ideal engineered system to model microbial communities fulfilling key eco-environmental services, as well as reflecting changes in ecosystem structure and function. Based on quantitative metagenomic approaches that enable absolute quantification of microbial taxon, we uncovered the microbiota spatio-temporal dynamics, key processes driving community assembly, and navigation of immigrating microorganisms through the world's largest water diversion project.

Microbiota composition and biomass are dynamic along the canal

Both bacterial and micro-eukaryotic communities in the source water showed marked variations in taxonomic composition upon entering the canal, which could be attributed to the

356 readily comprehensible differences in the environmental conditions between the natural
357 reservoir and its receiving engineered canal and potential influences exerted by the approach
358 channel through which source water enters the canal. Meanwhile, we observed intriguingly
359 decreased water microbial diversity after water transportation through an inverted siphon (S09
360 vs. S09p, Fig. S5 and S6). Taken together, these suggest that hydraulic structures along the
361 canal apply considerable influence on the microbial community diversity and composition,
362 which needs to be fully considered when setting baseline communities for long-term
363 microbial monitoring in the water diversion canal.

364 The prevalence of uncultured actinobacterial hgcI clade and CL500-29 marine group
365 echoed what was previously reported in other freshwater habitats (Keshri et al., 2018; Yang et
366 al., 2017). While both groups of yet-to-be-cultured lineages have been shown to favor lower
367 level of dissolved organic carbon and temperature (Lindh et al., 2015), the hgcI clade was
368 additionally implicated to be competitively advantageous in utilizing low-concentration
369 organic carbon (Glöckner et al., 2000) and to harbor potentially photoheterotrophic species
370 showing preferential utilization of nitrogen-rich organic compounds in freshwater (Ghylin et
371 al., 2014), implying its potential role in the nutrient uptake and water auto-purification.

372 Unlike bacterial communities, micro-eukaryotic communities in the canal have not yet
373 been explored before. By virtue of the high-throughput molecular analyses, we detected not
374 only *Rotifera*, copepods and protozoa previously microscopically observed as abundant in the
375 Danjiangkou Reservoir (Li et al., 2012), but also thousands of micro-eukaryotic populations

376 (as ASVs), of which 6.7% are uncultured or unclassified even at the class level, implying
 377 detection of numerous novel populations in the canal water. Notably, metazoan *Calanoida*
 378 was repeatedly found to show drastic increase in abundance in the micro-eukaryotic
 379 communities of canal head, compared with in the source water in both October and August.
 380 This big order of aquatic crustaceans belonging to *Copepoda* inhabiting marine and
 381 freshwater environments are found as the most successful among zooplankton colonizing
 382 pelagic habitats (Blanco-Bercial et al., 2011; Bradford-Grieve, 2002). We further noted that
 383 the SAR clade and *Cryptophyceae* showed substantially decreased relative abundance as a
 384 result of high enrichment of *Calanoida* compared with the source water. However, their
 385 absolute abundance or biomass, as we estimated as their 16S rRNA gene copies, remained
 386 comparable (2.78 vs. 3.43×10^8 16S-copies L^{-1} for the SAR clade, and 3.68 vs. 4.25×10^8
 387 16S-copies L^{-1} for *Cryptophyceae*). This strong decoupling pattern between relative and
 388 absolute abundance cannot have been resolved by currently popular sequence-based relative
 389 measures alone which have been shown to provide evidently misleading information on the
 390 variation of absolute abundance (Stämmler et al., 2016; Vieira-Silva et al., 2019). It also well
 391 demonstrates the power of our quantitative metagenomics approaches as an alternative to a
 392 more direct but difficult-to-achieve quantitative PCR assay approach (due to the lack of 18S
 393 rRNA gene universal primers) for estimating total micro-eukaryotic biomass.

394 **Microbiota assembly is deterministically driven by seasonally dependent habitat** 395 **filtering effects**

396 Elucidating mechanisms and factors shaping biogeographic patterns has become a central
397 goal in microbial ecology (Hanson et al., 2012). Environment exerts influence on community
398 composition by selecting for populations that better adapted to the corresponding ecological
399 niches. We found that temperature, pH, dissolved oxygen, total nitrogen and fluoride all
400 showed significant effects on the community dynamics in the main canal. Fluoride affects
401 bacterial growth and metabolic activity through targeting enzyme inhibition (Liao et al., 2015).
402 Fluoride has been reported to alter microbial community in high-fluoride groundwater (Zhang
403 et al., 2019a) and to exhibit inhibitory effect on bacterial populations involved in various
404 wastewater treatment processes (Ochoa-Herrera et al., 2009). Our results showed that fluoride
405 with concentration falling within the range of 0.14-0.22 mg L⁻¹ (Table S3) could also affect
406 microbial community structure in a drinking water diversion system, where fluoride could be
407 mainly contributed by natural processes, such as mineral weathering (Nong et al., 2019), and
408 atmospheric pollutants (Pu et al., 2017). Interestingly, we found positive response of the hgcI
409 clade to elevated fluoride concentration (Table S6). While the mechanisms of their fluoride
410 resistance still await exploration, such evidence is in line with their favorable distribution in a
411 wide range of water ecosystems.

412 Although bacterial and micro-eukaryotic communities are both significantly influenced
413 by the examined environmental variables and spatial segregation, their overall variation were

414 explained more by seasonality. Meanwhile, the strong co-influence of temporal and
 415 environmental factors revealed by variation partitioning analysis could be at least partially
 416 explained by seasonal variation of the examined variables (Table S5), suggesting
 417 season-driven habitat filtering effects on microbiota. These results together imply a strong
 418 interplay between temporal, spatial and environmental factors on influencing microbial
 419 communities in the canal water, as previously noticed in natural lakes (Hu et al., 2017).
 420 Although potential contribution from unmeasured environmental factors cannot be ruled out,
 421 the remaining unexplained variation could possibly be generalized and linked to the influence
 422 of stochastic processes.

423 **Contrasting proportion of stochasticity contributes to bacterial and micro-eukaryotic** 424 **community assembly**

425 Microbial community assembly is typically governed by four processes: selection, drift,
 426 speciation and dispersal (Vellend and Agrawal, 2010). While selection is inherently
 427 deterministic and drift is stochastic, speciation and dispersal could involve both deterministic
 428 and stochastic mechanisms (Chase and Myers, 2011). For the MRP main canal, an
 429 investigation period of one year is probably short for resolving speciation. Thus, selection
 430 (deterministic processes), drift (stochastic processes) and dispersal (involving both) are
 431 expected to shape the observed community assembly patterns, and their relative contributions
 432 could be disentangled to help answer when, where, why and how much each process
 433 contribute to the observed patterns (Hanson et al., 2012).

434 Based on NST estimation and neutral modeling, we found that relative importance of
 435 stochastic and deterministic processes in microbial community assembly differ between
 436 bacteria and micro-eukaryotes, and among seasons and the canal sections. Neutral processes
 437 explained ~67% to 76% of the community patterns in October and March, but notably less in
 438 May (~41% and 13% for bacteria and eukaryotes, respectively). Liu et al. (2018a) reported a
 439 prominent shortage in water storage of Danjiangkou Reservoir related to seasonal flood
 440 control in May, which could have possibly imposed higher environmental selection effect to
 441 the microbiota in the reservoir and across the canal, making the observed decreased
 442 community stochasticity (or increased determinism) plausible. Although ecological
 443 stochasticity adds to challenge to predict microbial communities, the new knowledge
 444 produced in this study undoubtedly contribute to an improved understanding on when the
 445 canal water microbiota is more prone to stochastic processes, or under which conditions
 446 stochasticity is of more relevance.

447 The NST values suggested that the relative contribution of deterministic processes is
 448 larger to bacterial community assembly compared with micro-eukaryotic communities, where
 449 stochasticity prevails. Recently, Gweon et al. (2021) reported that stochastic processes played
 450 a more important role in the planktonic bacterial communities in the River Thames,
 451 presumably due to the influence of various natural and anthropogenic sources of the surface
 452 water. What we found showed that in a water ecosystem almost isolated from other sources
 453 than resource water, bacterial communities are predominantly shaped by deterministic abiotic
 454 and biotic processes. The micro-eukaryotes, however, possess structurally and functionally

455 more complex cells than prokaryotes (Massana and Logares, 2013), which could facilitate
 456 niche adaptation and diminish the environmental filtering effect at the community level. This
 457 was supported by our observation that the micro-eukaryotes were distributed with higher
 458 occurrence frequency and lower variability across the canal compared to bacteria. The
 459 different controlling mechanisms between bacterial and micro-eukaryotic community
 460 assembly found here has been observed in other natural habitats, such as Antarctic (Logares et
 461 al., 2018) and Tibetan lakes (Liu et al., 2020b). Our comparative analyses between bacteria
 462 and micro-eukaryotes across canal sections and over seasons thus provide first informative
 463 insights into the domain-specific, and space- and season-dependent patterns in the canal water
 464 community assembly.

465 **Divergent population lifestyles underly microbiota dynamics revealed by absolute** 466 **quantification**

467 A synthesis of population and community ecological perspectives can provide valuable
 468 insights into how microorganisms drive ecosystem processes (Koch et al., 2018). Microbial
 469 populations in this giant system displayed interestingly divergent lifestyles reflecting the
 470 difference in their adaptability to the canal environment. The Sloan neutral model sorted the
 471 microbial ASVs to above-prediction, below-prediction (jointly non-neutral) and as-predicted
 472 (neutral) groups, which were different in community composition (Fig. 5C and 5D),
 473 indicating taxonomically dependent competitive fitness in the investigated canal. In addition,
 474 we observed contrasting low (~20%) contribution of neutral group to community abundance

despite their high proportion in richness (~80%) for both bacteria and micro-eukaryotes (Fig. 5B). Chen et al. (2019) reported for micro-eukaryotic communities in a subtropical river as source of drinking water the dominance of neutral groups in both richness and abundance of the overall communities as well as across taxonomic groups. These contrasting patterns suggested that microorganisms are prone to more selective processes in an engineered canal compared with natural habitats.

In particular, we demonstrate the use of local growth factor (LGF) as a meaningful metric to infer growth of immigrating microbial taxa in the canal by employing absolute quantification of microbial populations. This leads to the first estimation that 67.1% of bacterial and 82.3% of micro-eukaryotic populations were capable of sustaining growth (LGF > 0), implying again better acclimation of eukaryotes to the environmental conditions in the canal. It is worth clarifying that the microbial growth estimated here was aimed to characterize fate of immigrating microorganisms across the canal, calculated based on relative fluctuation in absolute abundance at local sites compared with canal head, and thus is not equivalent to per-capita or population growth in the Sloan neutral model (Sloan et al., 2006). *Limnohabitans* sp., ubiquitous in a wide variety of freshwater habitats, showed remarkable growth across the canal. Members of *Limnohabitans* typically display high growth rate as well as metabolic flexibility, and they actively utilize alga-derived dissolved organic substances and are vulnerable to protist grazing (Horňák et al., 2017; Šimek et al., 2013; Šimek et al., 2011). *Cryptophyceae* were among the micro-eukaryotes that displayed prominent growth throughout the canal, which are small flagellates as important food of many

496 protists and micro-crustaceans (Horňák et al., 2017; Posch et al., 2015), and are perceived as
 497 characteristic of clean water (Lackey, 1941). Thus, *Limnohabitans* and *Cryptophyceae* are
 498 potentially important constituents of water food webs in the investigated canal. Two
 499 cyanobacterial lineages (*Cyanobum* PCC-6307) and *Acinetobacter* spp., on the contrary, were
 500 clearly depressed in the canal downstream sections, demonstrating the elimination of these
 501 microorganisms of water security concern along the canal. *Acinetobacter* spp. are ubiquitous
 502 in nature of major ecological and clinical significance, featuring, for instance,
 503 multi-antimicrobial resistance and rapid resistance development (Jung and Park, 2015). The
 504 diminishing of the versatile *Acinetobacter* spp. along the canal could possibly be attributed to
 505 unfavorable environmental conditions (Al Atrouni et al., 2016) or composite intra- and
 506 inter-domain biological interactions. Further research efforts with genome-centric
 507 metagenomic approaches are needed to elaborate the mechanisms behind such die-off patterns.
 508 It is noteworthy that due to the restriction in taxonomic resolution of the ribosomal RNA gene
 509 sequencing analysis employed in this study, species-level confirmation is still needed to
 510 evaluate the relevance of these potentially pathogenic microorganisms. Together, our results
 511 demonstrated the power of local growth factor to identify key players in the investigated
 512 system and facilitate predicting fate of the microbes traversing such thousand-kilometer water
 513 diversion canal.

514 **Conclusions**

515 The main canal of the Middle Route Project of the South-to-North Water Diversion projects is

516 an engineered system where the aquatic ecosystem is still at its early forming stage (since
517 2014). Various aspects of hydrology, geochemistry and ecology need to be intricately
518 elaborated to jointly contribute to sustained system's eco-environmental services, especially
519 water auto-purification ensuring its quality and competence for designed social-economic
520 aims. The present study uncovered the underlying microbial community assembly
521 mechanisms and population dynamics in a giant engineered water diversion water canal, such
522 as the sharp decline of potentially harm-producing microbes (e.g., *Cyanobacteria*) and
523 potential pathogens (e.g., *Acinetobacter* spp.), and the favorable enrichment of alga-derived
524 organic scavengers (e.g., *Limnohabitans*) as potential key microbial food-web players,
525 demonstrating the valuable insights provided by microbial community and population
526 assessments for water monitoring and their potential in indicating ecosystem disturbance.
527 Furthermore, the bacterial community shift driven by combined environmental effects as well
528 as the stochasticity in micro-eukaryotic community assembly needs to be considered in water
529 quality monitoring along the canal, towards an appropriate microbe-based biomonitoring
530 which enables deeper understanding of processes behind water quality parameters.
531 Considering possibly occasional pollution into the canal water caused by, for example, traffic
532 accidents on highway bridges across the canal, and the central roles microorganisms play in
533 the water quality maintenance, future efforts are needed to explore the metabolic functions of
534 microbial community contributing to water purification (e.g., carbon, nitrogen, and
535 phosphorus removal) during diversion and sustaining water quality.

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757

758 **Figure Legends**

759 **Fig. 1** Four sampling campaigns at 19 sites along the 1432-km main canal of the MRP of the
760 SNWD project selected for microbial and physicochemical analyses. Colored fans indicate
761 months of sampling at each site. The map was created using QGIS (version 3.10.5,
762 www.qgis.org).

763 **Fig. 2** Seasonal variation of bacterial and micro-eukaryotic communities in the canal. (A-F)
764 Ribosomal RNA gene copies (A and B), Chao1 richness (C and D) and Shannon diversity
765 index (E and F) across seasons. Different letters above boxes indicate statistically significant
766 differences between months of sampling (Mann-Whitney *U* test with the Benjamini-Hochberg
767 correction, $P < 0.05$). (G-H) Ordination analysis of communities using non-metric
768 multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities. Trajectories indicate
769 locations of sampling sites along the canal.

770 **Fig. 3** Microbial community composition and diversity along the MRP main canal (~1432 km)
771 of the SNWD project in October 2018. (A) Bacterial composition with abundant phyla (> 1%)
772 and 10 most abundant genera shown. (B) Micro-eukaryotic composition with abundant phyla
773 (> 1%) and 10 most abundant orders shown.

774 **Fig. 4** Redundancy analysis (RDA) and variation partitioning analysis (VPA) demonstrating
775 the relative influence of seasonality, spatial and environmental factors on the canal microbial
776 communities. The RDA biplots show factors impacting bacterial (A) and micro-eukaryotic (B)
777 communities. The VPA diagrams show the percentage contribution of seasonal, spatial and

778 environmental factors to bacterial (C) and micro-eukaryotic (D) community variations. Only
 779 those factors identified to significantly influence bacterial and micro-eukaryotic communities
 780 are shown in the RDA and included in the VPA analyses. PCNMs, geographic factors
 781 generated by principal coordinate analysis of neighborhood matrices. T, temperature; F,
 782 fluoride; TN, total nitrogen; DO, dissolved oxygen.

783 **Fig. 5** Normalized stochasticity ratio (NST) estimation and microbiota partitioning by
 784 population compliance to the neutral model prediction. (A) NST estimated stochasticity in
 785 bacterial and micro-eukaryotic community assembly. (B) Proportions in richness (ASV
 786 number) and abundance (sequence number) of the three fitting groups (occurrence frequency
 787 above prediction, as predicted and below prediction) within the microbial communities to the
 788 Sloan neutral model. (C and D) Taxonomic composition of the three fitting partitions in
 789 richness (Ri) and abundance (Ab) for bacterial (C) and micro-eukaryotic (D) communities.
 790 Only abundant bacterial phyla (> 1%) and 10 most abundant micro-eukaryotic orders are
 791 shown.

792 **Fig. 6** Microbial population proliferation across canal sections evaluated by local growth
 793 factor analysis. Distribution patterns of population local growth factors in the different canal
 794 sections are shown with violin plots for bacteria (A) and micro-eukaryotes (B). Each dot
 795 represents an ASV with colors indicating its average abundance across the canal.

796

Bacteria

Micro-eukaryotes

(A)

(B)

(A) **(B)** **(C)** **(D)**

(A)

(B)