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1 **Prospects for Multi-omics in the Microbial Ecology of Water Engineering**

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28 **ABSTRACT**

29 Advances in high-throughput sequencing technologies and bioinformatics approaches over
30 almost the last three decades have substantially increased our ability to explore
31 microorganisms and their functions – including those that have yet to be cultivated in pure
32 isolation. Genome-resolved metagenomic approaches have enabled linking powerful
33 functional predictions to specific taxonomical groups with increasing fidelity. Additionally,
34 related developments in both whole community gene expression surveys and metabolite
35 profiling have permitted for direct surveys of community-scale functions in specific
36 environmental settings. These advances have allowed for a shift in microbiome science away
37 from descriptive studies and towards mechanistic and predictive frameworks for designing and
38 harnessing microbial communities for desired beneficial outcomes. Water engineers,
39 microbiologists, and microbial ecologists studying activated sludge, anaerobic digestion, and
40 drinking water distribution systems have applied various (meta)omics techniques for
41 connecting microbial community dynamics and physiologies to overall process parameters
42 and system performance. However, the rapid pace at which new omics-based approaches
43 are developed can appear daunting to those looking to apply these state-of-the-art practices
44 for the first time. Here, we review how modern genome-resolved metagenomic approaches
45 have been applied to a variety of water engineering applications from lab-scale bioreactors to
46 full-scale systems. We describe integrated omics analysis across engineered water systems
47 and the foundations for pairing these insights with modeling approaches. Lastly, we
48 summarize emerging omics-based technologies that we believe will be powerful tools for water
49 engineering applications. Overall, we provide a framework for microbial ecologists specializing
50 in water engineering to apply cutting-edge omics approaches to their research questions to
51 achieve novel functional insights. Successful adoption of predictive frameworks in engineered
52 water systems could enable more economically and environmentally sustainable
53 bioprocesses as demand for water and energy resources increases.

54

55 **KEYWORDS**

56 *microbial ecology, metagenomics, metatranscriptomics, metaproteomics, metabolomics,*
57 *metabolic modelling, water engineering*

58

59 **INTRODUCTION**

60 Microorganisms and complex microbial communities are an integral and essential component
61 of engineered water systems designed to improve public health, maintain ecosystem health,
62 and provide optimal water infrastructure and quality. Activated sludge (AS), anaerobic
63 digestion (AD), and drinking water systems (DWSs) are all designed to harness or manage
64 microbial communities for desired process outcomes (**Figure 1**). AS systems are designed to
65 enrich for specific microbial lineages to remove nutrients, mostly organic carbon, phosphorus,
66 and nitrogen species to prevent downstream receiving water eutrophication (Ardern and
67 Lockett, 1914). AD processes are applied to various organic waste streams to recover biogas
68 and other valuable products as a source of renewable energy (Angelidaki et al., 2011;
69 Weiland, 2010). While biofilms in drinking water treatment plants (DWTPs) can leverage
70 microbial communities for pollutant removal (Kirisits et al., 2019), DWSs apply
71 physicochemical approaches to minimize microbial abundance in tap water (Berry et al.,
72 2006). All of these systems are the foundation of modern urban water infrastructure, and are
73 intertwined through their dependence on adequately managing different microbial
74 communities through a water-microbiome continuum (Raskin and Nielsen, 2019). As many of
75 the key microorganisms underpinning these water cycles remain uncultured to date (Lloyd et
76 al., 2018; Steen et al., 2019), our understanding of the microbial lineages involved in these
77 engineered ecosystems have largely relied on cultivation-independent approaches.

78 Tools for characterizing the structure and function of microbial communities have
79 greatly expanded since the development of methods for surveying uncultivated
80 microorganisms based on the use of the 16S ribosomal RNA (rRNA) gene (Fox et al., 1980;
81 Lane et al., 1986; Woese and Fox, 1977). Building on the identification of specific 16S rRNA
82 gene sequences, uncultivated microorganisms could be directly visualized using fluorescently
83 labelled oligodeoxynucleotide probes (DeLong et al., 1989) or microautoradiography

84 (Giovannoni et al., 1988). Although advances in high-throughput sequencing technologies
85 have allowed for large-scale 16S rRNA gene amplicon surveys instead of constructing labor-
86 intensive clone libraries (Sogin et al., 2006; Caporaso et al., 2012; Kozich et al., 2013;
87 Thompson et al., 2017), operationally defined taxonomical units cannot be linked to functional
88 capabilities in mixed communities with fidelity, especially for poorly characterized lineages.
89 Metagenomics comprises a group of methods for solving this problem. The term metagenome
90 is defined as the entire genetic content of the collective microbial genomes contained within a
91 given environmental sample (Handelsman et al., 2002, 1998). Early metagenomic approaches
92 cloned fragments of extracted microbial DNA into a host backbone (e.g., *E. coli*) to screen for
93 particular sequences (Beja et al., 2000; Henne et al., 1999), functionally characterize novel
94 enzymes through heterologous expression systems (Brady and Clardy, 2000; Rondon et al.,
95 2000), or perform shotgun sequencing (Tyson et al., 2004; Venter et al., 2004). All of these
96 approaches have the potential to link functional observations to phylogenetic identity, and
97 therefore provide a powerful approach to characterize the ecology and evolution of
98 uncultivated microorganisms.

99 Metagenomic sequencing approaches can be defined under two categories, read-
100 based and assembly-based. Read-based metagenomic approaches map the resulting reads
101 back to reference genomes to assess the taxonomic composition and functional potential of a
102 mixed microbial sample (Quince et al., 2017b; Sharpton, 2014). However, read-based
103 approaches heavily rely on reference databases, and therefore under-explored environments
104 such as engineered water systems may not be well-represented in these databases.
105 Additionally, this approach cannot directly link specific functions to individual microbial guilds
106 or populations. Assembly-based approaches such as genome-resolved metagenomics extract
107 individual genomes *de novo* from a mixed community through a process termed binning of
108 assembled contigs or scaffolds (Albertsen et al., 2013; Tyson et al., 2004). The resulting
109 genomes can be termed draft bins, metagenome-assembled genomes (MAGs), and/or
110 population genomes, as they most often represent a mixed consensus sequence of closely
111 related microbial cells (Bowers et al., 2017). These genomes can then be used as a foundation

112 for integrated multi-omics analyses using insights from metatranscriptomics, metaproteomics,
113 and/or metabolomics that attempt to directly tie metabolic guilds and functional activities to
114 specific microorganisms (Anantharaman et al., 2016; Woodcroft et al., 2018; Wrighton et al.,
115 2012).

116 In recent years, there have been calls for microbiome science as a whole to shift away
117 from mere descriptive studies and towards more mechanistic and predictive frameworks for
118 designing and harnessing diverse microbial communities for desired beneficial outcomes
119 (Alivisatos et al., 2015; Lawson et al., 2019). Successful adoption of predictive frameworks in
120 engineered water systems could enable more economically and environmentally sustainable
121 bioprocesses as demand for water and energy resources increases (Raskin and Nielsen,
122 2019; Sheik et al., 2014). To achieve this, an interdisciplinary approach combining theoretical
123 microbial ecology and engineering principles must be applied. Genome-resolved
124 metagenomic approaches have been widely adopted in water engineering applications to
125 explore the diversity, metabolic potential, and spatiotemporal dynamics of microorganisms
126 inhabiting water systems. Integrating multi-omics approaches within an eco-systems biology
127 framework is a promising approach for creating more predictable, controllable biotechnology
128 applications for water microbiomes (Narayanasamy et al., 2015).

129 Here, we review existing work and prospects for multi-omics applications in engineered
130 water systems. We summarize pioneering work in genome-resolved metagenomic
131 approaches over the last two decades in AS, AD, and DWDS. We then describe how
132 mechanistic modeling and time-series forecasting of microorganisms in water systems can
133 integrate genome-resolved metagenomics to enhance their predictive capabilities. Finally, we
134 highlight emerging omics technologies that will pave the way for remarkable advances not
135 only in water systems but microbiome science as a whole.

136

137 **1. GENOME-RESOLVED METAGENOMIC APPROACHES FOR UNCOVERING**

138 **PRINCIPLES OF MICROBIAL ECOLOGY IN WATER SYSTEMS**

139 Numerous molecular methods have been applied to engineered water systems to explore the
140 diversity and ecophysiology of microorganisms, including 16S rRNA gene amplicon
141 sequencing and *in situ* hybridization approaches. However, these approaches alone are
142 fundamentally limited in their ability to connect structure-to-function for any natural microbial
143 community. Here, we review how early applications of molecular approaches have provided
144 valuable insights into the microbial ecology of water systems. We then broadly summarize
145 genome-resolved metagenomic approaches that span the methodologies of metagenomic
146 binning, metatranscriptomics, and metaproteomics, and highlight their use in water
147 engineering applications.

148

149 **1A. Early Insights from Molecular Approaches**

150 The demonstrable success of molecular biology in the 1970s-80s in developing new
151 methods for isolating, sequencing, and manipulating nucleic acid sequences naturally led
152 microbial ecologists to apply these techniques to identifying uncultivated microbes in
153 environmental samples (see extensive contemporary reviews in Amann et al., 1995; Olsen et
154 al., 1986). Once the small subunit rRNA was identified as a suitable marker gene for
155 phylogenetic analysis of taxa (Woese and Fox, 1977), several landmark studies demonstrated
156 the feasibility of surveying the composition of natural microbial communities through
157 comparing sequences of clone libraries and amplicons (Giovannoni et al., 1990; Schmidt et
158 al., 1991). The utility of 16S rRNA gene profiling increased dramatically with the development
159 of second-generation sequencing technologies initially with Roche 454 and subsequently
160 Illumina, along with the development of related bioinformatics workflows (Bolyen et al., 2019;
161 Callahan et al., 2016; Schloss et al., 2009). 16S rRNA gene amplicon sequencing surveys
162 have been applied to infer the diversity and dynamics of specific microbial populations in full-
163 scale WWTP configurations over space and time, revealing contributions of core microbiome
164 members and rare taxa in these systems (Lawson et al., 2015; Saunders et al., 2016; Wu et
165 al., 2019; Zhang et al., 2012). Based on 16S rRNA gene sequencing surveys, the Microbial
166 Database for Activated Sludge (MiDAS) field guide was developed to connect functional

167 information to manually curated taxonomical assignments for abundant microbial lineages in
168 activated sludge (McIlroy et al., 2015), and was further updated to include anaerobic digestion
169 systems (McIlroy et al., 2017).

170 The advent of 16S rRNA gene sequencing led to using radioactively-labeled
171 oligonucleotide probes binding to rRNA structures to quantify the abundance of specific
172 microbial populations (Giovannoni et al., 1988; Stahl et al., 1988). Panels of oligonucleotide
173 probes were constructed for major subclasses of *Proteobacteria* and specific members of the
174 Cytophaga-Flavobacter-Bacteroidetes (CFB) supergroup (Manz et al., 1996, 1992).
175 Development of group-specific hybridization probes allowed for quantifying the relative
176 abundance of methanogens in anaerobic digestion systems (Raskin et al., 1994a, 1994b),
177 which were subsequently used to connect population dynamics to process performance
178 parameters (Griffin et al., 1998; McMahon et al., 2001). Modifications to these methods
179 including combining fluorescent *in situ* hybridization (FISH) with rRNA-targeted
180 oligonucleotide probes and adding radioactively-labeled substrates through
181 microautoradiography to visualize spatial co-localization and metabolic activities of microbial
182 communities (Lee et al., 1999). These methods were particularly appealing for studying the
183 dense, biofilm-like floc aggregates characteristic of activated sludge systems, such as
184 quantifying and localizing filamentous and nitrifying bacteria within individual flocs (Mobarry et
185 al., 1996; Wagner et al., 1994a). Subsequently, FISH-based methods combined with isotopes
186 were used for understanding *in situ* activities (Amann, 1995; Wagner and Haider, 2012), and
187 could also be used for verifying metabolic potentials of microorganisms made through
188 hypotheses with reconstructed genomes (McIlroy et al., 2016).

189 Although both 16S rRNA gene sequencing and oligonucleotide probe applications
190 have provided valuable insights into engineered water systems and continue to be used for
191 novel insights, they carry significant limitations. 16S rRNA gene amplicon sequencing can be
192 used as a high-throughput and low-cost tool to characterize microbial community dynamics
193 through time and space; however, associated functional predictions cannot be captured *a*
194 *priori* using this approach. Additionally, specific microbial traits underlying key biogeochemical

195 transformations have been shown to be horizontally transferred and therefore not easily
196 inferred from 16S rRNA gene identity (Anantharaman et al., 2018; Lawson and Lückner, 2018;
197 McDaniel et al., 2020; Palomo et al., 2018; Parsons et al., 2020). The analysis of 16S rRNA
198 gene amplicons remains complex due to conceptual difficulties surrounding the definition of
199 OTUs (Edgar, 2018; Stackebrandt and Goebel, 1994), the propensity of OTU-clustering to
200 generate misleading artifacts, limitations of cross-referencing short, noisy amplicon
201 sequences across multiple studies, and the challenges of distinguishing closely related strains
202 harboring similar 16S rRNA gene sequences. Some of these limitations have been addressed
203 by constructing ecosystem-specific databases that can resolve amplicons at the species level
204 due to using full-length 16S rRNA genes from long-read sequencing technologies (Nierychlo
205 et al., 2020).

206 Read-based metagenomic approaches were initially used in engineered water
207 systems to compare *in situ* species to existing reference genomes and measure the extent of
208 similarity and population heterogeneity (Albertsen et al., 2011; MacIlroy et al., 2013). However,
209 read-based metagenomic approaches depend on comprehensive reference databases to
210 ensure accuracy of results. Although improvements in metagenomic sequencing efforts have
211 increased the amount of reference genomes from environmental ecosystems (Nayfach et al.,
212 2020; Parks et al., 2017), the fraction of high-quality genomes from engineered water systems
213 still remains small (Hull et al., 2019; Nayfach et al., 2020). Therefore, substantial efforts are
214 still needed to construct high-quality reference databases of genomes from anaerobic
215 digestion, activated sludge, and drinking water system microbiomes. Although thorough
216 methodological reviews and best practices in metagenomics exist elsewhere (Dick, 2018;
217 Knight et al., 2018; Quince et al., 2017b), we provide brief overviews of existing and newly
218 developed workflows and toolkits in genome-resolved omics to provide water engineers and
219 scientists with the most recent advances and guidelines in a field subject to very rapid
220 development. Lastly, we outline current technologies and approaches for applying integrated
221 multi-omics approaches to explore the dynamics and activities of microbial communities
222 underpinning engineered water systems.

223

224 **1B. Genome-Resolved Metagenomics: From Individual Genomes to Large-Scale**
225 **Reference Databases**

226 Genome-resolved metagenomic approaches apply *de novo* assembly to extract
227 representative population genomes from a given system through metagenomic binning, and
228 subsequently use draft bins with multi-omics approaches to gain functional insights (**Figure**
229 **2**). The first objective of any genome-resolve approach is to assemble the representative
230 members of a given community into MAGs. This process is termed “binning”, in which short
231 reads are *de novo* assembled into longer contigs, with groups of contigs hypothesized to arise
232 from the same genomic context subsequently clustered based on sequence composition,
233 differential coverage across time and/or space, or a combination of both parameters (**Figure**
234 **3**) (Albertsen et al., 2013; Alneberg et al., 2014; Dick et al., 2009; Sharon et al., 2013). These
235 groups of “binned” contigs can be considered as working models of whole genomes, also
236 referred to as population genomes, as the phylogenetic composition of assembled bins can
237 be operationally defined by the minimum sequence identity of reads mapping back to
238 assembled contigs (Jain et al., 2018; Olm et al., 2020). For example, bins are clustered and/or
239 dereplicated at the 95% sequence identity threshold, and therefore a bin representing an
240 “individual species” may actually be composed of multiple, closely-related strains that could
241 not be assembled individually due to computational constraints (Jain et al., 2018; Olm et al.,
242 2017). Draft bins can then be curated to verify uniform read coverage of all contigs
243 (Bornemann et al., 2020; Eren et al., 2015), estimate approximate genome completion and
244 contamination estimates by detecting universal single-copy genes (Parks et al., 2015; Seppey
245 et al., 2019), dereplicate redundant sets of bins resulting from the use of binning results from
246 multiple assemblies or binning algorithms (Olm et al., 2017; Sieber et al., 2018), or manually
247 scaffold bins to close gaps and potentially recover completely circular MAGs (Chen et al.,
248 2020; Lui et al., 2020).

249 After curation, taxonomical assignments and functional annotations are performed on
250 all MAGs to connect structure to potential function. Currently, two main approaches exist for

251 assigning taxonomy to draft genomes; an automated approach based on whole-genome
252 sequence similarity against publicly available genomes (Chaumeil et al., 2019; Parks et al.,
253 2018), and approaches based on clustering of ribosomal protein sequences followed by
254 phylogenetic reconstruction with select reference genomes (Hug et al., 2016; Lee, 2019;
255 McDaniel et al., 2019), both achieved through concatenating alignments of single copy marker
256 genes. After assigning taxonomical classifications to genomes, specific metabolic pathways
257 can be investigated based on gene presence and/or absence. Numerous pipelines exist for
258 assigning functional annotations to draft genomes, and mostly differ in the underlying
259 database(s) from which they draw functional annotations (Aramaki et al., 2019; Hanson et al.,
260 2014; Seemann, 2014). Recently, several pipelines have been developed for exploring
261 specific metabolic guilds within MAG datasets based on curated databases or marker profiles
262 (McDaniel et al., 2019; Neely et al., 2020; Shaffer et al., 2020; Zhou et al., 2019). Furthermore,
263 ecological and evolutionary insights can be made through comparative genomics approaches,
264 such as hypotheses of niche differentiation based on gene content (Camejo et al., 2017;
265 Flowers et al., 2009; Koch et al., 2015; Oyserman et al., 2016a; Skennerton et al., 2015; Speth
266 et al., 2012), or analyzing microdiversity and population heterogeneity (Albertsen et al., 2011;
267 Leventhal et al., 2018).

268 Some of the first demonstrations of genome-resolved metagenomics are from
269 laboratory-scale wastewater enrichment bioreactors. The first representative genome of the
270 polyphosphate accumulating organism (PAO) '*Candidatus Accumulibacter phosphatis*' UW-1
271 was assembled from American and Australian enrichment bioreactors (Martín et al., 2006).
272 Remarkably, both bioreactors happened to be enriched with similar *Accumulibacter*
273 populations at the time of sequencing. Genomes of multiple *Ca. Accumulibacter* strains have
274 since been assembled to date (Albertsen et al., 2016; Camejo et al., 2019; Flowers et al.,
275 2013; Gao et al., 2019; Mao et al., 2014; Qiu et al., 2020; Skennerton et al., 2015), including
276 high-quality genomes assembled from full-scale WWTPs (Law et al., 2016; Srinivasan et al.,
277 2019), even though it remains to be isolated in pure-culture. The uncultivated bacterium
278 *Kuenenia stuttgartiensis* (Strous et al., 2006) underlying anaerobic ammonium oxidation

279 (anammox) was assembled from a complex bioreactor community, and subsequently other
280 anammox species within the *Planctomycetes* have been assembled (Lawson et al., 2017;
281 Speth et al., 2016). The genome of the nitrite-oxidizing bacterium '*Candidatus Nitrospira*
282 *defluvii*' was assembled from an activated sludge enrichment bioreactor (Lücker et al., 2010),
283 followed by other *Nitrospira* and *Nitrospina* species (Koch et al., 2015; Lücker et al., 2013).
284 Remarkably, complete ammonium oxidation (comammox) was discovered by two groups
285 through enrichment of *Nitrospira inopinata* from the pipe of a deep oil exploration well and two
286 distinct *Nitrospira* species from a recirculation aquaculture system biofilter, respectively
287 (Daims et al., 2015; Van Kessel et al., 2015).

288 Before the development of automatic binning methods, reconstruction of genomes
289 from uncultured microbial lineages within a mixed population was mostly limited to a few
290 abundant members to study their metabolism in detail, as the assembly and curation of any
291 single MAG relied on largely manual approaches (Tyson et al., 2004). Albertsen et al. first
292 demonstrated that MAGs of low abundant microorganisms within an activated sludge
293 enrichment bioreactor could be captured using differential-coverage and composition based
294 binning (Albertsen et al., 2013). Since then, numerous automatic binning algorithms that
295 implement differential coverage profiles in addition to sequence composition signatures have
296 been developed, which include the programs CONCOCT (Alneberg et al., 2014), MetaBAT2
297 (Kang et al., 2015) and MaxBin (Wu et al., 2014).

298 Genome-resolved metagenomic surveys now routinely consist of aiming to assemble
299 all representative species within a community for which medium- to high-quality MAGs can be
300 reconstructed (Anantharaman et al., 2016; Bowers et al., 2017; Woodcroft et al., 2018).
301 Recent examples of whole-community genome-resolved metagenomic analyses in
302 engineered water systems include metagenomic assembly of *Ca. Accumulibacter* and 39 non-
303 PAO "flanking" community members in a denitrifying enrichment bioreactor (Gao et al., 2019),
304 investigating the impact of disinfection on microbial community structure and metabolism in a
305 full-scale *drinking water distribution system* (DWDS) (Dai et al., 2019; Sevillano et al., 2020),
306 nitrogen metabolism in drinking water reservoirs (Potgieter et al., 2020), and tracking the

307 microbial community through a pilot plant for potable water reuse of wastewater (Kantor et al.,
308 2019). Representative genomes from full-scale systems have only recently been constructed
309 through large-scale sequencing efforts and curation of system-specific MAG databases, such
310 as the collection of 1,600 MAGs from anaerobic digestion biogas reactors (Campanaro et al.,
311 2020) and 2,045 MAGs assembled from a collection of 114 activated sludge full-scale WWTPs
312 (Ye et al., 2020).

313 The use of MAG-based methods has allowed for the recovery of a large number of
314 genomes from the uncultivated majority, however, current methods have several significant
315 limitations. The most significant relates to the fractionated nature of MAGs assembled from
316 short read data, which is a consequence of the limited read length and the inability to
317 reconstruct regions containing complex repeat structures or share relatively high similarity
318 between sub-species or strains, such as in ribosomal RNA operons (Chen et al., 2020; Meziti
319 et al., 2021). This results in genomes that are comprised between ten to hundreds of contigs,
320 which can complicate the accurate estimation of genome completeness and contamination
321 (Orakov et al., 2021). Furthermore, recovering genomes of individual strains from complex
322 communities still remains a significant limitation of current methods, despite the highly
323 sophisticated bioinformatics algorithms that have been developed for this problem (Olm et al.,
324 2021; Quince et al., 2017a; Segata et al., 2012). All of these limitations are particularly acute
325 in highly complex microbial communities such as those inhabiting full-scale activated sludge
326 and anaerobic digester communities. According to the Minimum Information about a
327 Metagenome-Assembled Genome (MIMAG), a high-quality MAG draft contains all three
328 ribosomal rRNA genes, at least 18 tRNAs, and completion of >90% and contamination <5%
329 calculated by the presence of single-copy marker genes (Bowers et al., 2017; Parks *et al.*,
330 2015). A recent large-scale assembly and binning effort of publicly available metagenomes in
331 the Integrated Microbial Genomes (IMG) system recovered less than 20% high-quality MAGs
332 in the ~52,000 MAG (Nayfach et al., 2020). Approximately 3,400 of these MAGs were
333 recovered from engineered water systems (**Figure 4**), and demonstrate that the majority of
334 these MAGs are of medium-quality according to MIMAG standards. Although a large portion

335 of these MAGs contain >90% completeness and <5% redundancy, they do not meet the high-
336 quality standards due to missing rRNA and/or tRNA genes. As discussed below, obtaining
337 high-quality MAGs is essential for ensuring accuracy of functional hypotheses and further
338 validating these hypotheses with experiments. Using composite MAGs of medium-quality or
339 low-quality can lead to inaccurate ecological inferences due to either inflating relative
340 abundance calculations or missing genes conferring key biogeochemical transformations
341 (Shaiber and Eren, 2019).

342 At the time of writing, there are several near-automated workflows available for
343 recovery and evaluation of MAGs from metagenome data, for example, using MetaWRAP
344 (Uritskiy *et al.*, 2018) and the MAG annotation workflow MAGPy (Stewart *et al.*, 2019). The
345 anvi'o platform, an open source data analysis and visualization system, is another major
346 development for enabling MAG based analyses, including multi-omics data (Eren *et al.*,
347 2015). Such analyses can be greatly facilitated by the use so-called containerized
348 computational workflows, such as Snakemake (Koster and Rahmann, 2012) and Nextflow
349 (<https://www.nextflow.io>), which enable portability across systems, extensibility and
350 reproducible research practice. We also highlight the emerging role that machine learning
351 methods have in MAG recovery and characterization, based on several recent publications
352 (Ye *et al.*, 2020; Nissen *et al.*, 2021).

353 In summary, MAG recovery from short read metagenomic data, while currently is
354 routinely and relatively easily applied, is still subject to many caveats and substantive
355 limitations.

356

357 **1C. Integrated Multi-omics Approaches in Water Systems**

358 Integrated multi-omics analyses consist of applying genome-resolved metagenomics in
359 combination with one or more additional omics-based approaches, such as
360 metatranscriptomics, metaproteomics, and/or metabolomics (**Figure 2**). Although each
361 integrated approach can enhance our understanding beyond obtaining genomic sequences
362 alone, these methodologies are currently less developed than metagenomic binning

363 workflows, and may require more specific knowledge bases as well as system-specific
364 methodological development to be performed effectively. It is important to note that in the case
365 of metatranscriptomics and metaproteomics, the acquired data can be referenced directly
366 back to recovered genome sequences. However, this is not the case for metabolomic data,
367 which requires known or putative metabolic reaction pathways to be known, or predicted, in
368 order to link detected compounds with the corresponding genes that encode cognate enzymes
369 for producing, or utilizing, these compounds.

370 Therefore, in both genome-resolved metatranscriptomics and metaproteomics
371 approaches, short cDNA reads or peptide sequences are competitively mapped against a
372 collection of genomes to identify which genes are actually being expressed and to provide
373 estimates of relative expression levels (Shakya et al., 2019; Wilmes and Bond, 2009;
374 Woodcroft et al., 2018). Successfully conducting metatranscriptomics and metaproteomics is
375 substantially more complex than encountered in metagenomics. The extraction and handling
376 of RNA and protein are complicated by the fact that both molecules are far less stable than
377 DNA (Moran et al., 2013). Furthermore, because approximately 80-90% of the RNA in a given
378 cell is mostly ribosomal RNA (Westermann et al., 2012), then the remaining ~10-20% fraction
379 of the reads from total RNA sequencing are from protein-coding genes. At the whole
380 community level, this can result in noise-effected or false-negative calls for genes in all but the
381 most abundant genomes. Accordingly, rRNA-depletion procedures are best applied following
382 the extraction of total RNA (Culviner et al., 2020; He et al., 2010b; Stewart et al., 2010).
383 Collectively, these issues require complex bench workflows, requiring a high degree of
384 molecular biological knowledge and skill. At the data analysis level, because sequencing-
385 based gene expression estimates are generally relative, it can be challenging to understand
386 the relationship between changes in gene expression and fluctuations in taxon abundance.
387 Therefore, co-extraction of DNA, RNA, and/or protein from the same aliquot is recommended,
388 combined with recovery of MAGs from the DNA fraction, with the aim of providing accurate
389 reference sequences to support the analysis and interpretation of the corresponding RNA-Seq
390 data (Roume et al., 2013a, 2013b).

391 While in the case of AS and AD samples, biomass concentration is high enough that
392 extraction procedures will yield adequate amounts of nucleic acid, in the case of DWDS, the
393 low biomass concentration requires either size selection methods and/or DNA amplification
394 procedures to sufficient concentration and amount to initiate extraction procedures (Kantor
395 et al., 2019; Vosloo *et al.* 2021)

396 Traditionally, metatranscriptomics analyses employ either gene-centric or genome-
397 centric approaches, both of which depend on identifying highly, differentially, or co-expressed
398 genes (Shakya et al., 2019). Gene expression dynamics of *Accumulibacter* were first
399 elucidated using oligonucleotide microarrays (He et al., 2010a), with a more detailed metabolic
400 reconstruction of this lineage generated using time-series RNA sequencing (Oyserman et al.,
401 2016b), and ecological roles for co-existing *Accumulibacter* clades inferred through
402 comparative genome-resolved metatranscriptomics (McDaniel and Moya et al., 2020; Wang
403 et al., 2020). Genome-resolved metatranscriptomics has also been used to demonstrate
404 activities of specific genes, such as a complete denitrification pathway in *Accumulibacter* clade
405 IC (Camejo et al., 2019), and extracellular electron transfer activities of a wastewater-fed
406 microbial full cell community (Ishii et al., 2013) and anammox bacteria (Shaw et al., 2020) .
407 Expanding beyond the detailed study of a single microbial lineage, genome-resolved
408 metatranscriptomic approaches have been applied to whole communities for which there is
409 sufficient read coverage (Woodcroft et al., 2018) to infer putative interactions and connect
410 gene expression patterns to overall ecosystem functioning. Lawson et al. (2017) applied
411 genome-resolved metatranscriptomics to 17 assembled population genomes from an
412 anammox bioreactor to infer interactions between heterotrophic and anammox bacteria.
413 Scarborough et al. (2018) integrated genome-resolved metatranscriptomics and
414 thermodynamic analysis to elucidate microbial interactions contributing to medium-chain fatty
415 acid production of an anaerobic microbiome. Hao et al. (2020) reconstructed 182 MAGs from
416 two full-scale anaerobic digestors and applied genome-resolved metatranscriptomics to
417 explore gene expression programs in response to short-chain fatty acid stimulation.

418 Proteomics methods couple liquid chromatography (LC) with tandem mass-
419 spectrometry (MS) to profile the protein content of a single organism or microbial community
420 (Lipton et al., 2002; Wilmes and Bond, 2006). Overall, genome-resolved metaproteomics
421 approaches as a whole have not been applied as frequently as metatranscriptomics. This is
422 most likely due to both the technical difficulties in high-throughput mass spectrometry
423 methodologies and development of appropriate bioinformatics pipelines for competitively
424 mapping short peptide sequences to a collection of assembled genomes. However, landmark
425 studies have applied proteomics to whole communities to examine the functional activities of
426 dominant lineages (Lo et al., 2007; Ram et al., 2005; Roume et al., 2015a; Woodcroft et al.,
427 2018). In the context of engineered water systems, shotgun proteomics has been performed
428 on activated sludge enriched for *Accumulibacter* to test hypotheses about previously proposed
429 metabolic models in EBPR, and associate them with strain variants within the *Accumulibacter*
430 population (Wilmes et al., 2008). Shotgun proteomics, supported by shotgun metagenomics,
431 has also been used to analyze the proteome associated with membrane-bound respiratory
432 complexes in anammox bacteria (de Almeida et al., 2016).

433 Fully integrated studies across the genomic, transcriptomic, proteomic, and even
434 metabolomic landscapes have only recently been produced for engineered water systems.
435 Methodologies for sequential extraction of DNA, RNA, protein, and metabolites from a single
436 microbial community sample have enhanced the reproducibility of integrated omics
437 measurements (Roume et al., 2013a, 2013b). An integrated comparative 'omic analysis using
438 metagenomics, metatranscriptomics, and metaproteomics of the anoxic tank of a full-scale
439 WWTP was carried out to construct community-wide metabolic networks (Roume et al.,
440 2015b). Metabolomics paired with genomic, transcriptomic, and proteomic data was used to
441 understand lipid accumulation in WWTPs and the role of '*Candidatus Microthrix parvicella*'
442 (Muller et al., 2014). Genome-resolved metagenomic, metatranscriptomic, and metaproteomic
443 approaches were applied to a partial-nitration anammox lab-scale bioreactor to explore
444 operational parameters and microbial interactions affecting nitrogen removal (Wang et al.,
445 2019). Recently, an integrated time-series genome-resolved metagenomics,

446 metatranscriptomics, metaproteomics, and metabolomics investigation of full-scale WWTP
447 microbial community recovered 1,364 MAGs and demonstrated patterns of resistance and
448 resilience to natural environmental perturbations (Herold et al., 2020). As technologies and
449 bioinformatics algorithms improve, integrated multi-omics approaches have the potential to
450 enable powerful ecological insights into engineered water microbiomes and create the
451 foundation for more predictable and controllable systems (Muller et al., 2013).

452

453 **2. PAIRING OMICS-DRIVEN INSIGHTS WITH MODELING APPROACHES**

454 Obtaining genome sequences for uncultivated microbial lineages and integrating
455 spatiotemporal metatranscriptomic and metaproteomic data has become increasingly
456 commonplace in research conducted by microbial ecologists and water engineers. Looking
457 ahead, effective prediction and control of microbial communities for desired outcomes in
458 engineered water systems is likely to rely in part on the ability to integrate vast amounts of
459 omics data into process modeling frameworks. In this section, we describe two overarching
460 aims of broad modeling approaches used to 1) interrogate metabolic networks and 2) predict
461 the temporal dynamics of complex communities. For metabolic modeling approaches, we
462 summarize how emerging techniques in metabolomics and stable isotope probing are being
463 used in engineered water systems. For modeling population dynamics, we briefly review how
464 16S rRNA gene sequencing approaches have been used for time-series forecasting in water
465 engineering, and how these studies can be strengthened by applying genome-resolved multi-
466 omics approaches.

467

468 ***2A. Metabolic Modeling with Pathway Flux, Stable Isotope Probing and Metabolomics***

469 The metabolic capabilities of a microorganism depend on the available reaction networks,
470 putative intracellular storage compounds, and environmental conditions. The past few
471 decades have seen major developments in metabolic modeling methods, fueled by more
472 quantitative data from the application of different omics approaches as well as increased
473 computational power. These approaches have ranged from “simple” stoichiometric models to

474 complex spatiotemporal, multi-scale simulations of ecosystems (Bauer et al., 2017).
475 Annotation and generation of genome-scale stoichiometric models is becoming a more
476 automated process with different tools available for binning, functional annotation, and
477 metabolic model generation (Hamilton and Reed, 2014; Machado et al., 2018). Overall, these
478 predictions are based on 1) the metabolic reaction networks of different microorganisms,
479 which can be derived from annotated genomes, at least within the limits of known biochemistry
480 (Feist et al., 2009; Hamilton and Reed, 2012) 2) expression levels of the required enzymes
481 and their activities (Hamilton et al., 2017; Molenaar et al., 2009), and 3) the kinetics of the
482 extracellular environment and metabolite concentrations (Herold et al., 2020).

483 The exploration of metabolic network functionality has been enabled by stoichiometric
484 modeling approaches, building on the availability of whole-genome sequences, namely flux
485 balance analysis (FBA) (Orth, Thiele and Palsson, 2010) or flux variability analysis (FVA)
486 toolkits (Gudmundsson and Thiele, 2010). These approaches were first developed for steady-
487 state systems and were able to predict biological phenomena such as overflow metabolism in
488 a single organism (Basan et al., 2015), as well as cross-feeding consortia (Stolyar et al., 2017;
489 Carlson et al., 2018). Extensions to these approaches have been made to more dynamic and
490 cyclic conditions to correctly predict storage accumulation and depletion cycles in different
491 organisms (Reimers et al., 2017). For example, applications of FBA and FVA in engineered
492 water systems include the study of *Ca. Accumulibacter* to highlight the flexibility of anaerobic
493 metabolism due to intracellular accumulation of different polymers (Guedes da Silva et al.,
494 2020). Genome-scale models can also be used to understand the gain insight into
495 thermodynamic constraints that may pattern metabolic activity, as seen by an analysis of
496 genome-scale metabolic network reconstructions of an anaerobic syntrophic coculture formed
497 by the bacterium *Syntrophobacter fumaroxidans* and the methanogenic
498 archaeon *Methanospirillum hungatei* (Hamilton et al., 2015). Weinrich et al. (2019) developed
499 a framework to incorporate FBA into the IWA Anaerobic Digester Model No.1 to predict
500 thousands of intracellular metabolite fluxes of individual species in anaerobic digesters,
501 providing an opportunity to couple process modeling with multi-omics datasets. Integrated

502 omics approaches can also aid in improving genome-scale metabolic models and
503 thermodynamic analyses. Scarborough et al. used genomic and transcriptomic data to
504 understand interactions and substrates of an anaerobic microbiome producing medium-chain
505 fatty acids (Scarborough et al., 2018). They subsequently used time-series transcriptomic data
506 and metabolomics to understand the environmental conditions favoring medium-chain fatty
507 acid production (Scarborough et al., 2020b) and construct unicellular and guild-based
508 metabolic models of the community (Scarborough et al., 2020a).

509 Stable isotope probing (SIP) and tracing approaches have been applied to highly
510 enriched and complex microbial communities to identify metabolically active populations
511 (**Figure 5**). Metabolically active populations can be further taxonomically and functionally
512 characterized by pairing heavy labelled ^{13}C isotopes with metagenomic sequencing (Dumont
513 and Murrell, 2005; Radajewski et al., 2000) or metaproteomic sequencing (Jehmlich et al.,
514 2008; Von Bergen et al., 2013). Incorporation of the ^{13}C isotope into a given carbon source
515 then allows for ^{12}C and ^{13}C labelled fractions to be resolved by density-gradient centrifugation
516 (for DNA/RNA-SIP) and separately characterized by amplicon or shotgun metagenomic
517 sequencing approaches (Chemerys et al., 2014; Chen and Murrell, 2010; Coyotzi et al., 2016;
518 Neufeld et al., 2007; Saidi-Mehrabad et al., 2013), or by mass-spectrometry approaches for
519 protein-SIP (Sachsenberg et al., 2015). Genome-resolved DNA-SIP approaches can be
520 achieved by exploiting abundance patterns between ^{12}C and ^{13}C density fractions and applying
521 differential coverage binning to metagenomic samples (**Figure 5**). Genome-centric SIP
522 approaches were demonstrated to elucidate populations that could degrade oleate (Ziels et
523 al., 2018) and butyrate (Ziels et al., 2019) in anaerobic digesters. Insights into novel syntrophic
524 acetate oxidizers in full-scale anaerobic digesters were also obtained by coupling genome-
525 resolved metagenomics with metaproteomics-SIP (Mosbæk et al., 2016).

526 Isotopic tracers can also be used in metabolic flux analyses to measure the *in vivo* flux
527 of metabolites in individual organisms (Ando and García Martín, 2019; Matsuoka and Shimizu,
528 2014) or cocultures (Gebreselassie and Antoniewicz, 2015). Quantifying metabolic flux
529 changes under different environmental conditions using isotopic tracers can provide insights

530 into metabolic regulation (Antoniewicz, 2015; Long and Antoniewicz, 2019; Nielsen, 2003).
531 Lawson et al. used a combination of time-series ^{13}C and ^2H isotopes, metabolomics, and
532 isotopically nonstationary metabolite flux analysis to resolve the central carbon metabolism of
533 a highly enriched anammox bioreactor community (Lawson et al., 2020). Although metabolic
534 flux analyses are difficult to perform for complex microbial communities, advances in assigning
535 individual peptides and metabolites to a given species has started to pave the way for
536 metafluxomics (Beyß et al., 2019; Ghosh et al., 2014).

537 Recent advances in high-throughput mass spectrometry-based technologies have
538 allowed for metabolomics approaches to shift from targeted characterization of known
539 metabolites to untargeted surveys of numerous metabolites and other small molecules
540 (Schrimpe-Rutledge et al., 2016). Although both targeted and untargeted strategies have their
541 shortcomings and challenges, untargeted approaches enable discovery-based studies that
542 can be subsequently tested with targeted approaches (Cajka and Fiehn, 2016). Metabolomics
543 can be combined with other multi-omics and modeling approaches to compare results from
544 the level of gene regulation to metabolite production of a given system or process (Herold et
545 al., 2020; Lawson et al., 2020; Muller et al., 2014; Roume et al., 2015a; Scarborough et al.,
546 2020a). Recent advances in methods for simultaneous, nondestructive extraction of DNA,
547 RNA, and metabolites from a given sample have paved the way for these types of intrasample
548 datasets (Roume et al., 2013a, 2013b). These multi-omics approaches integrated with
549 metabolic modeling approaches can greatly enhance our ability to predict the environmental
550 conditions that are favorable for desired outcomes in engineered water systems.

551

552 ***2B. Genome-Resolved Omics Applied to Time-Series***

553 Time-series data have been applied to numerous ecosystems to unravel the spatiotemporal
554 dynamics of microbial communities in the context of environmental parameters (Shade et al.,
555 2013). For example, longitudinal studies have been used to identify marine seasonal microbial
556 community dynamics (Gilbert et al., 2012), community succession patterns in the developing
557 infant gut (Palmer et al., 2007), and characterize patterns of microbial community recovery

558 and resilience after ecosystem disturbances (Shade et al., 2012). Microbial community time-
559 series have also been used to address important questions in engineered water systems such
560 as community assembly in full-scale wastewater treatment plants (Griffin and Wells, 2017; Lee
561 et al., 2015), population dynamics and immigration of anaerobic digestion communities (Griffin
562 et al., 1998; Kirkegaard et al., 2017), and monitoring bacterial community migration dynamics
563 in drinking water distribution systems (Boers et al., 2018; Pinto et al., 2014). However, most
564 time-series resolved data have been generated using 16S rRNA gene amplicon sequencing
565 surveys, which have significant shortcomings. Assigning metabolic functions to 16S rRNA
566 gene based OTUs is not entirely intuitive for most guilds, such as heterotrophic bacteria that
567 span diverse phyla (Frigon and Wells, 2019; Marques et al., 2017). In addition to some taxa
568 in engineered water systems remaining unclassified, 16S rRNA gene based OTUs usually
569 cannot be resolved more specifically than the genus-level (Kirkegaard et al., 2017).

570 Although long-read sequencing technologies (discussed below) can improve the
571 resolution of resulting 16S rRNA gene based OTUs (Karst et al., 2021; Nierychlo et al., 2019),
572 time-series data integrating metagenomics and integrated genome-resolved omics methods
573 can be a powerful approach (Faust et al., 2015). Metagenomes collected over time can be
574 used to improve binning efforts based on differential coverage profiles (Kang et al., 2015), and
575 subsequently identify metabolic guilds and abundance patterns (Linz et al., 2018). If full-length
576 16S rRNA gene sequences are retrieved from assembled MAGs, defined microbial guilds
577 based on metabolic reconstructions can be connected to 16S rRNA amplicon sequencing
578 surveys of a given system (Nierychlo et al., 2019; Petriglieri et al., 2020; Singleton et al., 2020).
579 Pérez et al. assembled 173 MAGs from a full-scale WWTP and assessed associated
580 functional potential and the dynamics of rRNA gene operons during operational disturbances
581 (Pérez et al., 2019). In conjunction with 16S rRNA amplicon sequencing, assembled MAGs
582 were used to characterize functional profiles between operational periods (Pérez et al., 2019).
583 Integrated genome-resolved omics approaches can also be applied in longitudinal analyses
584 to understand community dynamics and functional profiles. Time-resolved omics approaches

585 were recently applied to a full-scale wastewater treatment plant and demonstrate the power
586 of fully integrated omics methodologies (Herold et al., 2020; Martínez Arbas et al., 2021).

587 Other promising developments have combined time-series omics data with
588 biogeochemical models to predict the fate of nutrients in environmental systems (Louca et al.,
589 2016; Reed et al., 2014). Such ecosystem level models have been constructed based on the
590 abundance of genes in metagenomes (Reed et al., 2014), as well as transcript and protein
591 abundances (Louca et al., 2016). The underlying model structure of such gene-centric
592 modeling approaches is amenable to their adaptation into existing water engineering models
593 (e.g. IWA, ASM, and ADM). However, due to the fact that omics data are relative
594 measurement, novel methods are needed to accurately infer relative or absolute taxa
595 abundances so the stoichiometry can be incorporated within process models. While some
596 recent methods show promise, such as using internal quantitation standards for metagenomes
597 (Hardwick et al., 2018), this task is not trivial due to differences in cell size, composition,
598 biomass concentration, and complexity of biomass morphology.

599

600 **3. EMERGING TECHNOLOGIES IN OMICS AND WATER ENGINEERING**

601 Over the last few years, advances in sequencing technologies and novel methodologies have
602 emerged that have direct implications for improving genome-resolved metagenomic insights.
603 Although there have been various novel, cultivation-independent inventions for broadly
604 characterizing microbial communities, we focus on two fields that have exhibited tremendous
605 growth and opportunity for leveraging multi-omics approaches specifically in engineered water
606 systems. First, we summarize how improvements in the quality and substantial decrease in
607 costs of long-read sequencing technologies have allowed for obtaining complete, closed, and
608 high-quality reference genomes of microorganisms from mixed communities. We then
609 highlight emerging cell-sorting techniques that can be applied in conjunction with multi-omics
610 for novel functional insights.

611

612 ***3A. High-Quality Genomes Enabled by Advances in Sequencing Technologies***

613 Although next-generation sequencing technologies have greatly enhanced high-throughput
614 surveys of microbial communities, the emergence of so-called “third-generation” sequencing
615 technologies using single molecule sequencing to produce longer reads are poised to
616 revolutionize *de novo* assembly of complex genomes and metagenomes (Lee et al., 2016).
617 Primarily, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) have
618 emerged as the main technologies for long-read metagenomic surveys of microbial
619 communities (Arumugam et al., 2021, 2019; Bertrand et al., 2019; Moss et al., 2020; Singleton
620 et al., 2021; Somerville et al., 2019; Stewart et al., 2019). The greatly enhanced read length
621 (routinely greater than 10k bp) presents substantial advantages in the process of genome
622 assembly, by permitting the successful reconstruction of genome regions containing complex
623 repeats, such as multiple intragenomic rRNA operons. Such repeat regions typically contribute
624 to the high degree of fragmentation observed in assemblies constructed with short read data.
625 In the case of gDNA sequencing from culture isolates, the use of long read technologies will
626 typically result in the generation of a complete closed genome, represented a single,
627 continuous sequence construct (Frank et al., 2018).

628 The major disadvantage of current long read methods relates to reduced sequence
629 quality compared to short read sequencing, with error rates cited as being as high as 10-30%
630 (Amarasinghe et al., 2020), compared to short read sequencing which holds as error rate of
631 no more than 1%. Rapid progress in sequencing chemistry has led to reduction in these error
632 rates, with some reports of error rates <5% error using ONT sequencing and <1% with PacBio
633 (as discussed by Amarasinghe et al., 2020). The impact of high error rates can readily be seen
634 in protein coding sequences, where the occurrence of frame-shift errors (insertion/deletion, or
635 indels) can be up to 80 times higher than that observed in Illumina sequences (Huson et al.,
636 2018). Mitigating the impact of error rates requires the application of complex data correction
637 procedures (Morisse et al., 2020), either self-correction of the long read data themselves,
638 approaches that make use of complementary higher-accuracy short reads, or correction via
639 the use of sequence alignment to reference sequence databases (Huson et al., 2018). At the
640 time of writing, the development of base-calling methods and new error-correction procedures

641 is a highly active research topic (Morisse et al., 2019). Several datasets also exist, including
642 mock communities, which provide co-assayed reference data from both long and short read
643 sequencing technologies, and which can be used as comparative reference data sets for
644 comparing the two different modalities (Sevim et al., 2019; Nicholls et al., 2019; Sommerville
645 et al., 2019).

646 In the context of water engineering, Arumugam and colleagues (Arumugam et al.,
647 2021, 2019) demonstrated the recovery of complete genomes (single chromosome), and in
648 some cases, circularized, closed genomes, of the most abundant community members from
649 an enrichment bioreactor (22 in total, including key PAO, GAO, and filamentous species,
650 among others; of which 10/22 were circularized). Recently Singleton et al. analyzed
651 approximately 1Tbp of Nanopore data generated from 23 full scale WWTPs in Denmark, and
652 obtained 1,083 high quality MAGs from 581 distinct prokaryotic species, including 57 with
653 circularized closed genomes (Singleton et al., 2021). Notwithstanding these advances, there
654 remain many emerging problems that will need attention with long read MAG recovery, such
655 as the need for long read specific binning procedures (Singleton et al., 2021) and the impact
656 of sequencing error rate on coding sequence quality (Arumugam et al., 2020).

657 In addition to technology advancements that directly allow for longer-reads from single
658 molecules, novel, synthetic long-read approaches such as chromosome conformation capture
659 (also referred to proximity ligation), linked-reads, and optical mapping (Amarasinghe et al.,
660 2020) also have enormous potential for applications in water systems. Chromosome
661 conformation capture (3C) methods apply formaldehyde cross-links prior to DNA lysis and
662 extraction to create interaction points between nearby loci (Dekker et al., 2002). Crosslinked
663 chromatin is then digested with specific restriction enzymes to obtain long-range interaction
664 pairs, and used to analyze the contact frequencies of these pairs (Lieberman-Aiden et al.,
665 2009; Sati and Cavalli, 2017). Originally, variants of 3C technologies were developed to study
666 the three-dimensional organization of chromosomes and genome structures (Hsieh et al.,
667 2015; Rao et al., 2014). Recently, this method has been adapted for generating genomes from
668 semi-complex and complex microbial communities, such as assembling a yeast hybrid from a

669 beer sample, population genomes from the human gut, and used in addition to standard
670 Illumina shotgun sequencing to obtain ~900 genomes from the cow rumen (Burton et al., 2014;
671 Marbouty et al., 2014; Press et al., 2017; Smukowski Heil et al., 2018; Stewart et al., 2018).
672 Since this method produces linkages between genetic content within the same cell, this
673 approach can also produce associations between antibiotic resistance genes (ARGs),
674 plasmids, and mobile elements to the host genome with fidelity. This was recently
675 demonstrated in a microbial community from a full-scale WWTP to link ARGs and plasmids to
676 specific populations (Stalder et al., 2019), while also highlighting the challenges associated
677 with implementing Hi-C approaches in complex and poorly studied microbial communities.

678 Although this review mainly covers genome-resolved approaches, long-reads have
679 also been applied to improve amplicon sequencing surveys. Briefly, these high-throughput
680 pipelines generate thousands to millions of full-length 16S rRNA genes (or any other amplicon
681 of choice) from mixed microbial communities without primer-bias (Karst et al., 2021, 2018).
682 The recently updated release of the MiDAS Field Guide with full-length 16S rRNA references
683 allows for species-level classifications of microorganisms in activated sludge and anaerobic
684 digestion systems (Nierychlo et al., 2019). Singleton et al. also demonstrated the ability to
685 connect structure to function with fidelity by comparing full-length 16S rRNA sequences from
686 large-scale microbial surveys to corresponding high-quality MAGs (Singleton et al., 2020).
687 Full-length 16S rRNA sequences from high-quality MAGs were also used in Singleton et al.
688 2020 to construct novel FISH probes to visualize the dynamics of specific and understudied
689 microorganisms in full-scale WWTPs (Singleton et al., 2020). Advances in long-read
690 sequencing technologies will allow for the rapid recovery of high-quality genomes from
691 complex microbial communities to use in conjunction with detailed experiments (**Figure 6**).

692

693 ***3B. Omics Approaches Enabled through Cell-Sorting Techniques***

694 Cell-sorting methodologies involve the separation of cells from a complex sample into simpler
695 subsets for various downstream purposes. Sorting can be accomplished through various
696 strategies, such as fluorescently or physically tagging cells with specific probes or dyes,

697 randomly dividing groups of cells, or scanning cells for the presence of specific polymers or
698 storage products (Malmstrom and Eloë-Fadrosh, 2019). Advances in cell-sorting technologies
699 have allowed for both fundamental discoveries and applied practices in engineered water
700 systems. Flow cytometry has been commonly used to monitor microbial communities in
701 DWDS (Besmer et al., 2014; Douterelo et al., 2014), sometimes in conjunction with next-
702 generation sequencing approaches (Prest et al., 2014). In relation to omics-based
703 approaches, cell-sorting methods allow for downstream applications such as sequencing of
704 simpler “mini-metagenomes” of a complex community, performing targeted cultivation of
705 specific microbial lineages, and characterizing individual microbial cells exhibiting specific
706 traits or physiologies (**Figure 6**).

707 Complex microbial communities can be subjected to cell-sorting techniques prior to
708 DNA extraction and sequencing, leading to sequencing a “mini-metagenome” (Ji et al., 2017).
709 This could entail randomly dividing cells by microfluidic sorting (Yu et al., 2017) or
710 nonrandomly by fluorescently-activated cell sorting (FACS) (Schulz et al., 2018). Single
711 amplified genomes (SAGs) are obtained through non-specific staining prior to sorting and
712 screened for sequencing (Clingenpeel et al., 2014; Rinke et al., 2013). However, genomes
713 from single-cell sequencing efforts are usually incomplete, contain high amounts of
714 contamination, and can be expensive to obtain for low abundant microorganisms (Rinke et al.,
715 2013; Swan et al., 2011; Xu and Zhao, 2018). Targeted approaches employ FISH probes to
716 stain specific microbial lineages prior to FACS (Kalyuzhnaya et al., 2006), which can also
717 allow access to low abundant microorganisms (Podar et al., 2007; Tan et al., 2019; Yilmaz et
718 al., 2010). Improvements in FISH-based methods for staining specific microbial clades have
719 led to higher quality genomes reconstructed from these efforts (Grieb et al., 2020). Intriguingly,
720 a reverse genomics-based approach implementing engineered antibodies to capture specific
721 microorganisms from a complex community was applied to culture members of the
722 Saccharibacteria (TM7) (Cross et al., 2019). Live cell sorting was applied to marine nitrite-
723 oxidizing enrichments to isolate novel *Nitrospinae* strains and investigate their nitrite affinity
724 and metabolic differences (Mueller et al., 2020). Targeted cell sorting through fluorescent

725 labeling combined with single-cell genomics was used to enrich for low abundant Chloroflexi
726 species from a full-scale wastewater treatment plant (Dam et al., 2020).

727 In addition to targeting specific microorganisms based on phylogenetic identity,
728 microbial populations can be screened and selected based on specific metabolic activities.
729 Raman microspectroscopy methods quantify the scattering of light given off by chemical
730 bonds to generate a spectral profile of specific biomarkers at the single-cell level (Huang et
731 al., 2004; Neufeld and Murrell, 2007). Raman spectroscopy can be combined with stable
732 isotope probing (SIP) and FISH methods to connect the phylogenetic identity of cells exhibiting
733 specific metabolic activities (Huang et al., 2007). These methods have been used to retrieve
734 cells based on specific functions before sequencing (Lee et al., 2020) or confirming metabolic
735 activities of microorganisms based on genome sequences (Fernando et al., 2019). Raman
736 cell-sorting in conjunction with mini-metagenomics was applied to a mouse gut microbial
737 community to identify commensals that incorporate mucus-derived monosaccharides and
738 rationally design a probiotic microbial consortia (Pereira et al., 2020). In engineered water
739 systems, Raman microspectroscopy-based methods have been particularly used to identify
740 polyphosphate accumulating organisms (PAOs) based on quantifying intracellular storages of
741 polyphosphate (Fernando et al., 2019). In addition to the model PAO *Ca. Accumulibacter*,
742 members of the actinobacterial genus *Tetrasphaera*, multiple species of *Dechloromonas*, the
743 filamentous microorganism *Ca. Microthrix*, and the novel *Ca. Methylophosphatis* have been
744 shown to incorporate polyphosphate intracellularly using Raman microspectroscopy imaging
745 and quantification methods (Fernando et al., 2019; Petriglieri et al., 2021, 2020; Singleton et
746 al., 2020). Enzymatic tagging using click-chemistry based approaches followed by cell sorting
747 can be used to sequester low abundant populations for targeted metagenomic analyses
748 (Sakoula et al., 2021). *Bioorthogonal non-canonical amino acid tagging* (BONCAT) is one
749 such substrate analogue probing technique that labels newly translated proteins via azide-
750 alkyne click-chemistry, and can be used to selectively sort active microorganisms from
751 complex communities and environments (Couradeau et al., 2019; Hatzenpichler et al., 2016;
752 Reichart et al., 2020). Advances in cell-sorting methods provide powerful approaches to apply

753 identity- or function-based tagging to specific microbial populations in a complex community
754 such as engineered water systems. These approaches can be used to construct mini-
755 communities or synthetic consortia designed to perform a desired function, allowing for more
756 controllable and predictable dynamics and outputs (Lawson et al., 2019; Pereira et al., 2020).

757

758 **4. CONCLUSIONS**

759 In the release of *Microbial Ecology of Activated Sludge* in 2010 (Seviour and Nielsen, 2010),
760 with extensive field updates to the 1999 publication of *The Microbiology of Activated Sludge*
761 (Seviour and Blackall, 1999), the editors noted that the greatest future challenge would be “to
762 communicate with engineers to convince them that all this elegant science can be applied
763 productively to improve how they design and operate their activated sludge systems” (Seviour
764 and Nielsen, 2010). In the decade since, numerous breakthroughs and technological
765 advancements in water-related microbiome science as a whole have stemmed from the desire
766 to unravel the ecology and evolution of the microorganisms underpinning these systems.
767 Clearly, the water engineering community is more convinced than ever that state-of-the-art
768 molecular and omics tools can provide valuable insights into the microbiology of their systems.
769 However, applying these insights to improve the design and operation of engineered water
770 systems remains a grand challenge. Ultimately, successfully harnessing engineered water
771 microbiomes for improved process design cannot be accomplished without effectively bridging
772 engineering principles with microbial ecology theory (Lawson et al., 2019). Advances in
773 genome-resolved multi-omics approaches provide an exciting new avenue for water engineers
774 and microbial ecologists to tackle these interdisciplinary grand challenges.

775

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785

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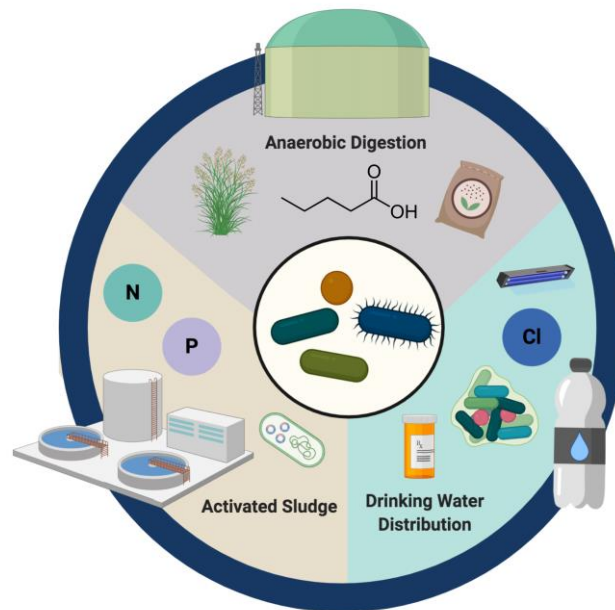
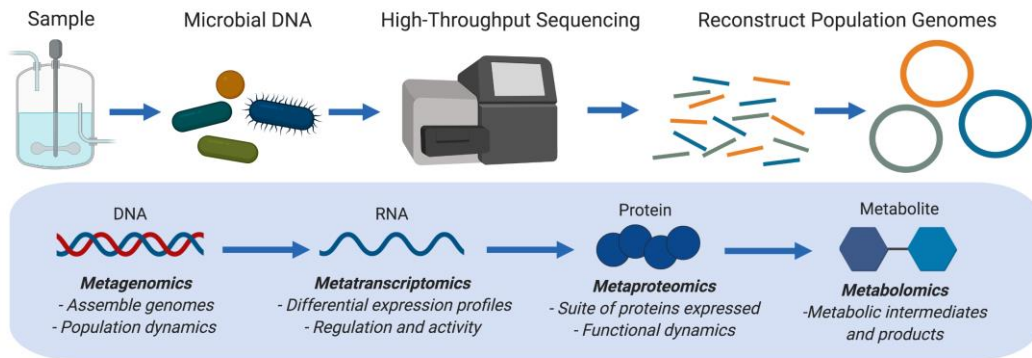


Figure 1. Engineered water systems unified through microbial ecology: engineered water systems are unified through microbial ecology in that they all aim to manage diverse microbial communities for desired outcomes. *Anaerobic Digestion* (AD) systems apply microbial consortia to produce valuable bioenergy products from waste streams. *Activated Sludge* (AS) wastewater treatment systems harness the unique metabolic physiologies of specific microorganisms to remove nutrients such as nitrogen and phosphorus. *Drinking Water Distribution Systems* (DWDS) aim to manage microbial pathogens and biofilms to provide safe, clean drinking water. Created with BioRender.com.

1967

Figure 2



1968

1969 **Figure 2. Genome-resolved multi-omics approaches:** genome-resolved approaches can
1970 be applied individually at the genomic, transcriptomic, and proteomic levels, or in conjunction
1971 with another. Microbial DNA is extracted from the environment of interest and used for high-
1972 throughput shotgun sequencing. Short reads are assembled into longer contigs, which are
1973 used to bin into population genomes based on sequence composition and differential
1974 coverage through space and/or time. Typical workflows for binning and downstream analyses
1975 of assembled genomes are outlined in Figure 3. Population genomes from short-read data
1976 usually represent a consensus sequence of closely related strains in a sample. Created with
1977 BioRender.com.

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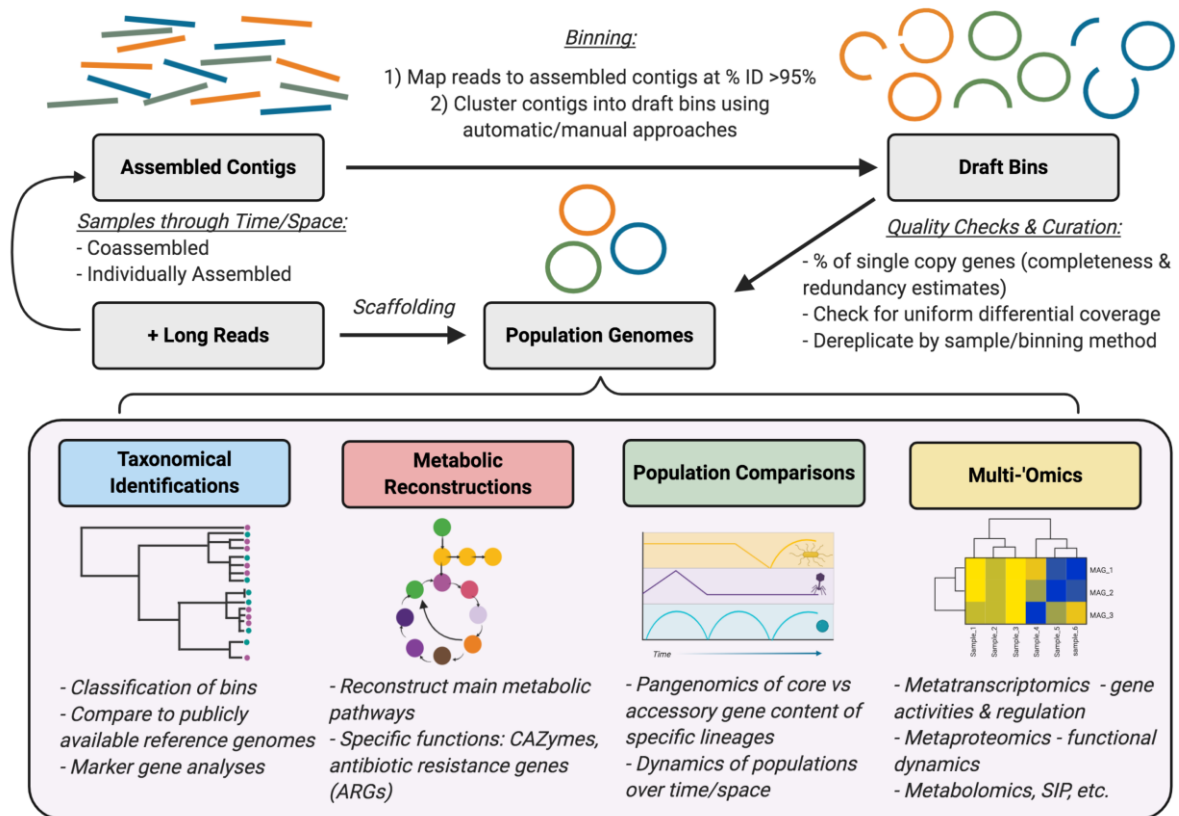
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1987 **Figure 3. Workflow for binning population genomes:** from sequenced metagenomes,

1988 samples are either assembled individually or coassembled together into longer contigs. These

1989 contigs are binned into draft bins by mapping reads back to assembled contigs at a percent

1990 identity >95%, and clustered by differential coverage profiles over space and/or time and/or

1991 using nucleotide signatures. These draft bins are then quality checked and curated based on

1992 the presence of single copy genes, checked for uniform differential coverage across all

1993 contigs, and dereplicating by sample or binning method. The resulting set of population

1994 genomes represent non-redundant, “species”-level bins that can be recovered from the

1995 community. Long reads can be incorporated either during initial assembly process with

1996 polishing tools to improve contigs lengths, used for scaffolding individual bins, or potentially

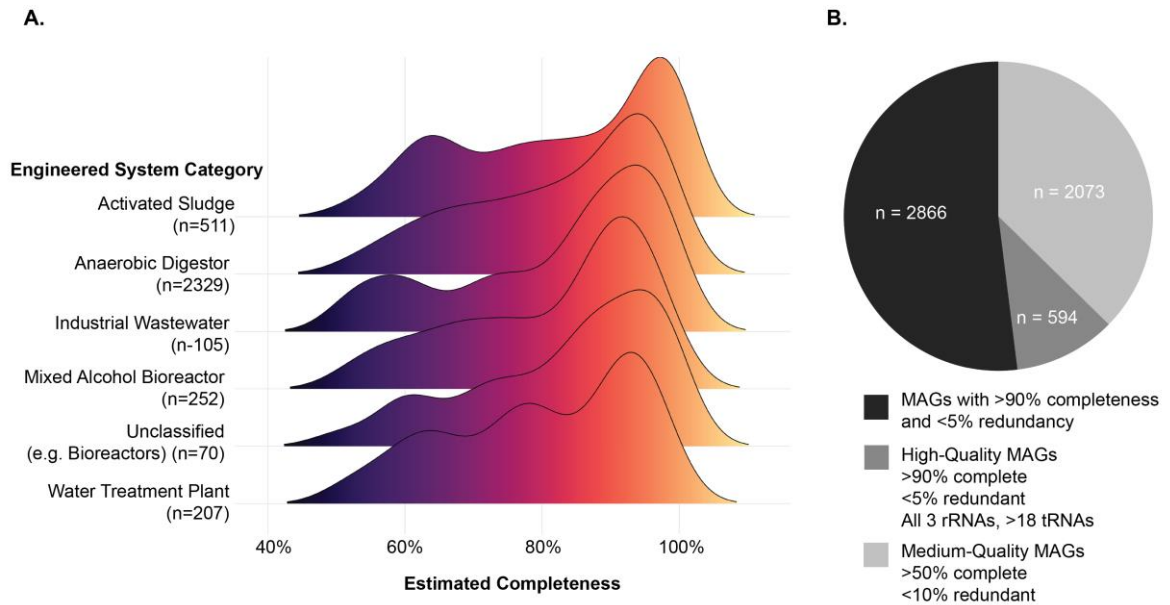
1997 as the basis for genome recovery (not shown). Population genomes can then be used for a

1998 variety of analyses to understand the given ecosystem – phylogenomic comparisons,

1999 metabolic reconstructions, dynamics of specific populations, and/or integrating with other

2000 multi-omics approaches. Created with BioRender.com.

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2003 **Figure 4. Quality of MAGs from engineered water systems:** genome information from the

2004 Genomes from Earth's Microbiomes (GEM) catalog (Nayfach et al., 2020). From this dataset,

2005 genomes were subset by ecosystem and ecosystem category to only include genomes from

2006 engineered water systems (activated sludge, anaerobic digestion, drinking water systems, and

2007 lab-scale bioreactors simulating these processes). From 52,515 assembled MAGs, 3,474

2008 MAGs were determined to be recovered from engineered water systems. **A.** Distribution of

2009 estimated completeness of all MAGs from different environmental categories of engineered

2010 water systems. **B.** Distribution of MAGs from engineered water systems that fall under the

2011 categories high-quality, medium-quality, and genomes that are >90% complete and <5%

2012 redundant but do not contain all 3 rRNA genes and at least 18 tRNAs, as defined by the

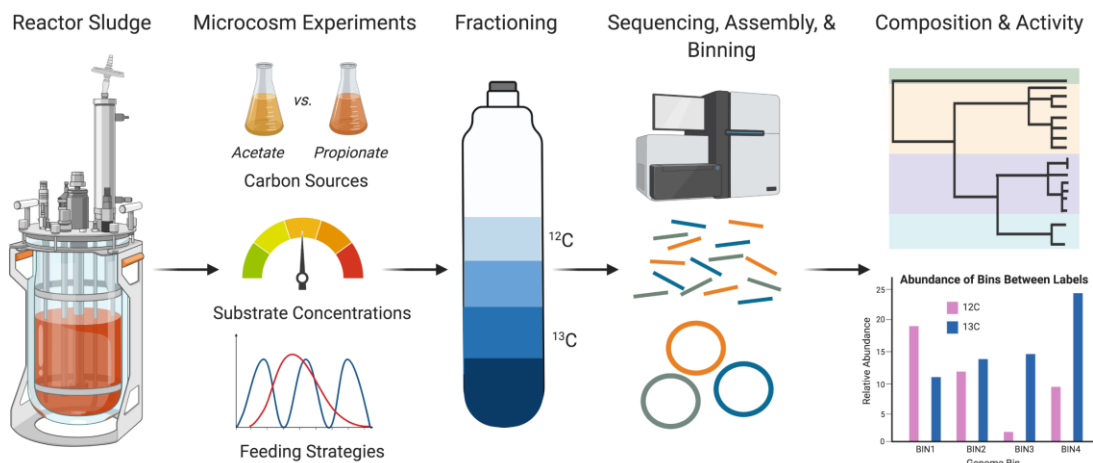
2013 MIMAG standards (Bowers et al., 2017).

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2019 **Figure 5. DNA Stable Isotope Probing (SIP) paired with genome-resolved**

2020 **metagenomics:** DNA stable isotope probing (SIP) can identify active populations in a mixed

2021 community. Reactor sludge is inoculated into microcosms used for incubation experiments

2022 such as testing differences between carbon sources, substrate concentrations, or feeding

2023 strategies on community dynamics. Labelled DNA is density centrifuged and separated

2024 between light and heavy labelled DNA. The fractions are sequenced and assembled.

2025 Individual assembly of metagenomic contigs and differential coverage binning can then yield

2026 insights into the activities of specific populations between conditions. Created with

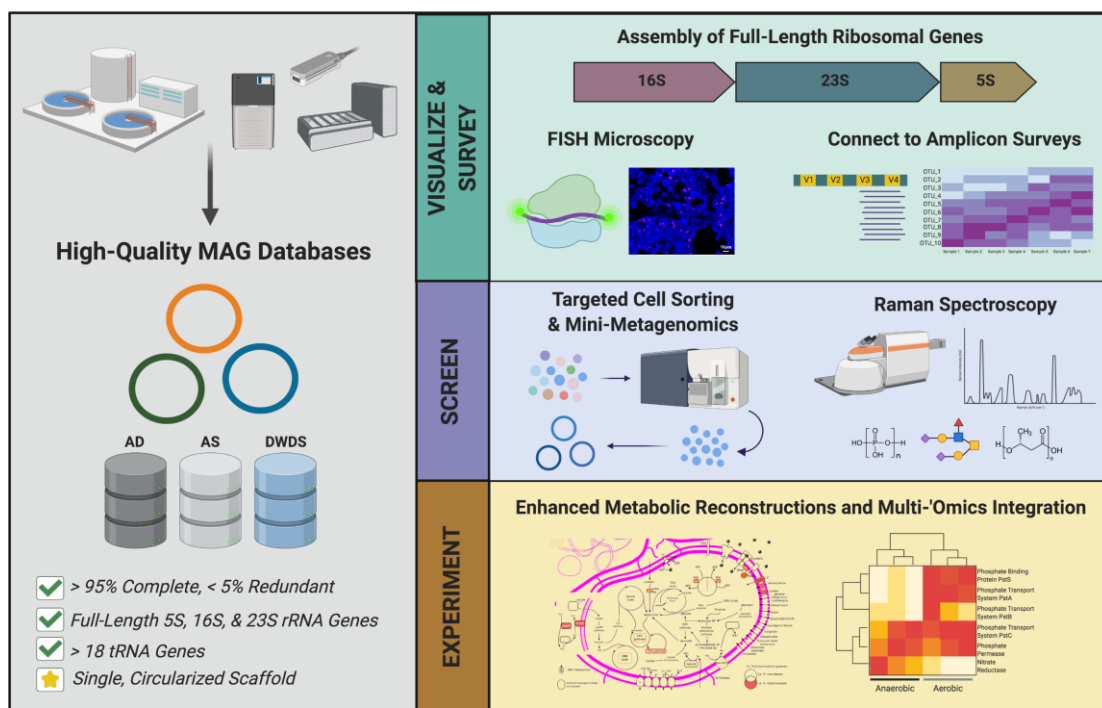
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2033 **Figure 6. Emerging technologies and future prospects for multi-omics of engineered**

2034 **water microbiomes:** improvements and decreasing costs in long-read sequencing

2035 technologies paired with novel experimental technologies have paved the way for exciting

2036 discoveries in engineered water systems. With genome-resolved metagenomic sequencing

2037 surveys of AD, AS, and DWDS ecosystems, high-quality genome databases can be

2038 constructed for downstream experiments. The assembly of full-length ribosomal genes from

2039 MAGs will allow for enhanced FISH probe construction and comparisons to amplicon

2040 sequencing studies. These genomes can then be used for the basis for screening for specific

2041 activities by targeted cell sorting or Raman spectroscopy. Multi-omics experiments can then

2042 be applied to understand the metabolism, regulation, and dynamics of key microbial guilds in

2043 these systems. We envision that these approaches will become commonplace and iterative

2044 of each other, such as demonstrated in Singleton et al. 2020. Created with BioRender.com.

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