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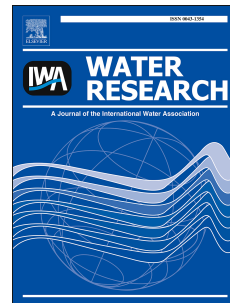
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1 **Position Paper:**

2

3 **Extracellular polymeric substances of biofilms:**

4 **suffering from an identity crisis**

5

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26

27

28 **Abstract**

29 Microbial biofilms can be both cause and cure to a range of emerging societal
30 problems including antimicrobial tolerance, water sanitation, water scarcity and
31 pollution. The identities of extracellular polymeric substances (EPS) responsible for
32 the establishment and function of biofilms are poorly understood. The lack of
33 information on the chemical and physical identities of EPS limits the potential to
34 rationally engineer biofilm processes, and impedes progress within the water and
35 wastewater sector towards a circular economy and resource recovery. Here, a
36 multidisciplinary roadmap for addressing this EPS identity crisis is proposed. This
37 involves improved EPS extraction and characterization methodologies, cross-
38 referencing between model biofilms and full-scale biofilm systems, and functional
39 description of isolated EPS with *in situ* techniques (e.g. microscopy) coupled with
40 genomics, proteomics and glycomics. The current extraction and spectrophotometric
41 characterization methods, often based on the principle not to compromise the integrity
42 of the microbial cells, should be critically assessed, and more comprehensive methods
43 for recovery and characterization of EPS need to be developed.

44 **Introduction**

45 Often described in a cursory manner as the slime, the extracellular polymeric
46 substances (EPS) are key to the formation, persistence and physicochemical behavior
47 of microbial biofilms across clinical, environmental and industrial settings (Seviour et
48 al. 2012b). Moreover, increased tolerance to antimicrobials is the result of the ability
49 of certain pathogens to produce EPS, which hence constitutes a global threat to the
50 consequences of multidrug resistance (Frieri et al. 2017).

51

52 EPS also play significant roles in the successful implementation of water reclamation
53 and purification technologies that have arisen to meet increasing demands for water of
54 different purities, water scarcity (predicted by the United Nations to be the biggest
55 global problem in the coming decade), land shortage and the water-energy nexus. EPS
56 provide structure for anaerobic and aerobic granular sludges, which have emerged
57 over the last thirty years, along with activated sludge and fixed biofilm systems (i.e.
58 trickling filters), as alternatives for biological treatment of industrial and domestic
59 used waters with lower land and energy footprints (Bengtsson et al. 2018). Advances
60 in membrane technologies have made it possible to create drinking water either from
61 sources that were previously considered not available for drinking water production
62 (i.e. brackish water seawater, or wastewater) (Le and Nunes 2016), or without the
63 addition of chemical disinfectants (Derlon et al. 2012, Madaeni 1999). However, the
64 hydraulic throughput of these technologies is often limited by membrane fouling,
65 which in many instances is due to biofilm growth.

66 Biofilms, therefore, feature prominently in many of the challenges facing water
67 technology implementations. As the number of antimicrobial-resistant strains
68 increases, and the range of water reclamation and purification technologies grows, so
69 too does the need to control or predict EPS production. Yet, despite decades of
70 research, we know very little about the molecular composition and function assigned
71 to individual EPS components, and we are not in a position to control the formation
72 and composition with any meaningful predictable outcome. This limits our ability to
73 manage biofilms effectively. We need to enhance our efforts to deliver improved
74 analytical methods and unravel biochemical production pathways, and most
75 importantly, discontinue the use of methods that misrepresent the roles and
76 significance of EPS. The current practice of dismissing EPS, or relegating them to

77 merely a perfunctory study as a footnote in process optimization, should be
78 abandoned. It is essential to identify and reveal how EPS composition determines the
79 microscopic and macroscopic behavior of biofilm systems.

80 We propose that identifying functional biofilm EPS is the critical path to address key
81 questions in biofilm control. This will not be possible if we persist with the current
82 practice of applying general, superficial and correlative characterizations alone.
83 However, prior to suggesting a roadmap for achieving an in depth understanding of
84 EPS, it is first necessary to explain why so little progress has been made in identifying
85 and characterizing extracellular polymers present in biofilms.

86 **The extracellular matrix**

87 The EPS of biofilms are a complex mixture of interlaced biological polymers. They
88 provide mechanical stability and scaffolds that allow biofilm cells to establish
89 synergistic microconsortia, enhance water retention and nutrient sorption, provide
90 protection against viruses, predation, antimicrobials and disinfectants, and ultimately
91 act as nutrient recycling yards (Flemming and Wingender 2010). These functions can
92 be provided by a large variety of biopolymers, particularly polysaccharides, proteins
93 and nucleic acids. EPS compounds originate from different community members and
94 a specific organism can produce different polymers as a function of time or condition.
95 Moreover, EPS produced by a given microbial population can persist long after the
96 population producing it has disappeared. All of these different components contribute
97 to the function and organization of the matrix. Additionally, many of the biopolymers
98 produced by the cells are processed by extracellular enzymes embedded in the
99 extracellular matrix (Whitfield et al. 2015). It is currently not possible to track the
100 production of specific EPS components over time or attribute them to the specific host

101 organism in mixed species biofilm communities, nor do we have the means to
102 effectively manipulate EPS quantity or composition. A better understanding of the
103 EPS would derive from metabolic labelling approaches (Liang et al. 2017). For
104 example, EPS biosynthesis compounds could be tracked to identify the organisms
105 producing them, when and where they are released, and their fate over time. This
106 could be monitored in real-time using state-of-the-art laser microscopes and
107 nanoscopes to generate high-resolution three-dimensional image data sets. Limitations
108 in our current understanding of the EPS, however, render such methodologies
109 presently beyond our reach.

110 Structural and functional assignment of key biofilm EPS is confounded by their
111 compositional complexity, but also by the challenges in processing and isolating EPS
112 components. The diversity of biofilm EPS is described in Figure 1, in terms of the
113 number of types of molecules observed across a range of biofilms (i.e. rather than in
114 any single biofilm). See Box 1 for a description of each EPS. Biofilms and many of
115 the EPS described in Figure 1 are poorly soluble in aqueous systems. Unless methods
116 are developed to extract the entire spectrum of biofilm EPS, our understanding of EPS
117 will be skewed by solubility and characterization biases. Mechanical and chemical
118 methods have been applied for EPS extraction from these biofilms with the objective
119 of maximizing extraction yield and minimizing cell lysis (Ras et al. 2011). In most
120 cases these methods have not been verified to assess whether they extract the
121 structural polymers from the biofilms. While potentially effective on some biofilms,
122 these extraction protocols are often only partially or marginally effective, which
123 results in the characterization of EPS that are not important for the biofilm structure
124 (Felz et al 2016). This is particularly the case for stratified and dense aggregates such
125 as fixed biofilms or granular sludge.

126 **A solution for the insoluble?**

127 The range of techniques required to extract and solubilize known biopolymers, such
128 as the polysaccharides cellulose, chitin and alginate (examples of neutral, cationic and
129 anionic polysaccharides respectively), highlights the need for even harsh extraction
130 methods (i.e. non-aqueous, extreme pH or temperature) (Zhang et al. 2017).
131 Combinations of mechanical pre-treatments (grinding, ultrasonication,
132 homogenizers), acidification (demineralization), enzymatic hydrolyses, alkalization
133 (for deproteination or deprotonation), novel solvents like ionic liquids and heat
134 treatments are typically invoked in order to extract such polysaccharides (Kumari and
135 Rath 2014, Seviour et al. 2015b, Younes and Rinaudo 2015). While cytosolic protein
136 extraction is possible through cell lysis, the task is far more problematic for structural
137 proteins. These are often large (Julio and Cotter 2005) and/or have a tendency to
138 fibrillate, whereby alkalization or acidification may be required to solubilize them,
139 often in conjunction with enzymatic treatments (Le et al. 2016).

140 Given the analytical challenges of identifying and characterizing functional EPS of
141 biofilm assemblages, we should sometimes be prepared to apply methods that damage
142 cells rather than prioritizing cell integrity (Felz et al. 2016) in order to resolve the
143 contributions of a broader range of key extracellular polymers. This approach would
144 then include the subsequent step of retrospectively identifying whether extracted
145 polymers are extracellular, as accomplished by microscopic techniques (Neu and
146 Lawrence 2014, Wagner et al. 2009). The target extracellular polymers can be
147 recovered from solution by fractional precipitation (e.g. using anti-solvent addition or
148 pH neutralization), and purified further by, for example, electrophoretic or
149 chromatographic techniques (Seviour et al. 2010a). Complementary biophysical
150 assays can then be undertaken on biofilm and isolates to elucidate function (Seviour et

151 al. 2015a). Detailed structural and functional characterization of novel relevant
152 extracellular polymers requires significant quantities of a sufficiently pure compound,
153 which is a common and often insurmountable hurdle to achieve the ultimate goal of
154 resolving more precisely the identity of key extracellular polymers.

155 **Do the same extracellular polymers provide the same functions**
156 **across systems?**

157 Despite the complexity and diversity of EPS in multi-species biofilms, we assume that
158 particular roles performed by EPS are conserved across biofilms, e.g. gel formation
159 and adhesion (Lin et al. 2013). The more information we acquire on the mechanical,
160 biophysical and structural aspects of the extracellular polymers contributing to these
161 functions, the easier it will be to identify and monitor their expression. This could
162 involve information derived from metaproteomic analysis, specific labelling of
163 functional groups in polymers (e.g. by lectins) and observation by microscopy (Neu &
164 Kuhlicke 2017), or quantifying polymers with greater accuracy. The list of reference
165 polymers is limited to those isolated from a relatively small number of models, often
166 clinical organisms (e.g. *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas*
167 *aeruginosa*) (Colvin et al. 2012, Marvasi et al. 2010). These bacteria are uncommon
168 in biofilms found in water treatment biofilms and their polymers are unlikely to be
169 representative of biofilm EPS in water treatment systems. Another approach for
170 understanding whether EPS perform common functions across different biofilm
171 system, currently neglected in EPS research, could be to screen interactions between
172 EPS and known glycan-binding proteins in order to infer function and identity (i.e.
173 glycomics) (Cummings and Pierce 2014, Lipke 2016). This would create a database

174 for EPS comparison, identification of new sugar-binding proteins for visualization of
175 novel sugars, and potentially facilitate the identification and analysis of glycoproteins.

176 **Agreeing on model biofilms for EPS characterization**

177 Full-scale biological systems in the water sector are often represented by highly
178 diverse microbial communities (Saunders et al. 2016). We would expect the EPS to be
179 similarly complex at a molecular level. Hence, full-scale systems may not be the ideal
180 starting point for isolating and characterizing reference polymers. We should
181 therefore improve the resolution of characterization of EPS from biofilms comprising
182 organisms known to contribute to key water and wastewater biofilm functions, such
183 as nitrification, enhanced biological phosphate removal, floc and filament formation
184 and the Anammox process. The microbial community composition of model systems
185 can be tracked and compared to full-scale systems. Biomass samples from these
186 model systems should be made broadly available to the water sector and act as a
187 common reference point for initial EPS characterization. There are a few examples of
188 EPS isolated from bacteria found in biofilms related to wastewater treatment,
189 including granulan (Seviour et al. 2012a), alginate-like exopolysaccharide (ALE) (Lin
190 et al. 2010), acid soluble polysugars (Pronk et al. 2017) and glycosylated proteins
191 (Lin et al. 2018). However, we still need to understand how widespread these EPS are
192 in biofilms, as well as identify new extracellular polymers from other key systems to
193 expand our database of identified, characterized and relevant EPS.

194 **Sequencing approaches for EPS characterization**

195 The application of next generation DNA-sequencing methods in conjunction with
196 bioinformatic analyses may allow for the identification of signature extracellular
197 polymers across a vast number of environmental biofilms, and to elucidate their

198 regulation. Metagenome assembled genomes (MAGs) representing individual
199 community species can be described relatively inexpensively (Albertsen et al. 2013),
200 and when coupled with long-read sequencing technologies, such as PacBio and
201 Nanopore sequencing, closed genomes from mixed communities can be constructed
202 (Hao et al. 2017, McIlroy et al. 2017). MAGs provide blueprints for the proteins
203 (enzymes, transporter, and chaperones) that are involved in the biogenesis of all
204 cellular components and EPS. In the case of proteinaceous EPS, MAGs provide the
205 exact recipe for how to synthesize them. Genetically encoded systems for EPS
206 biogenesis can be predicted by bioinformatic approaches such as genome annotation
207 and pathway modeling. However, EPS identified purely through bioinformatics and
208 molecular methods remain theoretical extracellular polymers only. Hence, validation
209 through biophysical and chemical characterization of isolated reference compounds
210 will be required.

211 Sequencing and molecular techniques can enable recombinant model systems to to be
212 designed to produce extracellular proteins for chemical and biophysical
213 characterization, where the proposed extracellular proteins are expressed and isolated
214 from bacteria with little or no biofilm production, as is the case for common
215 laboratory strains of *E. coli* and *B. subtilis* (Dueholm et al. 2010). Such proteins can
216 even be used to generate antibodies that can be applied for *in situ* analyses.
217 Furthermore, identifying the genetic blueprints for the synthesis of reference polymers
218 would allow us to identify related systems by homology searches (Dueholm et al.
219 2012) and employ transcriptomics to determine how such genes are regulated in
220 response to environmental factors. Liquid chromatography combined with tandem
221 mass spectrometry (LC-MS/MS) could confirm that theoretical extracellular proteins
222 are expressed in complex samples (Cox et al. 2014). LC-MS/MS may also provide

223 information on chemical modifications, which could be relevant for their functions.
224 However, while methods for high throughput protein identification are well-
225 established, the same advances have not been achieved for extracellular
226 polysaccharide analysis due to the structural diversity of carbohydrates (Wang et al.
227 2017, Zhao and Jensen 2009). Furthermore, the reliability of current methods, e.g. for
228 polysaccharide quantification by colorimetric methods, is impaired by other
229 chromogenic compounds (i.e. interference) and non-representative reference sugars
230 (Le and Stuckey 2016).

231 ***In situ* approaches have an important role to play**

232 New and combined imaging techniques offer the opportunity to link the production of
233 specific EPS components with specific bacterial groups *in situ*, as well as validate
234 whether the isolated polymers are indeed extracellular. Imaging provides a link
235 between genomic information and how the EPS are distributed throughout the biofilm
236 (i.e. with regards to location), whereby changes in microbial cells and matrix
237 composition can be monitored over time and together with changes in environmental
238 parameters. Advanced imaging techniques can be combined with increasingly
239 sophisticated computational analyses to describe microbial behavior quantitatively
240 with greater precision (Neu et al. 2010). Laser scanning microscopy coupled with
241 fluorescent staining has proven to be the most flexible approach for imaging biofilm
242 EPS (Neu and Lawrence 2014). Key fluorescence approaches include selective
243 fluorogenic staining (e.g. TOTO-1 for DNA (Okshevsky and Meyer 2014), NileRed
244 for lipids (Rumin et al. 2015), Sypro/NanoOrange and epicocconone for proteins
245 (Randrianjatovo et al. 2015, Zubkov et al. 1999), lectins for analysis of EPS
246 glycoconjugates (Neu and Kuhlicke 2017), and EPS specific antibodies, e.g., WO1

247 for amyloid proteins (Poul et al. 2007)). By combining EPS microscopy with
248 fluorescence *in situ* hybridization (FISH), EPS production can potentially be linked to
249 specific bacterial taxa (Bennke et al. 2013, Tawakoli et al. 2017).

250 Finally, chemical imaging could become a key tool for analyzing complex microbial
251 communities, and bridging isolation and *in situ* characterization studies. Particularly
252 relevant techniques include FTIR imaging, Raman microscopy, nanoSIMS and ToF-
253 SIMS as well as synchrotron-based imaging such as STXM, although some problems
254 still need to be addressed, such as correlated imaging (suitable mounting and probes)
255 and scale of observation (covered area and depth) (Gowen et al. 2015, Lawrence et al.
256 2003, Marshall et al. 2014).

257 **Can EPS recovery help us to move towards a circular economy?**

258 A better understanding of the EPS matrix will lead to improved strategies for both
259 resource recovery and biofilm management in water and wastewater treatment
260 systems. The growing interest in renewable resources highlights a focus on the
261 production of EPS from waste biomass, and their conversion into bioproducts and
262 biomaterials, as an appealing route for contributing to a reduced economic
263 dependence on fossil fuels (More et al. 2016) and enhanced sustainability and
264 economics of wastewater treatment (Lin et al. 2015). EPS-like polymers
265 (hydrocolloids) cannot, in general, be derived from oil-based chemicals, and hence
266 supply relies solely on natural resources. Wastewater derived hydrocolloids could be
267 an important new supply route. A better understanding of the metabolic pathways
268 involved in EPS biosynthesis, molecular composition, interactions with other
269 materials and structure-function relationships would lead to the identification of new
270 applications and markets for EPS, ensure stable and cost-effective production of

271 biopolymers from waste biomass and wastewater, and provide a step towards
272 successful development of extracellular polymer-based bioproducts.

273 **Improved bioprocess control through EPS management**

274 The optimum strategy for biofilm control depends largely on whether EPS production
275 is beneficial (e.g. granular sludges) or detrimental (e.g. membrane bioreactors,
276 infections or biofouling). For both outcomes, altering the mechanical properties of
277 biofilms may improve the process management. Changing either the EPS constituents
278 that are present or how they interact with each other, will modify biofilm cohesive
279 strength, viscosity or elasticity. This can allow for easier removal of biofilms from
280 filters by backwashing or to select for rapid settling of granular sludge in high
281 throughput wastewater processes. There are several strategies available to change the
282 mechanical stability of biofilms, including the use of enzymes, (e.g., lipases,
283 hydrolases, proteases), oxidants (e.g., Cl_2), chelators (e.g., EDTA), or temperature
284 (Jones et al. 2011, Stewart 2014). The current shortcomings in our understanding of
285 EPS make these approaches highly empirical and less effective. A better
286 understanding of the EPS composition, configuration, and interactions among
287 constituents will inform on more effective and targeted chemical interventions.

288 If we understood more about which EPS are present, what they are doing and how
289 their expression is regulated, another strategy targeting biofilm mechanics could be to
290 modulate EPS secretion. This would allow for biofilms to be engineered to have more
291 desirable properties, such as reduced adhesion and increased permeability. Thus,
292 membrane reactor performances are improved. EPS secretion could be regulated by
293 applying different growth or operating conditions. Certain growth conditions, such as
294 nutrient-limitation, feast-famine or extended solid retention time, may increase

295 exopolysaccharide secretion. In membrane biofilters, excessive exopolysaccharide
296 production reduces biofilm permeability and thus throughput of drinking water
297 (Desmond et al. 2018). Supplementing process waters with phosphorus can increase
298 biofilm permeability and reduce membrane head loss (Lauderdale and Brown 2010).
299 In conventional membrane systems, however, phosphorus limitation may prevent
300 microbial growth and biofouling (Vrouwenvelder et al. 2010). While hydraulic
301 conditions are known to influence biofilm morphology (Fish et al. 2017, van
302 Loosdrecht et al. 1995), the exact relationship between reactor hydraulics and EPS
303 production has not yet been elucidated. A better understanding the genomic regulation
304 of EPS formation and the factors that influence it could yield a real breakthrough.
305 This might allow for advanced control of mixed microbial communities with respect
306 to EPS, as is currently under development for pure cultures (Ha and O'Toole 2015).

307 Establishing the means to control biofilm EPS is thus crucial for improved
308 management of our water resources and to stave off the emergence of multi-drug
309 resistant pathogens. Before we can benefit from better control and engineering of
310 biofilm-based systems in water treatment, identify alternative antimicrobial therapies,
311 and recover EPS as a bioresource, we need to go beyond describing the EPS in terms
312 of the exopolymer classes present and identify exactly which molecules contribute to
313 specific biofilm functions. This involves an integrated, multidisciplinary approach on
314 biofilms and molecular isolates (summarized in Figure 2). Improved EPS extraction
315 methods, advanced imaging, chemical characterization, and genetic and biophysical
316 analyses, need to be applied to biofilms and EPS isolates alike.

317

318 **Conclusions**

319 A better understanding of the EPS will increase the breadth of strategies available for
320 controlling biofilms in water, wastewater and medical systems alike, which are
321 currently unreliable, empirical and binary (at best). A variety of complementary
322 approaches is required, to overcome extraction and analysis biases, as well as
323 knowledge constraints regarding, for example, exopolymer references in databases.

324 Required developments include:

- 325 - Extraction methods targeting full solubilization of key structural and
326 functional EPS, with a preparedness to use harsh methods if necessary,
327 contingent on using methods to verify the intra- or extra-cellular origin of the
328 analyzed molecules;
- 329 - Chemical characterization methods to identify the exact molecular structure;
- 330 - *In situ* methods for verifying the identity, distribution and function of the EPS
331 (biophysical, imaging with fluorescent or nanoparticle-based probes and
332 chemical profiling); and
- 333 - Model biofilm systems to cross-reference industrially and medically-relevant
334 systems.

335

336

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347 **References**

348 Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K.L., Tyson, G.W. and
349 Nielsen, P.H. (2013) Genome sequences of rare, uncultured bacteria obtained by
350 differential coverage binning of multiple metagenomes. *Nature Biotechnology*
351 31, 533.

352 Bengtsson, S., de Blois, M., Wilén, B.-M. and Gustavsson, D. (2018) Treatment of
353 municipal wastewater with aerobic granular sludge. *Critical Reviews in*
354 *Environmental Science and Technology* 48(2), 119-166.

355 Bennke, C.M., Neu, T.R., Fuchs, B.M. and Amann, R. (2013) Mapping
356 glycoconjugate-mediated interactions of marine Bacteroidetes with diatoms.
357 *Systematic and Applied Microbiology* 36(6), 417-425.

358 Borlee, B.R., Goldman, A.D., Murakami, K., Samudrala, R., Wozniak, D.J. and
359 Parsek, M.R. (2010) *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated
360 adhesin to reinforce the biofilm extracellular matrix. *Molecular Microbiology*
361 75(4), 827-842.

362 Colvin, K.M., Irie, Y., Tart, C.S., Urbano, R., Whitney, J.C., Ryder, C., Howell, P.L.,
363 Wozniak, D.J. and Parsek, M.R. (2012) The Pel and Psl polysaccharides provide
364 *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix.
365 *Environmental microbiology* 14(8), 10.1111/j.1462-2920.2011.02657.x.

366 Cox, J., Hein, M.Y., Lubber, C.A., Paron, I., Nagaraj, N. and Mann, M. (2014) Accurate
367 Proteome-wide Label-free Quantification by Delayed Normalization and Maximal

- 368 Peptide Ratio Extraction, Termed MaxLFQ. *Molecular & Cellular Proteomics* :
369 MCP 13(9), 2513-2526.
- 370 Cummings, Richard D. and Pierce, J.M. (2014) The Challenge and Promise of
371 Glycomics. *Chemistry & Biology* 21(1), 1-15.
- 372 Derlon, N., Peter-Varbanets, M., Scheidegger, A., Pronk, W. and Morgenroth, E.
373 (2012) Predation influences the structure of biofilm developed on ultrafiltration
374 membranes. *Water Research* 46(10), 3323-3333.
- 375 Desmond, P., Best, J.P., Morgenroth, E. and Derlon, N. (2018) Linking composition
376 of extracellular polymeric substances (EPS) to the physical structure and
377 hydraulic resistance of membrane biofilms. *Water Research* 132, 211-221.
- 378 Dueholm, M.S., Albertsen, M., Otzen, D. and Nielsen, P.H. (2012) Curli Functional
379 Amyloid Systems Are Phylogenetically Widespread and Display Large Diversity
380 in Operon and Protein Structure. *PLOS ONE* 7(12), e51274.
- 381 Dueholm, M.S., Petersen, S.V., Sønderkær, M., Larsen, P., Christiansen, G., Hein,
382 K.L., Enghild, J.J., Nielsen, J.L., Nielsen, K.L., Nielsen, P.H. and Otzen, D.E. (2010)
383 Functional amyloid in *Pseudomonas*. *Molecular Microbiology* 77(4), 1009-1020.
- 384 Felz, S., Al-Zuhairy, S., Aarstad, O.A., van Loosdrecht, M.C.M. and Lin, Y.M. (2016)
385 Extraction of Structural Extracellular Polymeric Substances from Aerobic
386 Granular Sludge. *Journal of Visualized Experiments : JoVE* (115), 54534.
- 387 Fish, K., Osborn, A.M. and Boxall, J.B. (2017) Biofilm structures (EPS and bacterial
388 communities) in drinking water distribution systems are conditioned by
389 hydraulics and influence discoloration. *Science of The Total Environment* 593-
390 594, 571-580.
- 391 Flemming, H.-C. and Wingender, J. (2010) The biofilm matrix. *Nature Reviews*
392 *Microbiology* 8, 623.

- 393 Frieri, M., Kumar, K. and Boutin, A. (2017) Antibiotic resistance. Journal of
394 Infection and Public Health 10(4), 369-378.
- 395 Gowen, A.A., Feng, Y., Gaston, E. and Valdramidis, V. (2015) Recent applications
396 of hyperspectral imaging in microbiology. Talanta 137, 43-54.
- 397 Ha, D.-G. and O'Toole, G.A. (2015) c-di-GMP and its effects on biofilm formation
398 and dispersion: a *Pseudomonas aeruginosa* review. Microbiology spectrum 3(2),
399 10.1128/microbiolspec.MB-0003-2014.
- 400 Hao, L.-P., Mcllroy, S.J., Kirkegaard, R.H.H., Karst, S.M., Fernando, W.E.Y., Aslan, H.,
401 Meyer, R.L., Albertsen, M., Nielsen, P.H. and Dueholm, M.S. (2017) Novel
402 prosthecate bacteria from the candidate phylum Acetothermia revealed by
403 culture-independent genomics and advanced microscopy. bioRxiv.
- 404 Hiroshi, N., Kenji, K. and Luel, L.B. (2012) Lipoproteins in bacteria: structures
405 and biosynthetic pathways. The FEBS Journal 279(23), 4247-4268.
- 406 Jennings, L.K., Storek, K.M., Ledvina, H.E., Coulon, C., Marmont, L.S., Sadovskaya,
407 I., Secor, P.R., Tseng, B.S., Scian, M., Filloux, A., Wozniak, D.J., Howell, P.L. and
408 Parsek, M.R. (2015) Pel is a cationic exopolysaccharide that cross-links
409 extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. Proceedings of
410 the National Academy of Sciences 112(36), 11353-11358.
- 411 Jones, W.L., Sutton, M.P., McKittrick, L. and Stewart, P.S. (2011) Chemical and
412 antimicrobial treatments change the viscoelastic properties of bacterial biofilms.
413 Biofouling 27(2), 207-215.
- 414 Julio, S.M. and Cotter, P.A. (2005) Characterization of the Filamentous
415 Hemagglutinin-Like Protein FhaS in *Bordetella bronchiseptica*. Infection and
416 Immunity 73(8), 4960-4971.

- 417 Kumari, S. and Rath, P.K. (2014) Extraction and Characterization of Chitin and
418 Chitosan from (*Labeo rohita*) Fish Scales. *Procedia Materials Science* 6, 482-489.
- 419 Lauderdale, C.V. and Brown, J.C. (2010) Low-level phosphorus supplementation
420 enhances biofiltration hydraulic performances and treatment efficiency.
421 *Proceeding of the Water Environment Federation* 2010(7), 472-475.
- 422 Lawrence, J.R., Swerhone, G.D.W., Leppard, G.G., Araki, T., Zhang, X., West, M.M.
423 and Hitchcock, A.P. (2003) Scanning Transmission X-Ray, Laser Scanning, and
424 Transmission Electron Microscopy Mapping of the Exopolymeric Matrix of
425 Microbial Biofilms. *Applied and Environmental Microbiology* 69(9), 5543-5554.
- 426 Le, C., Kunacheva, C. and Stuckey, D.C. (2016) "Protein" Measurement in
427 Biological Wastewater Treatment Systems: A Critical Evaluation. *Environmental
428 Science & Technology* 50(6), 3074-3081.
- 429 Le, C. and Stuckey, D.C. (2016) Colorimetric measurement of carbohydrates in
430 biological wastewater treatment systems: A critical evaluation. *Water Research*
431 94, 280-287.
- 432 Le, N.L. and Nunes, S.P. (2016) Materials and membrane technologies for water
433 and energy sustainability. *Sustainable Materials and Technologies* 7, 1-28.
- 434 Liang, H., DeMeester, K.E., Hou, C.-W., Parent, M.A., Caplan, J.L. and Grimes, C.L.
435 (2017) Metabolic labelling of the carbohydrate core in bacterial peptidoglycan
436 and its applications. *Nature Communications* 8, 15015.
- 437 Lin, Y., de Kreuk, M., van Loosdrecht, M.C.M. and Adin, A. (2010) Characterization
438 of alginate-like exopolysaccharides isolated from aerobic granular sludge in
439 pilot-plant. *Water Research* 44(11), 3355-3364.
- 440 Lin, Y., Reino, C., Carrera, J., Pérez, J. and van Loosdrecht, M.C.M. (2018)
441 Glycosylated amyloid - like proteins in the structural extracellular polymers of

- 442 aerobic granular sludge enriched with ammonium - oxidizing bacteria.
443 MicrobiologyOpen 0(0), e00616.
- 444 Lin, Y.M., Nierop, K.G.J., Girbal-Neuhauser, E., Adriaanse, M. and van Loosdrecht,
445 M.C.M. (2015) Sustainable polysaccharide-based biomaterial recovered from
446 waste aerobic granular sludge as a surface coating material. Sustainable
447 Materials and Technologies 4, 24-29.
- 448 Lin, Y.M., Sharma, P.K. and van Loosdrecht, M.C.M. (2013) The chemical and
449 mechanical differences between alginate-like exopolysaccharides isolated from
450 aerobic flocculent sludge and aerobic granular sludge. Water Research 47(1), 57-
451 65.
- 452 Lipke, P.N. (2016) Glycomics for Microbes and Microbiologists. mBio 7(4).
- 453 Liu, Y., Defourny, K.A.Y., Smid, E.J. and Abee, T. (2018) Gram-Positive Bacterial
454 Extracellular Vesicles and Their Impact on Health and Disease. Frontiers in
455 Microbiology 9(1502).
- 456 Madaeni, S.S. (1999) The application of membrane technology for water
457 disinfection. Water Research 33(2), 301-308.
- 458 Marshall, M.J., Belchik, S.M., Tucker, A.E., Chrisler, W.B., Thomas, M., Renslow,
459 R.S., Kuprat, A.P., Dohnalkova, A.C. and Hirschmugl, C.J. (2014) Chemical Imaging
460 of Biofilms: The Integration of Synchrotron Imaging, Electron Microscopy and
461 Nuclear Magnetic Resonance (NMR) Technologies. Microscopy and
462 Microanalysis 20(S3), 1178-1179.
- 463 Marvasi, M., Visscher, P.T. and Casillas Martinez, L. (2010) Exopolymeric
464 substances (EPS) from *Bacillus subtilis* : polymers and genes encoding their
465 synthesis. FEMS Microbiology Letters 313(1), 1-9.

- 466 Mcllroy, S.J., Kirkegaard, R.H., Dueholm, M.S., Fernando, E., Karst, S.M., Albertsen,
467 M. and Nielsen, P.H. (2017) Culture-Independent Analyses Reveal Novel
468 Anaerolineaceae as Abundant Primary Fermenters in Anaerobic Digesters
469 Treating Waste Activated Sludge. *Frontiers in Microbiology* 8(1134).
- 470 More, T.T., Yan, S., Tyagi, R.D. and Surampalli, R.Y. (2016) Biopolymer Production
471 Kinetics of Mixed Culture Using Wastewater Sludge as a Raw Material and the
472 Effect of Different Cations on Biopolymer Applications in Water and Wastewater
473 Treatment. *Water Environment Research* 88(5), 425-437.
- 474 Nakao, R., Ramstedt, M., Wai, S.N. and Uhlin, B.E. (2012) Enhanced Biofilm
475 Formation by *Escherichia coli* LPS Mutants Defective in Hep Biosynthesis. *PLOS*
476 *ONE* 7(12), e51241.
- 477 Neu, T. and Kuhlicke, U. (2017) Fluorescence Lectin Bar-Coding of
478 Glycoconjugates in the Extracellular Matrix of Biofilm and Bioaggregate Forming
479 Microorganisms. *Microorganisms* 5(1), 5.
- 480 Neu, T.R. (1996) Significance of bacterial surface-active compounds in
481 interaction of bacteria with interfaces. *Microbiological Reviews* 60(1), 151-166.
- 482 Neu, T.R. and Lawrence, J.R. (2014) *Microbial Biofilms: Methods and Protocols*.
483 Donelli, G. (ed), pp. 43-64, Springer New York, New York, NY.
- 484 Neu, T.R., Manz, B., Volke, F., Dynes, J.J., Hitchcock, A.P. and Lawrence, J.R. (2010)
485 Advanced imaging techniques for assessment of structure, composition and
486 function in biofilm systems. *FEMS Microbiology Ecology* 72(1), 1-21.
- 487 Ogawa, H., Amagai, Y., Koike, I., Kaiser, K. and Benner, R. (2001) Production of
488 Refractory Dissolved Organic Matter by Bacteria. *Science* 292(5518), 917-920.

- 489 Okshevsky, M. and Meyer, R.L. (2014) Evaluation of fluorescent stains for
490 visualizing extracellular DNA in biofilms. *Journal of Microbiological Methods*
491 105, 102-104.
- 492 Poul, L., Lund, N.J., Simonsen, D.M., Ronald, W., Daniel, O. and Halkjær, N.P.
493 (2007) Amyloid adhesins are abundant in natural biofilms. *Environmental*
494 *microbiology* 9(12), 3077-3090.
- 495 Pronk, M., Neu, T.R., van Loosdrecht, M.C.M. and Lin, Y.M. (2017) The acid soluble
496 extracellular polymeric substance of aerobic granular sludge dominated by
497 *Deffluviicoccus* sp. *Water Research* 122, 148-158.
- 498 Randrianjatovo, I., Girbal-Neuhauser, E. and Marcato-Romain, C.-E. (2015)
499 Epicocconone, a sensitive and specific fluorescent dye for in situ quantification of
500 extracellular proteins within bacterial biofilms. *Applied Microbiology and*
501 *Biotechnology* 99(11), 4835-4844.
- 502 Ras, M., Lefebvre, D., Derlon, N., Paul, E. and Girbal-Neuhauser, E. (2011)
503 Extracellular polymeric substances diversity of biofilms grown under contrasted
504 environmental conditions. *Water Research* 45(4), 1529-1538.
- 505 Romero, D., Aguilar, C., Losick, R. and Kolter, R. (2010) Amyloid fibers provide
506 structural integrity to *Bacillus subtilis* biofilms. *Proceedings of the National*
507 *Academy of Sciences* 107(5), 2230-2234.
- 508 Rosenberg, E. and Ron, E.Z. (1999) High- and low-molecular-mass microbial
509 surfactants. *Applied Microbiology and Biotechnology* 52(2), 154-162.
- 510 Rumin, J., Bonnefond, H., Saint-Jean, B., Rouxel, C., Sciandra, A., Bernard, O.,
511 Cadoret, J.-P. and Bougaran, G. (2015) The use of fluorescent Nile red and
512 BODIPY for lipid measurement in microalgae. *Biotechnology for Biofuels* 8, 42.

- 513 Sand, W. and Gehrke, T. (2006) Extracellular polymeric substances mediate
514 bioleaching/biocorrosion via interfacial processes involving iron(III) ions and
515 acidophilic bacteria. *Research in Microbiology* 157(1), 49-56.
- 516 Saunders, A.M., Albertsen, M., Vollertsen, J. and Nielsen, P.H. (2016) The activated
517 sludge ecosystem contains a core community of abundant organisms. *The ISME*
518 *Journal* 10(1), 11-20.
- 519 Schurig, C., Smittenberg, R.H., Berger, J., Kraft, F., Woche, S.K., Goebel, M.-O.,
520 Heipieper, H.J., Miltner, A. and Kaestner, M. (2013) Microbial cell-envelope
521 fragments and the formation of soil organic matter: a case study from a glacier
522 forefield. *Biogeochemistry* 113(1), 595-612.
- 523 Secor, Patrick R., Sweere, Johanna M., Michaels, Lia A., Malkovskiy, Andrey V.,
524 Lazzareschi, D., Katznelson, E., Rajadas, J., Birnbaum, Michael E., Arrigoni, A.,
525 Braun, Kathleen R., Evanko, Stephen P., Stevens, David A., Kaminsky, W., Singh,
526 Pradeep K., Parks, William C. and Bollyky, Paul L. (2015) Filamentous
527 Bacteriophage Promote Biofilm Assembly and Function. *Cell Host & Microbe*
528 18(5), 549-559.
- 529 Serra, D.O., Richter, A.M. and Hengge, R. (2013) Cellulose as an Architectural
530 Element in Spatially Structured *Escherichia coli* Biofilms. *Journal of Bacteriology*
531 195(24), 5540-5554.
- 532 Seviour, T., Donose, B.C., Pijuan, M. and Yuan, Z. (2010a) Purification and
533 Conformational Analysis of a Key Exopolysaccharide Component of Mixed
534 Culture Aerobic Sludge Granules. *Environmental Science & Technology* 44(12),
535 4729-4734.
- 536 Seviour, T., Hansen, S.H., Yang, L., Yau, Y.H., Wang, V.B., Stenvang, M.R.,
537 Christiansen, G., Marsili, E., Givskov, M., Chen, Y., Otzen, D.E., Nielsen, P.H.,

- 538 Shochat, S.G., Kjelleberg, S. and Dueholm, M.S. (2015a) Functional Amyloids Keep
539 Quorum Sensing Molecules in Check. *Journal of Biological Chemistry*.
- 540 Seviour, T., Lambert, L.K., Pijuan, M. and Yuan, Z. (2010b) Structural
541 Determination of a Key Exopolysaccharide in Mixed Culture Aerobic Sludge
542 Granules Using NMR Spectroscopy. *Environmental Science & Technology* 44(23),
543 8964-8970.
- 544 Seviour, T., Malde, A.K., Kjelleberg, S., Yuan, Z. and Mark, A.E. (2012a) Molecular
545 Dynamics Unlocks Atomic Level Self-Assembly of the Exopolysaccharide Matrix
546 of Water-Treatment Granular Biofilms. *Biomacromolecules* 13(6), 1965-1972.
- 547 Seviour, T., Weerachanchai, P., Hinks, J., Roizman, D., Rice, S.A., Bai, L., Lee, J.-M.
548 and Kjelleberg, S. (2015b) Solvent optimization for bacterial extracellular
549 matrices: a solution for the insoluble. *RSC Advances* 5(10), 7469-7478.
- 550 Seviour, T., Yuan, Z., van Loosdrecht, M.C.M. and Lin, Y. (2012b) Aerobic sludge
551 granulation: A tale of two polysaccharides? *Water Research* 46(15), 4803-4813.
- 552 Sleytr, U.B., Schuster, B., Egelseer, E.-M. and Pum, D. (2014) S-layers: principles
553 and applications. *FEMS Microbiology Reviews* 38(5), 823-864.
- 554 Stewart, P.S. (2014) Biophysics of biofilm infection. *Pathogens and Disease*
555 70(3), 212-218.
- 556 Taglialegna, A., Navarro, S., Ventura, S., Garnett, J.A., Matthews, S., Penades, J.R.,
557 Lasa, I. and Valle, J. (2016) Staphylococcal Bap Proteins Build Amyloid Scaffold
558 Biofilm Matrices in Response to Environmental Signals. *PLOS Pathogens* 12(6),
559 e1005711.
- 560 Tawakoli, P.N., Neu, T.R., Busck, M.M., Kuhlicke, U., Schramm, A., Attin, T.,
561 Wiedemeier, D.B. and Schlafer, S. (2017) Visualizing the dental biofilm matrix by

562 means of fluorescence lectin-binding analysis. *Journal of Oral Microbiology* 9(1),
563 1345581.

564 Tielen, P., Kuhn, H., Rosenau, F., Jaeger, K.-E., Flemming, H.-C. and Wingender, J.
565 (2013) Interaction between extracellular lipase LipA and the polysaccharide
566 alginate of *Pseudomonas aeruginosa*. *BMC Microbiology* 13, 159-159.

567 Turnbull, L., Toyofuku, M., Hynen, A.L., Kurosawa, M., Pessi, G., Petty, N.K., Osvath,
568 S.R., Cárcamo-Oyarce, G., Gloag, E.S., Shimoni, R., Omasits, U., Ito, S., Yap, X.,
569 Monahan, L.G., Cavaliere, R., Ahrens, C.H., Charles, I.G., Nomura, N., Eberl, L. and
570 Whitchurch, C.B. (2016) Explosive cell lysis as a mechanism for the biogenesis of
571 bacterial membrane vesicles and biofilms. *Nature Communications* 7, 11220.

572 van Loosdrecht, M.C.M., Eikelboom, D., Gjaltema, A., Mulder, A., Tjihuis, L. and
573 Heijnen, J.J. (1995) Biofilm structures. *Water Science and Technology* 32(8), 35-
574 43.

575 Vrouwenvelder, J.S., Beyer, F., Dahmani, K., Hasan, N., Galjaard, G., Kruithof, J.C.
576 and Van Loosdrecht, M.C.M. (2010) Phosphate limitation to control biofouling.
577 *Water Research* 44(11), 3454-3466.

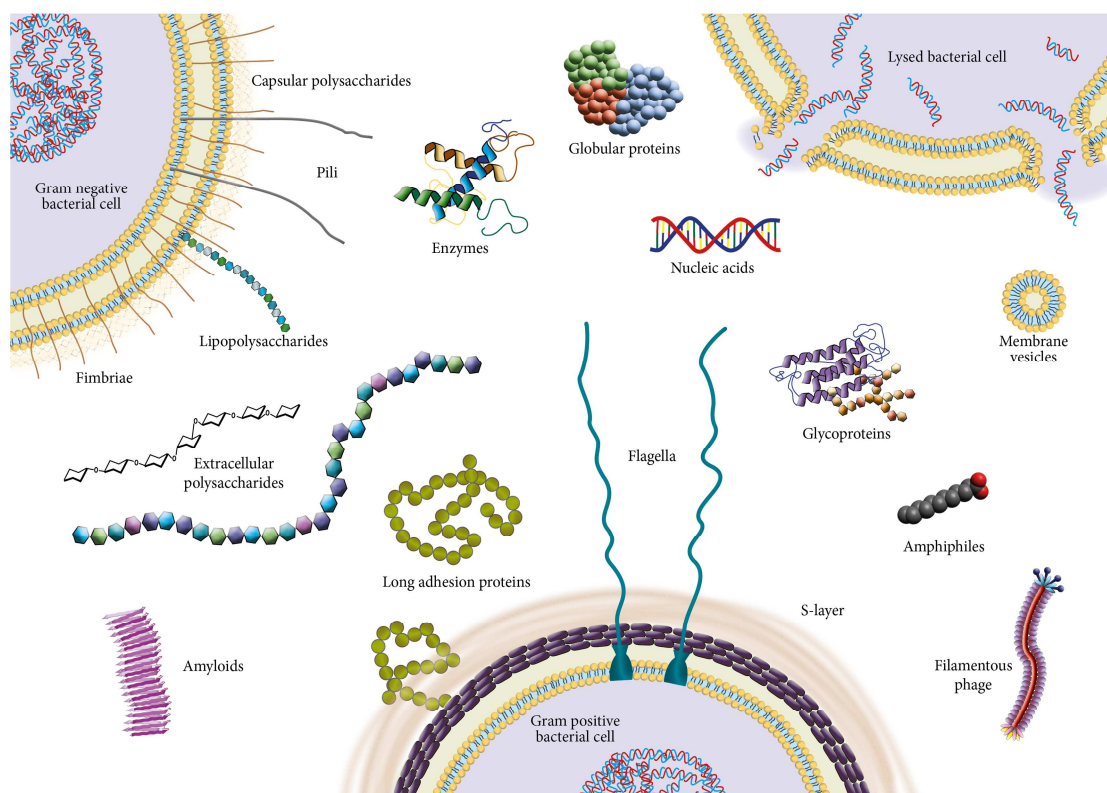
578 Wagner, M., Ivleva, N.P., Haisch, C., Niessner, R. and Horn, H. (2009) Combined
579 use of confocal laser scanning microscopy (CLSM) and Raman microscopy (RM):
580 Investigations on EPS – Matrix. *Water Research* 43(1), 63-76.

581 Wang, C., Gao, X., Chen, Z., Chen, Y. and Chen, H. (2017) Preparation,
582 Characterization and Application of Polysaccharide-Based Metallic
583 Nanoparticles: A Review. *Polymers* 9(12), 689.

584 Wang, H., Wilksch, J.J., Strugnell, R.A. and Gee, M.L. (2015) Role of Capsular
585 Polysaccharides in Biofilm Formation: An AFM Nanomechanics Study. *ACS*
586 *Applied Materials & Interfaces* 7(23), 13007-13013.

- 587 Whitfield, G.B., Marmont, L.S. and Howell, P.L. (2015) Enzymatic modifications of
588 exopolysaccharides enhance bacterial persistence. *Frontiers in Microbiology* 6,
589 471.
- 590 Younes, I. and Rinaudo, M. (2015) Chitin and Chitosan Preparation from Marine
591 Sources. Structure, Properties and Applications. *Marine Drugs* 13(3), 1133-1174.
- 592 Zhang, J., Wu, J., Yu, J., Zhang, X., He, J. and Zhang, J. (2017) Application of ionic
593 liquids for dissolving cellulose and fabricating cellulose-based materials: state of
594 the art and future trends. *Materials Chemistry Frontiers* 1(7), 1273-1290.
- 595 Zhao, Y. and Jensen, O.N. (2009) Modification - specific proteomics: Strategies
596 for characterization of post - translational modifications using enrichment
597 techniques. *PROTEOMICS* 9(20), 4632-4641.
- 598 Zubkov, M.V., Fuchs, B.M., Eilers, H., Burkill, P.H. and Amann, R. (1999)
599 Determination of Total Protein Content of Bacterial Cells by SYPRO Staining and
600 Flow Cytometry. *Applied and Environmental Microbiology* 65(7), 3251-3257.
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Box 1: Description of EPS found in the extracellular matrix of various biofilms

Amphiphiles (Neu 1996, Sand and Gehrke 2006): glycolipids (e.g. emulsan) and lipoproteins (Hiroshi et al. 2012), which along with microbially-derived humic-like compounds play key roles in interface interactions (Ogawa et al. 2001, Rosenberg and Ron 1999, Schurig et al. 2013).

Long adhesion proteins e.g. CdrA of *Pseudomonas aeruginosa* (Borlee et al. 2010), Biofilm associated protein of *Staphylococcus aureus* (Taglialegna et al. 2016).

Extracellular proteins: Exoenzymes e.g. lipase (Tielen et al. 2013), polypeptides.

Amyloids: e.g. Functional amyloids of *Pseudomonas* (Fap) (Dueholm et al. 2010), TasA of *Bacillus subtilis* (Romero et al. 2010) and curli of *Escherichia coli* (Dueholm et al. 2012).

Extracellular polysaccharides: anionic e.g. alginate-like exopolysaccharides (Lin et al. 2010), cationic e.g. Pel (Jennings et al. 2015), neutral e.g. cellulose (Serra et al. 2013), amphiprotic e.g. granulan (Seviour et al. 2010b).

Membrane vesicles: Enzyme-filled blebs from the outer membranes of G(-)(Turnbull et al. 2016) and G(+)(Liu et al. 2018) cells.

Nucleic acids: i.e. extracellular DNA (Turnbull et al. 2016).

Lipopolysaccharides: Involved in cell recognition and immunity (Nakao et al. 2012).

Filamentous phage: e.g. Pf4 bacteriophage in *Pseudomonas aeruginosa* (Secor et al. 2015).

Glycoproteins: e.g. Glycosylated amyloid-like proteins (Lin et al. 2018).

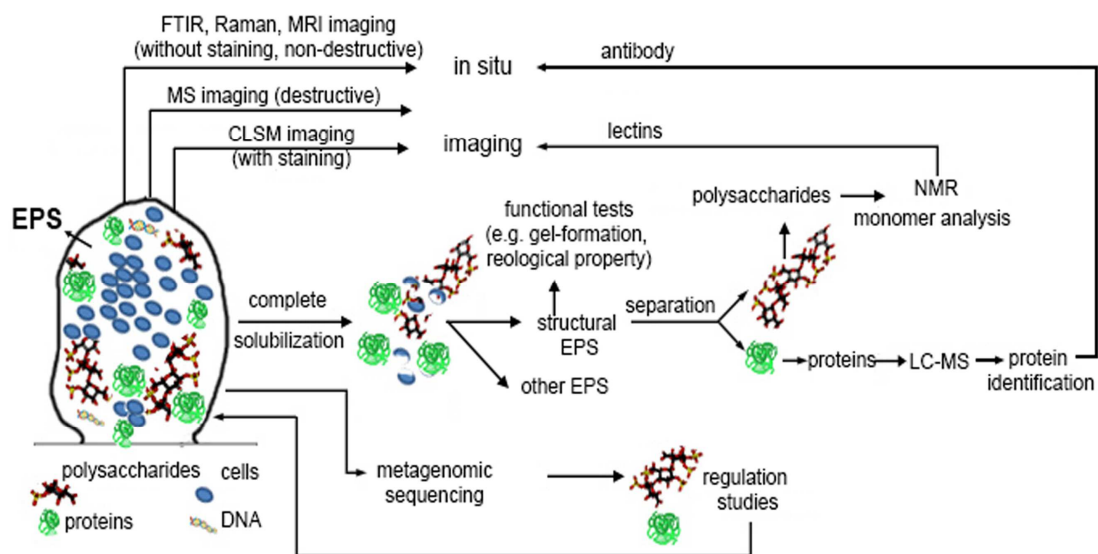
Capsular polysaccharides: i.e. surface-attached polysaccharides (Wang et al. 2015).

Pili: Hair-like appendage on bacterial surface composed of pilin proteins.

S-layer: external layer of cell envelope consisting of proteins or glycoproteins (Sleytr et al. 2014).

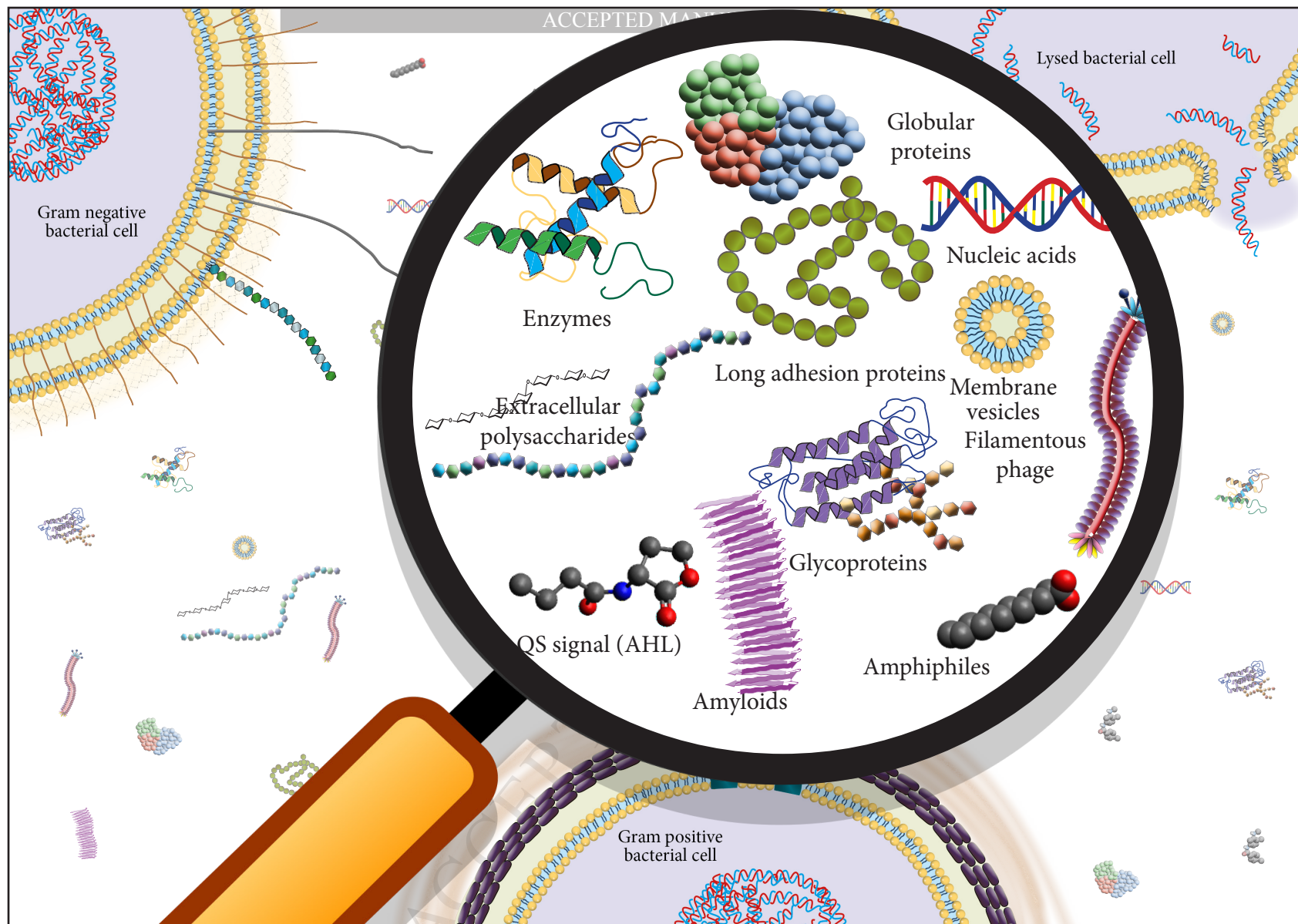
Figure 1: Illustration of exopolymers typically found in the extracellular matrix of biofilms. Note, such constituents have been identified from a range of biofilms, and not all matrices contain each of these components. Refer to Box 1 for a description of each exopolymer.

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Figure 2: Proposed multidisciplinary roadmap for resolving the identities and functions of extracellular polymeric substances in biofilms, involving complementary chemical, biophysical and 'omic' analysis of biofilms and isolated constituents.



Highlights

- Extracellular polymeric substances feature in key societal problems (clinical, environmental)
- Methods and standards of EPS recovery and characterization need to be critically assessed
- More emphasis should be placed on methods that enable identification (chemical and function)
- Integrated and multi-disciplinary analyses are required on biofilms and EPS isolates
- Will improve biofilm management and enable a more circular economy in water and waste