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Contribution of single-cell omics to microbial ecology

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19 **Highlights**

20 Microbes are involved in many ecosystems but remain understudied mostly due to
21 technical limitations.

22 Applying -omics technologies to microbes involve changes in the scales of ecological
23 studies.

24 Single-cell omics offer the opportunity to study microbes at a finer scale than meta-
25 omics tools.

26 Single microbial cell omics enable to assess and explore the dynamics of genetic
27 changes from individuals to higher levels of ecological complexity.

28

29 **Abstract**

30 Microorganisms play key roles in various ecosystems but many of their functions and
31 interactions remain undefined. To investigate the ecological relevance of microbial
32 communities, new molecular tools are being developed. Among them, single-cell
33 omics assessing genetic diversity at the population and community level and linking
34 each individual cell to its functions is gaining interest in microbial ecology. By giving
35 access to a bigger range of ecological scales (from individual to community) than
36 culture-based approaches and meta-omics, single-cell omics can contribute to
37 microorganisms genomes and functions identification but also to the testing of
38 concepts in ecology. Here, we discuss the contribution of single-cell omics to
39 possible breakthroughs in concepts and knowledge on microbial ecosystems and
40 eco-evolutionary processes.

41 **Main Text**

42 **Ecological scales**

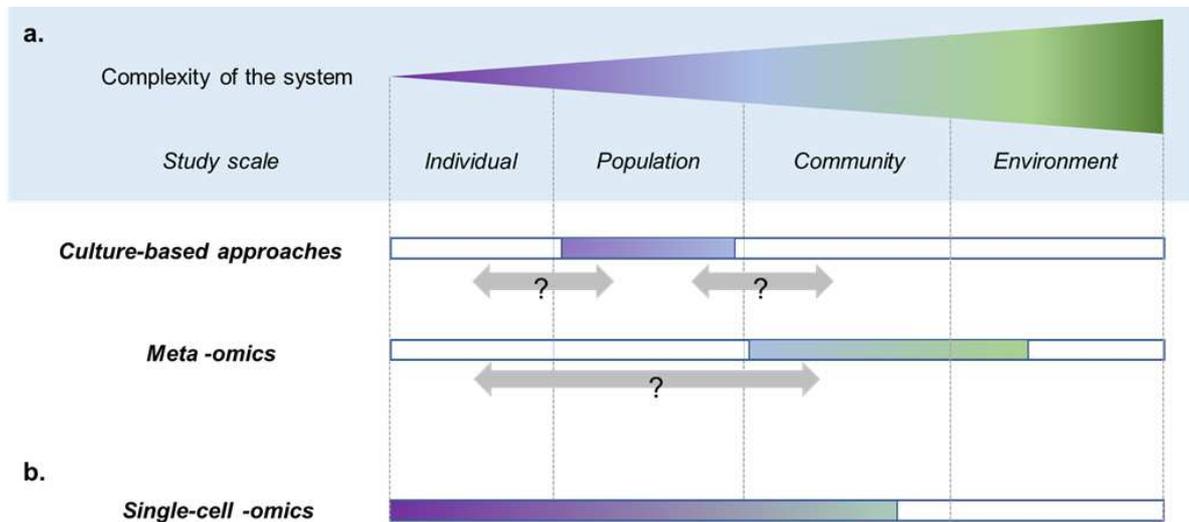
43 Interactions between organisms take place at all organizational levels, from
44 molecules to communities, within or between species, and shape ecosystem
45 dynamics. Ecological interactions are difficult to understand due to the number of
46 biotic and abiotic parameters involved. Assembling knowledge at various ecological
47 scales and from different standpoints is therefore crucial in the study of ecological
48 and evolutionary processes. This is particularly true in the case of microbes, which
49 individuals can be seen as metabolic units involved in complex metabolic networks
50 at much higher ecological scales (e.g. [1,2]). Therefore, accessing genetic and
51 metabolic information of microbes is a necessary step to understand ecosystem
52 functioning. In microbial metabolic units, “small” changes in genomes and metabolic
53 pathways may have significant impact on the microbial community organization and
54 hence on ecosystems. Deciphering large ecological scale processes therefore
55 requires observation of fine ecological scale (i.e. at the individual cell level), which is
56 the biggest challenge of current environmental microbiology [3] (Figure 1a).

57 A fundamental level of organization in ecology is the species. However, due to gene
58 flow between cells that increases with ecological overlap and genetic similarity [4],
59 microbial species concept and thus, **populations** (see Glossary), are not clearly
60 defined entities. Interactions and diversity at the populational level, i.e. between
61 individual cells of the same population (Box 1), is still obscure since it is not often
62 analyzed in environmental microbiology. Given the natural mutation rate in bacteria
63 ($\sim 10^{-7}$ substitution per nucleotide, e.g. [5]), even a single colony contains genetic
64 variations, i.e. variants within a cell population. The population has been suggested

65 to be more relevant than the species level for microbes [6,7] and species usually
66 contain genetically divergent microorganisms. Considering the hierarchical levels of
67 ecology, populations are keys to assessing genetic structure within species and over
68 time, changes therein. They thereby provide insights into eco-evolutionary
69 processes and advance our understanding of microbiota composition dynamics (Box
70 1).

71 Our understanding of the microbial world and its ecological roles is still very limited
72 [8]. Understanding the functions played by microbial cells in a complex community
73 remains a frontier in microbial ecology. Beyond the technical limits that microbiology
74 is facing, the information gathered from culture-based studies or from natural
75 ecosystems can be difficult to interpret (Figure 1a). Laboratory experiments attempt
76 to reproduce optimal ecological conditions for microbes by selecting among the
77 many biotic and environmental parameters [6] in order to understand specific
78 processes such as trait tradeoffs [9], interactions between strains [10], the production
79 of metabolites [11] or genome evolution [12]. Extrapolating observations obtained in
80 vitro, at restricted scales, to higher ecological scales such as natural communities
81 and ecosystems requires particular attention. Conversely, observations made from
82 environmental samples, including microbial community composition, diversity or
83 global functions are less specific and represent an average of the microbial
84 community. Ideally, we want to get the most information out of each level of
85 approach (i.e precise interactions and genetic dynamics from culture-based studies
86 coupled with global function and diversity of a community with meta-omics).
87 However, our ignorance of intricacies and interactions of ecological scales with one
88 another still jeopardize the assembly of the resulting information to answer specific
89 ecological questions in environmental microbiology. Moreover, the uncertainty of

90 community composition and the complexity of microbial interactions [2] make it even
 91 more difficult to target specific scientific hypotheses on natural communities and to
 92 choose the appropriate tools.



93

94 **Figure 1:** Gradient of ecological complexity, study scales and associated
 95 approaches.

96 Although other higher levels of ecology can be used, individual-population-
 97 community-environment scales describe much of the subject of ecology. a) The
 98 culture-based approaches aim at studying microbial populations or a very limited
 99 number of strains, therefore the outcoming data cannot be fully informative about
 100 higher levels of ecology. On the contrary, meta-omics approaches cover from the
 101 community to environmental scales of microbial ecology and does not provide
 102 finer information on the ecological gradient. In both cases, the individual scale is
 103 unattainable while being at the basis of ecological processes. b) Single-cell omics
 104 cover the scales from the individual microbe to the community from the same
 105 environmental sample which allows to connect the outcoming information of each
 106 ecological scale.

107

108 **Specific tools for specific ecological questions**

109 Like in any new field of exploration, ecological patterns within microbial communities
110 are firstly observed and described but poorly understood [13], testifying to the
111 enormous lack of knowledge concerning microbes [14]. The use of DNA- and RNA-
112 based methods to study natural microbial communities have demonstrated the
113 existence of a prodigious wealth of microorganisms which remained unsuspected
114 some years ago (e.g. [15]). Among **meta-omics** techniques, metagenomics and
115 metatranscriptomics are the most widely used methods to explore microbiota. These
116 techniques enabled a breakthrough in our understanding of microbial phylogenetic
117 relationships [16], species diversity and abundance [17], metabolic abilities [18] and
118 functional diversification [19]. The development and use of **metagenome-**
119 **assembled genomes (MAGs)** led to discoveries that advanced our understanding
120 of bacterial life and modified our perception of the tree of life [15,19]. Some studies
121 attempted to reconstitute population level genomes from metagenomes. For
122 example, Crits-Christoph et al. [20] investigated genetic variation within populations
123 of highly abundant soil bacteria by studying MAGs and observed spatial
124 differentiation of alleles. However, inferring population features from meta-omics
125 data remains limited, especially when genomes are inaccurately or incompletely
126 reconstructed from short sequence fragments (Figure 2, Key Figure). The use of
127 MAGs becomes challenging when microbial richness and diversity within a
128 community are high and taxa are phylogenetically close [21]. During genome
129 assembly, stitching of fragments from different individual genomes and/or
130 contaminant DNA can occur, creating chimeras that are irrelevant for the study of

131 populations. In this case, the approach would necessarily conceal a considerable
132 proportion of molecular diversity [22]. In addition, the molecular biology and
133 bioinformatics methods used in meta-omics approaches are varied and based on
134 different criteria and assumptions in the absence of a consensus, leading to
135 contrasting results and interpretations [23,24]. Overall, it might be difficult to directly
136 link the detected functions to their original microbial cell from meta-omics data,
137 thereby limiting the identification of signaling pathways and trade-offs in gene
138 regulation. Meta-omics approaches proved useful in describing communities using
139 large-scale sampling and make it possible to answer questions related to community
140 composition and its associated global functions but not to fully understand the
141 mechanisms underlying these patterns. Nevertheless, bioinformatics research has
142 developed algorithms aiming to identify genetic variations in microbial populations:
143 vertically and horizontally inherited genes can be differentiated, and from population-
144 specific sweeps, single nucleotide polymorphisms can be detected (e.g. DiscoSNP;
145 PopCOGenT) [4, 25].

146 To complement meta-omics data, modelling approaches are used to explore
147 microbial interactions, fluxes of metabolites, and to reconstruct ecological networks
148 in complex microbiomes [26-29]. These approaches provide a possible explanation
149 and scenarios of interactions in natural communities, but sometimes end in
150 contradictions with culture-based experiments [1]. Indeed, models can predict a
151 certain kind of interaction (e.g. cross-feeding) that is not verified or proven wrong in
152 an experimental setup due to the oversight of key parameters such as growing
153 conditions, space and (very often) time [1]. They also rely on the co-occurrence of
154 phenomena, which is more associated with correlations than cause-consequences
155 relationships. Culture-dependent approaches may help reach the population level in

156 simple community compositions through controlled and simplified laboratory-scale
157 experiments [30] and can be effective for testing patterns observed in meta-omics
158 studies and deconstructing mechanistic hypotheses (e.g. interactions, metabolism)
159 (e.g. [31]). The lack of information on individual cells and mostly on populations (i.e.
160 both functions and phylogeny) using existing methods limits our understanding of
161 observed processes. Many studies aim at unravelling microbiota diversity, primarily
162 in plants, soil, water, and animal bodies, but few explain associated community
163 assembly and evolutionary mechanisms [32-34]. In this context, microbiologists and
164 ecologists are searching for other technical possibilities or approaches such as
165 single-cell omics to complete the knowledge provided by current methods.

166 **The alternative scope of single -cell omics**

167 Single-cell whole genome sequencing (scWGS) and single-cell transcriptomics
168 (scRNA-seq) were first developed for eukaryotic cells and used in cancer research,
169 revealing both intra-population genetic diversity and heterogeneous genome
170 expression. As in cancer research where differentiation in space of the genome
171 expression among cell has been observed [35], a pioneer paper on *Pseudomonas*
172 *aeruginosa* biofilm using a fluorescent based approach (i.e. parallel sequential
173 fluorescence in situ hybridization (par-seqFISH)), revealed a differentiation in space
174 and time of cell-expression [36]. Because spatial single-cell microbial approaches
175 could allow the understanding of the drivers and mechanisms leading to the self-
176 organization of these microbial structures, new developments are expected to
177 expand in health science and many other fields of microbial ecology research.
178 Single-cell approaches are promising candidates for microbial studies, as they

179 provide a complementary view to metagenomics and metatranscriptomics that have
180 different strengths, but also weaknesses (Figure 2).

181 Single-cell omics technologies require additional steps to prepare a sample for
182 sequencing as compared to meta-omics techniques, especially with regards to cell
183 isolation, for which different technical options are available [37,38]. Once the cells
184 lysed, DNA and RNA content from a single cell is in the femtogram scale for
185 bacteria, i.e. 1,000-fold less than in animal cells. Preparing the sequencing library,
186 which typically requires ng ranges of material, will need an ultra-efficient prior
187 amplification step (e.g. Multiple Displacement Amplification (MDA), the most widely
188 used approach for bacteria [39]).

189 Single-cell approaches enable accurate access to genomic and transcriptomic
190 information for each cell, so that the assembled cell information is highly
191 representative of the original population (Figure 2). This enables the identification of
192 heterogeneity in gene assemblage, gene expression and metabolic pathways
193 between cells. Single-cell transcriptomic and genomic information provide a link
194 between phylogeny and functional traits and reveal the physiological status of an
195 individual cell, at the time of sampling. This is particularly important considering that
196 the individual gene expression profiles of genetically close cells may differ. What is
197 more, some cell isolation tools such as automated image-based isolation devices
198 (CellenONE Cellenion; icell8 Takara) make it possible to select cells based on their
199 integrity, their physiology and/or functional markers, and to minimize contamination
200 by the host or extracellular DNA. This is very promising for microbiology to, for
201 instance, select active cells in the studied sample at the time of sampling and reveal
202 which of them are taking part in the community productivity.

203 A seminal paper on single cell microbial genome analysis was published in 2005 [40]
204 and paved the way for further improvement of single-cell omics, notably on the
205 amplification method (here MDA) and lysis buffer. Recent studies using single-cell
206 omics have improved our understanding of intraspecific diversification and metabolic
207 capacities at a limited scale [41, 42]. Assessing the true individual cell gene
208 assemblage and expression using single-cell omics will make it possible to study the
209 hitherto unexplored microbial population level and the functioning of a given
210 microbiome by linking the different ecological scales (Figure 1b). Indeed, single-cell -
211 omics enable access to additional information than in culture-based and meta-omics
212 studies (the single individual ecological scale) whilst also, from the environmental
213 sample, giving information on the population and community interactions. To a
214 broader extent, this will enable better access to eco-evolutionary pressures and
215 evolutionary processes within microbial communities.

216 **Applications of single-cell omics in microbial ecology**

217 Single-cell omics provide information at the cell level by changing the camera angle
218 when studying environmental communities and can contribute to microbiology and
219 ecology at many levels by exploring microbial diversity or microbial interactions.

220 *The tremendous diversity of single-microbe genomes.*

221 Observations of microbiota can complement/validate the diversity observed by meta-
222 omics on fungi [43], human samples [8], and marine viruses [44], and resolve cryptic
223 bacterial species, which currently mainly rely on cultivable strains [45-47]. Single-
224 microbe -omics therefore contribute to the microbial inventory which is still in its
225 infancy in many ecosystems [48]. The use of single-cell based approaches could
226 demonstrate the existence of the discovered sequence-based lineages and discover

227 possible new branches. Isolating cells from environmental samples can also cast
228 light on rare organisms that might be obscured in millions of genome fragments
229 using meta-omics. These rare organisms are considered to play key roles in
230 community dynamics since they over-proportionally contribute to the functions of the
231 microbial community in fluctuating environments [49,50]. While most studies that
232 apply this approach use a limited number of cells, a recent survey of the marine
233 microbiome recovered no less than 12,000 genomes from single cells [51], revealing
234 a high degree of uniqueness and limited clonality in the analyzed samples of sea
235 water and providing evidence for the ecological roles of uncultured microbial groups.
236 Single-cell genomics, by looking at individual genomes instead of core genomes
237 from meta-omics, from natural microbial communities represent an unprecedented
238 opportunity to complete the identification and classification of microbes. This is a key
239 step sometimes missing in environmental microbiology [52]: knowing what to look at
240 and why to formulate hypotheses in ecology and better understand processes
241 involving microbes.

242 *Ecological and evolutionary hypothesis testing using single-cell -omics.*

243 Single-cell genomics and transcriptomics approaches therefore help to answer the
244 questions “What are these microbes?” and “What are they doing (or capable of
245 doing)?” but also to understand why and how observed patterns happen. Linking
246 environmental and community parameters to individual gene expression and
247 bacterial interactions enables a mechanistic understanding of underlying biotic and
248 abiotic conditions to patterns. This represents an opportunity to explore multiple
249 ecological theories and hypotheses, notably on interactions of microbes at many
250 levels: within the community, with external microbes (i.e. viruses) and with their host.

251 One of the hottest topics in microbial ecology is the link between diversity and
252 function, including the productivity of the ecosystem that relies on the **niche**
253 **partitioning** theory. This hypothesis states that species coexistence is enabled by
254 species specialization in different available resources (or combinations of resources),
255 thereby reducing interspecific competition [53] but likely modifying the microbial
256 population structure [54]. Specialization in specific resources raises many questions
257 concerning microbial interactions through the exchange of metabolites [55], loss of
258 traits [56] and genome reduction [57]. The Black Queen Hypothesis (BQH), one of
259 the ecological theories that conceptualized this phenomenon, states that microbial
260 community assembly and complexity are at least partially determined by functional
261 dependencies resulting from gene loss(es). Testing this hypothesis requires using
262 environmental samples to evaluate functional redundancy within communities, the
263 expression and distribution of the functions between interacting (micro-)organisms
264 and the impact of genotypic interactions on these functions. At the community level,
265 it is impossible to access this information through meta-omics since BQH evolution is
266 supposed to also occur at the population level [53,54] which is undetectable from
267 most of the meta-omics tools used so far. Eco-evolutionary processes can so far be
268 explicitly assessed only through ecological models [58] or from dedicated *in-vitro*
269 experiments [42] that require deciding which gene and which organisms to look at.
270 Single cell approaches can help to investigate such hypotheses and other theories in
271 ecology and highlight patterns of interactions that shape microbial communities.

272 *A breach to viral-host ecology.*

273 The diversity of microbial communities is also influenced by the viral infections to
274 which they are exposed, which is particularly difficult to evaluate in nature [59]. A

275 recent study assessed viral infections in the ocean thanks to single cell genomics by
276 identifying virus sequences in uncultured protist cells [44]. Such interactions are
277 widespread but generally missing in current microbial ecology analyses. Among
278 other roles, viruses are known to (1) control microbial community dynamics and drive
279 microbial-host evolution [60,61] and (2) impact ecosystem changes and
280 biogeochemical cycles [59,62]. Single-cell omics can help us understand the roles
281 played by viruses in microbial populations by making it possible to assess both the
282 prevalence of prophage sequences and possible lateral gene transfers [63,64] which
283 would reveal preferential association of specific viruses with specific bacteria.

284 *Single-cell insights into bigger scale interactions.*

285 Generally speaking, single-cell omics can highlight diverse levels of interaction in
286 natural habitats either between the microbes that comprise a microbiota or between
287 the microbes/microbiota and their host. Despite the great number of studies on the
288 composition of human microbiota, very little is known about interactions between the
289 microbes and with their host [65]. The single cell study of host-pathogen interactions
290 paves the way for understanding infectious processes through microbiota dynamics,
291 metabolic capacities, and host resistance [66]. Dysbiosis is often shown to display a
292 higher beta-diversity interpreted as a higher stochasticity in the microbiota assembly
293 [67]. Among other explanations, this apparent higher stochasticity may be the result
294 of drastic pathogen-induced changes in the habitat (i.e. transitory state in the
295 microbiota dynamics), modification of a component of the microbiota caused by
296 genetic change(s) and/or a functional modification expressing a modified phenotype
297 that leads to disequilibrium in the microbiota community. With the aim of
298 understanding how a disorder of the microbiota leads to disease or the reverse, i.e.

299 how a disease can modify the composition of a microbiota, single-cell microbiota
300 analyses of genomes and transcriptomes would help better define the characteristics
301 of dysbiosis (i.e. dysbiosis mechanisms). Using single cell genomics makes it
302 possible to address hypotheses related to changes in bacterial populations, while
303 microbial single-cell transcriptomics may be more appropriate to decrypt the
304 functions of microbes, metabolic abilities, and cellular states [68]. These two
305 strategies are necessary to understand how microbial interactions occur within
306 communities as well as their possible impact on the ecosystem [69,51].

307

308 Microbial single-cell omics are not solely expected to better understand functional
309 interactions but also the underlying evolutionary processes. Microbial single-cell
310 omics should also promote a shift in standpoint from observation to interpretation
311 and also offer new opportunities to test macroecological theories on microbes. The
312 development of microbial single-cell omics will have high impacts on our
313 understanding of microbial communities in many environments. For instance (i) in
314 freshwater and marine ecosystems, to define the interaction of bacteria and
315 phytoplankton through the exchange of metabolites, and to test links to blooms [70],
316 (ii) in soils, to better assess the provision of services by plant microbiota including
317 nutrient and water uptake and protection against pathogens [71], (iii) in plant, human
318 and animal health, to better decipher how dysbiosis could be cause or consequence
319 of a disease. However, it can't be ignored that the wide development of single-cell
320 omics applied to microorganisms is limited by technical limits.

321

322 **Limits of single-cell omics on microbes**

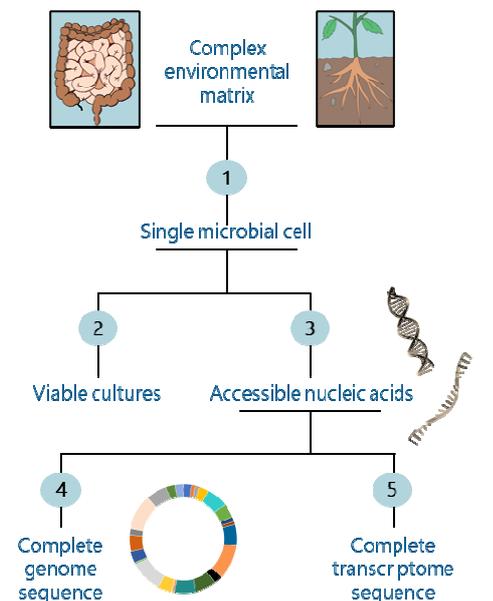
323 The current limited number of cells studied in the published papers questions the
324 representativeness of the analyses. Considering the number of microbial cells
325 contained in a given environmental sample, one can wonder how many cells need to
326 be isolated to cover the diversity of a sample, from hundreds [41], to thousands [51].
327 The limited number of analyzed cells is mostly due to technical problems and the
328 cost of such experiments. It has to be emphasized that the current use of single-cell
329 omics for microbes have to solve many technical obstacles (Box 2), reviewed in
330 [72,73] such as cell isolation, lysis, and a biased amplification step. The structure of
331 the microbial cell wall is complex, and unlike the animal cells, they do not break
332 easily. The diversity of cell wall composition across phylogeny and physiological
333 status (peptidoglycan layer(s), spores, capsules, etc.) makes it challenging to find a
334 universal lysis method able to breach each cell without damaging its content or
335 inhibiting enzymatic reactions downstream. Different protocols have been used in
336 recent microbial single-cell based studies, using either heat, temperature shocks,
337 sonication, enzymes, detergents, or combinations of these [74,75]. In addition to
338 technical issues, one can wonder how to be certain that cells are isolated and lysed
339 equally and not preferentially depending on their physical/physiological status. The
340 amplification step, usually made via MDA, has been reported to be imprecise
341 concerning the genome amplification uniformity, even though it presents a better
342 genome coverage than other approaches (i.e MALBAC [39]). This amplification step
343 is highly relevant, as it was suggested to be the cause of incomplete reconstruction
344 of single amplified genomes (SAGs) [76], although solutions are being developed
345 [77]. As the price of library preparation represents most of the cost of these new
346 single- cell omics for microorganisms, reduction of reaction volumes in
347 “nanolibraries” should be very cost-effective (Box 2). The probability of contamination

348 decreases with the miniaturization of the reagent volumes [78] and associated
 349 robotics. Working in nanovolumes seems to be a convenient solution to solve
 350 multiple problems, however this introduces new volume-related challenges such as
 351 pipetting or sample purification.

352 For these reasons, single-cell omics have been sometimes used in combination with
 353 meta-omics to combine the possibility of fine scale analysis with high throughput
 354 [76,79]. It also represents a good opportunity to validate multiple aspects of single-
 355 cell omics: 1) the isolation and lysis universality, 2) the sample preparation (genome
 356 amplification and library preparation for sequencing), 3) the lack of contamination,
 357 and 4) the representativeness of the sample covered by single -cell omics (See
 358 outstanding questions).

359 **Box 2:** Single microbial cell -omics approaches are technically challenging.

Challenge	Possible solution
1. Isolation of single microbial cells	
Community representative sampling	High throughput
Cell isolation from complex matrices, such as soil, sediments, host tissue, feces, mucus...	Sonication, filtration, density gradient centrifugation
Exhaustive/targeted labelling/detection	Fluorescence, antibodies
Maintenance of axenic conditions	(As) clean (as possible) room + Ⓟ
2. Culturomics experiments	
Maximum viability/cultivability	Gentle cell handling, liquid dispensing
Choice of culture conditions	High throughput media screening + Ⓟ
Assessment of monoclonality (= culture purity)	Microscopy, targeted sequencing
3. Microbial cell lysis/permeabilization	
Efficiency across phylogeny	(Ultra-) sonication, thermal shock, heat, enzymolysis, detergents...
Preservation of DNA/RNA quality and quantity	Gentle procedure, avoid purification
Minimum contamination from reagents and prevention of subsequent steps	Physical rather than biological/chemical + Ⓟ
4. Single-microbe WGS	



5 steps of single microbial approaches.

Super-efficient (100K- to 1 Mo-fold) amplification (1-10 fg DNA prokaryotic cell ⁻¹)	Ⓟ, molecular crowding, linear amplification (e.g. <i>in vitro</i> transcription)
Even/broad coverage, high fidelity, and no chimera creation	Minimum number of PCR cycles and/or primary template amplification (e.g. <i>in vitro</i> transcription)
Minimum contamination from reagents	Minimum reagent amount + Ⓟ
Cost reduction	Cell barcoding for multiplexing + Ⓟ
Bioinformatics	Dedicated tools for cell demultiplexing, monoclonality test...
5. Single-microbe RNA-seq	
Same as WGS (up to 100 fg RNA prokaryotic cell ⁻¹)	Ⓟ, molecular crowding
Amplification bias	Unique molecular identifier (UMI)
No poly-A tail on prokaryotic mRNA	RNA poly-A tailing, random priming, ribosomal RNA targeted depletion

360 **Table i.** Padlocks and possible keys offered by single microbial cell –omics
361 approaches.

362 Crucial, but solvable, issues (Table i) should be addressed at each step of single
363 microbe approaches (Fig. i). The reduction of reaction volumes down to
364 “nanovolumes” (see Ⓟ symbols), is often the most sensible solution, limiting
365 contamination probability and allowing high throughput and cost reduction.

366 **Concluding Remarks**

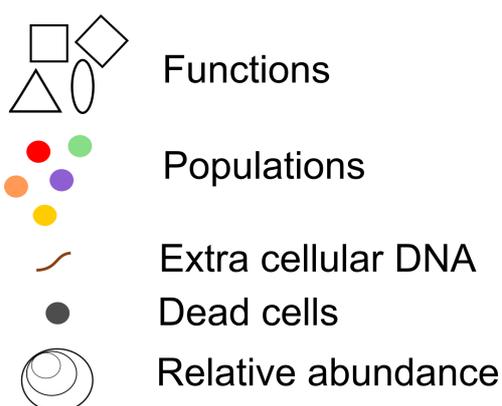
367 Soon, single-cell omics applied to microorganisms could become a gold standard in
368 microbial ecology thanks to the knowledge produced by focusing on individual
369 genomes and transcriptomes, and, possibly, individual proteomes and metabolomes.
370 Today, technical problems prevent the testing of broad ecological hypotheses.
371 Generalizing ecological single-cell studies on microbes requires the development of
372 robust high throughput techniques with a high-cost effectiveness ratio (See
373 Outstanding Questions). A knowledge upshot is expected in microbial interactions

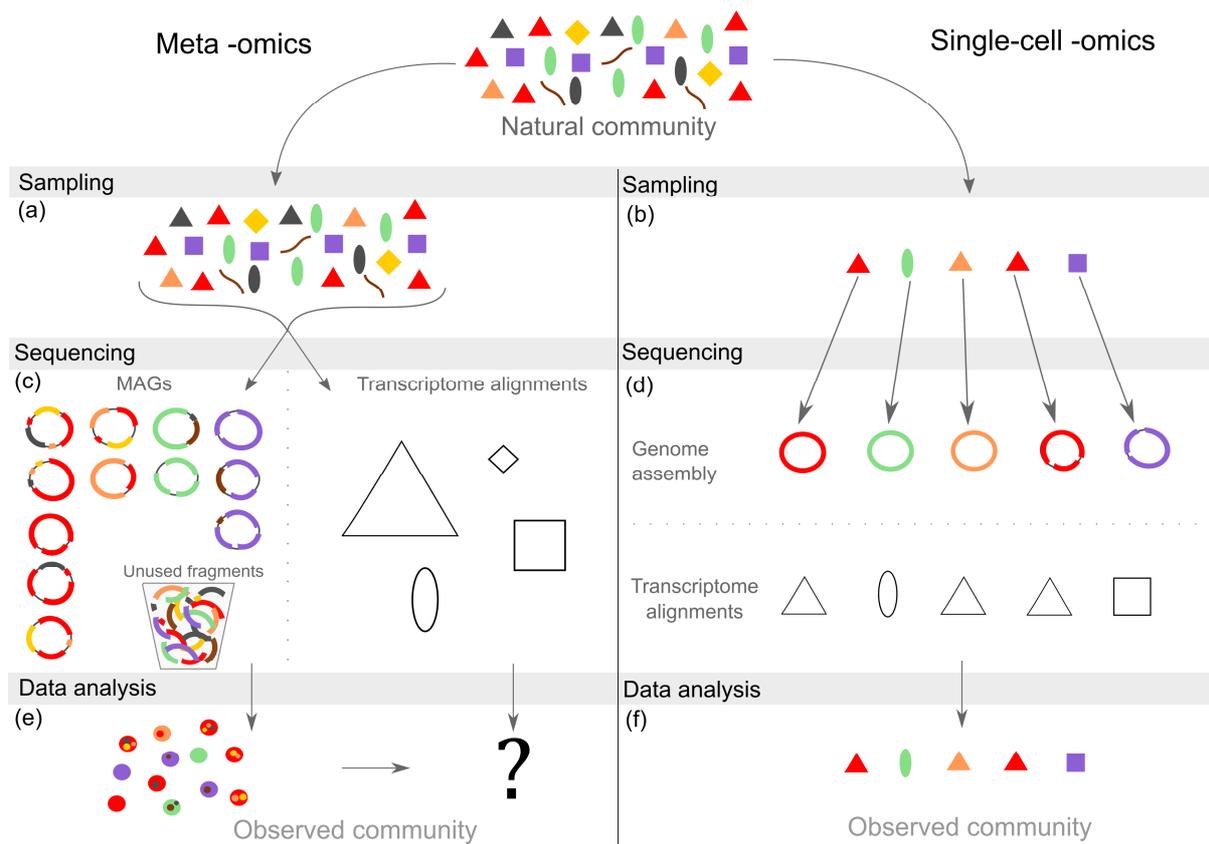
374 and eco-evolutionary boundaries through the enabling of mechanistic
375 characterization of deterministic populations and community assembly processes.
376 Currently, the use of metagenomics and single cell genomics in the same study
377 appears as the best solution, combining the strengths of the two approaches, i.e. (i)
378 high throughput and alpha/beta diversity, and (ii) fine scale analysis by scWGS
379 and/or scRNAseq [76,80]. Ideally, one would not over-interpret meta-omics data and
380 rather use those to build hypotheses on mechanisms, which can be tested using
381 single-cell approaches. Approaches that will allow more accurate assessment of
382 microbial genome diversity and genome functioning within complex microbiota, are
383 impatiently awaited. Still, the future of microbial single-cell omics will likely fuel a new
384 perception of the microorganisms world.

385 **Acknowledgements**

386 We would like to thank LABCOM 'Microscale' for funding.

387 **Key figure**





388

389 **Outstanding Questions**

390 To what extent can a single technical approach realistically reflect complex natural
 391 processes?

392 How many cells need to be isolated from a natural environment to accurately
 393 represent the population and/or community from which they originate? And how can
 394 this be assessed?

395 What criteria should be used to determine the scale of sampling in natural
 396 environments?

397 How can we use information obtained by single-cell omics for a better understanding
 398 of ecosystem functioning?

399 How can we make the application of proteomics, metabolomics and multi-omics
400 approaches to single microbes more realistic?

401

402 **Glossary**

403 **Metagenome-assembled genome (MAG):** in-silico reconstruction of an artificial
404 microbial genome obtained from one or multiple binned metagenomes that represent
405 the core genome of the population

406 **Meta-omics:** group of molecular biology technologies, extensively used to access
407 unculturable organisms, by studying the bulk pool of biomolecules from
408 environmental samples, to reveal genomes (metagenomics), transcriptomes
409 (metatranscriptomics), proteomes (metaproteomics) and metabolites
410 (metabolomics).

411 **Niche complementarity/partitioning:** ecological concept describing how species
412 differential specialization in different combinations of resource uses and functions
413 allow them to coexist in the same environment.

414 **Population:** applied to bacteria in natural communities, individuals with identical or
415 different genomes from the same species gathered in a specific environment or
416 sample. An isolated microorganism culture also comprises a population.

417

418 **Box 1: Microbial population**

419 In ecology, populations are individuals belonging to the same species, living in
420 the same environment, although the definition varies with different viewpoints
421 [81]. Microbial populations represent a unit of diversity and selection. Within
422 these populations, diversity can be either genetic or phenotypic. The diversity
423 within a population to some extent buffers an environmental stress because
424 existing variants are able to survive the stress and/or allow rapid phenotype
425 switching (e.g. Bet-Hedging, [82,31]) but positive selection of new variants can
426 also be induced by the stress. This organizational level is therefore a key to
427 understanding genetic structure and haplotype fitness, the dynamics of ecological
428 interactions, including associations of microbial species, symbioses, host-
429 pathogen interactions, and ecosystem functioning, resilience, and stability. For
430 instance, resistance to antibiotics can vary within populations [83] and the
431 virulence pathogens can vary across subpopulations [84]. Genetic diversity and
432 ecological features such as niches can vary between lineages [85], so that
433 subpopulations are able to co-exists through niche diversity. To capture the total
434 genetic and phenotypic diversity and get a holistic view of populations, the scale
435 must thus be tuned down to the individuals that comprise the population in a
436 given sample [84]. Otherwise, applying the current bacterial species concept to
437 make population level inferences may lead to false or partial interpretation of
438 ecological phenomena.

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706

707 **Figure 2 – Key Figure: Microbial communities observed through meta-**
708 **omics versus single-cell omics**

709 The key steps of meta-omics (left) and single-cell omics (right) approaches are
710 shown, resulting in contrasting representations of a natural microbial community.
711 Different genomes are represented by different colors, red, orange and yellow
712 show genomes of close relatives, i.e. intra-population genomic variants. Dead
713 cells are shown in grey, and extracellular or host DNA in brown. Different
714 functions are represented by different symbols (triangles, squares, diamonds, or
715 ovals). (a) After meta-omics sampling, the cell proportions are maintained but
716 transient DNA and dead cells are not filtered. (b) In single-cell omics, a smaller
717 proportion of the community is sampled, and dead cells can be excluded. (c) In
718 meta-omics, the unit sequenced is the complete extracted sample. Metagenome
719 assembled genomes (MAGs) are partial and include chimeras, i.e. unreal
720 collages of closely related genomes, dead cells, and extracellular material. Meta-
721 transcriptome analysis yields averaged relative abundances (represented by the
722 size of the symbol) of functions within the sample. (d) By contrast, in single-cell
723 omics, each cell is a sequenced unit and can be associated with its genome
724 and/or transcriptome. (e) The community observed through meta-omics is
725 representative of the composition of the whole community but not of the
726 associated genes and functions. (f) With genome and transcriptome information
727 from single cells, the observed community is under-sampled but is closer to the
728 natural community: if the sampling scale is appropriate, rare populations and
729 functions are more likely to be detected.