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Frontiers in Microbiology

Nano-sized and filterable Bacteria and Archaea: Biodiversity and Function

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Abstract

Nano-sized and filterable microorganisms are thought to represent the smallest living organisms on earth and are characterized by their small size (50-400 nm) and their ability to physically pass through <0.45 μm pore size filters. They appear to be ubiquitous in the biosphere and are present at high abundance across a diverse range of habitats including oceans, rivers, soils and subterranean bedrock. Small-sized organisms are detected by culture-independent and culture-dependent approaches, with most remaining uncultured and uncharacterized at both metabolic and taxonomic levels. Consequently, their significance in ecological roles remain largely unknown. Successful isolation, however, has been achieved for some species (e.g. *Nanoarchaeum equitans* and "*Candidatus* Pelagibacter ubique"). In many instances, small-sized organisms exhibit a significant genome reduction and loss of essential metabolic pathways required for a free-living lifestyle, making their survival reliant on other microbial community members. In these cases, the nano-sized prokaryotes can only be co-cultured with their 'hosts'. This paper analyses the recent data on small-sized microorganisms in the context of their taxonomic diversity and potential functions in the environment.

34 **1 Introduction**

35 Recent technological advances in microbiology have helped to reveal the enormous diversity

36 of prokaryotic life on our planet (Caporaso et al., 2011; Kuczynski et al., 2010; Thompson et

37 al., 2017). While this has enabled us to characterize and map prokaryote populations across a

38 diverse array of ecosystems, the functional role of most of these organisms remains unknown,

39 due to our inability to culture, and study them in the laboratory. Nevertheless, using culture-

40 independent approaches, e.g. metagenomics, many new candidate taxa that include nano-sized

41 and filterable organisms have been discovered.

42 Nano-sized microorganisms are termed 'ultra-micro bacteria', 'ultra-micro cells',

43 'dwarf cells', 'ultra-small bacteria', 'nanoorganisms', 'nanobacteria', nanoarchaea and

44 'nanobes' (Velimirov, 2001; Baker et al., 2010; Duda et al., 2012). The term nanoarchaea only

45 relates to the phylum *Nanoarchaeota* (Huber et al., 2002), although it is commonly erroneously

46 used within the literature. The exact definition of these terms is widely debated and no clear

47 set of guidelines currently exists, however, it is considered that the microorganism must be in

48 the "nano-range" (i.e. 50 to 400 nm) in size. It should also be noted that in regards to aquatic

49 systems, these ultra-small-sized organisms are not part of nanoplankton (2.0-20 μm in size),

50 but instead reside in the picoplankton (0.2-2.0 μm) or femtoplankton (0.02-0.2 μm)

51 communities (Sieburth et al., 1978; Fenchel, 1982; Azaml et al., 1983).

52 Previous studies have focused on detection of ultra-small-sized organisms in a wide

53 range of environmental conditions including: acid mine drainage settings (AMD) (Baker et al.,

54 2006), glacial ice (Miteva and Brenchley, 2005), permafrost (Suzina et al., 2015), freshwater

55 (Fedotova et al., 2012; Ma et al., 2016; Nakai et al., 2016), subterranean bedrock (Wu et al.,

56 2015), hypersaline lakes (Narasingarao et al., 2012), the open ocean (Venter et al., 2004;

57 Giovannoni et al., 2005; Glaubitz et al., 2013; Rogge et al., 2017), and the human body

58 (Kajander and Ciftcioglu, 1998; Kajander et al., 2003; He et al., 2015). The predictions from

59   genomic data from these environments suggest that there are many microorganisms that

60   contain small genomes and either are present as free-living organisms or form a symbiotic

61   relationship with other life forms, which adds another level of complexity to assess their

62   functional role in the environment.

63   As the review of Duda et al. (2012) discusses a number of issues related with

64   ultramicrobacteria, the aim of present review was to highlight the latest discoveries related to

65   (1) taxonomic diversity, (2) biogeography, (3) current experimental approaches to characterize

66   these organisms and (iv) potential role of ultra-small Bacteria and Archaea within a contrasting

67   range of environments.

68

69   **1.1   Overview of Terminology**

70   When considering ultra-small or nano-sized organisms, it is important to note the significance

71   of the terminology. There is no singular definition of what a nano-sized organism is (ultra-

72   small bacteria, ultra-micro bacteria, nanobes, nanoforms, ultramicrocells, etc.) and

73   consequently a variety of interpretations exists. Many of the terms are either synonymous, as

74   in the case of ultra-small and ultra-micro (Velimirov, 2001), or can be classified as separate

75   organisms, as in the case of nanobacterium and nanobe (Duda et al., 2012). Here we consider

76   three scenarios for their denotation (Fig. 1).

77   The first scenario that these microorganisms originated from known species, whose cell

78   size decreases over time due to either internal and/or external factors such as lack of nutrients

79   or ageing (Velimirov, 2001; Panikov, 2005; Duda et al., 2012). Such ability of bacteria and

80   archaea to change size in response to external stress is a well-studied phenomenon. For

81   example, under low nutrient conditions, *Staphylococcus aureus* reduced its size by 40%

82   (Watson et al., 1998; Chien et al., 2012), while the transfer of *Pseudomonas syringiae* from

83   laboratory culture media to plant leaves, induced the 50% reduction in cell size (Monier and

84   Lindow, 2003). This size reduction is an attribute of dwarf cells, midget cells, ultra-small,

85   ultramicro (Velimirov, 2001; Duda et al., 2012). For these cases, we advocate for the term

86   'ultramicrocells' *sensu* Duda et al. (2012).

87   The second scenario conjunctures that some distinct taxa, independently of growth

88   conditions, nutrients' availability or age of their culture do constantly exhibit small cell sizes.

89   One source describes these organisms in the following way: the microorganisms must be 0.1

90   μm^3 or smaller (<0.05-0.40 μm in diameter); the size must stay consistent under environmental

91   stressors and life cycles; and finally, its genome size must be within the range 0.58 Mbp to 3.2

92   Mbp (Duda et al., 2012). Under this definition, nano-sized microorganisms are associated with

93   terms like ultra-small, ultramicronanoarchaea, nanoforms, nanoorgansims, and nanobacteria

94   (Schut et al., 1995; Kajander and Ciftcioglu, 1998; Velimirov, 2001; Huber et al., 2002; Miteva

95   and Brenchley, 2005; Panikov, 2005; Comolli et al., 2009; Duda et al., 2012; Fedotova et al.,

96   2012; Luef et al., 2015; Giovannoni, 2017; Rogge et al., 2017). However, many standard-sized

97   microorganisms (i.e. cell volumes >0.1 μm^3) also possess small genomes (1.5-2.0 Mbp) and

98   would therefore fall into the 'ultra-small' category if based on these criteria alone.

99   The third scenario are microorganisms that have the ability to pass through membrane filter

100   pores with small diameters (0.45 or 0.22 μm) despite having larger cell sizes (above the

101   dimensions of 50-400 nm previously mentioned) (reviewed in Duda et al., 2012). This is often

102   due to the absence of a rigid cell wall, which allows these microorganisms to effectively

103   squeeze through small pores and as a result are commonly confused with nano-sized or

104   ultramicro-sized. 'Filterable' microorganisms is the most appropriate term to define such

105   microorganisms.

106   In this review, a unified definition for nano-sized organisms is proposed. We define them

107   as microorganisms that exhibit constant dimensions of 50-400 nm (volume ≤ 0.1 μm^3). All

108   microorganisms with synonymous names that fall under the definition provided are considered

109   nano-sized organisms. Viruses and prions, which are smaller than 50 nm in size, are not

110   considered to be living organisms (Fig. 2; Table 1). In aquatic systems, nano-sized organisms

111   are a part of the picoplankton and femtoplankton communities, along with viruses (Venter et

112   al., 2004; Tringe et al., 2005; Sieburth et al., 1978; Salcher, 2014).

113

114   **2   Microbial adaptations**

115   In the natural environments microorganisms use an arsenal of mechanisms to cope with, and

116   adapt to, constantly changing physio-chemical conditions, through changes in their gene

117   expression profile, physiology and morphology (Schulz and Jørgensen, 2001; Chien et al.,

118   2012). Here we highlight various survival strategies in prokaryotes, knowledge of which may

119   stimulate future discoveries pertaining to small-sized organisms.

120

121   **2.1   Extremely small size**

122   In general, microorganisms do not fit into one standard model of size or shape (morphology)

123   due to the impact environmental stressors (Young, 2006; Chien et al., 2012; Cesar et al., 2015;

124   Lever et al., 2016). The efficiency of nutrients' uptake is dependent on organism size and the

125   number of transporter systems on its surface (Button et al., 1998). Hence, in the case of cell

126   size reduction, the surface area-to-volume ratio tends to increase (Fig. 2). This, however, does

127   not imply that the percentage of genes encoding membrane-bound proteins in genomes is

128   higher in organisms with a larger surface area-to-volume ratio (Stevens and Arkin, 2000) (Fig.

129   2).

130   Under conditions of starvation and energy limitations, microorganisms can drastically

131   decrease in size, alter cellular morphology and motility to increase survivability (Torrella and

132   Morita, 1981; Cesar and Huang, 2017; Lever et al., 2015). For example, in low organic

133   phosphate conditions, *Caulobacter* spp. increase their surface area to volume ratio by growing

134 a prosthecae, stalk-like protrusions, in order to enhance organic phosphate uptake (Wagner et

135 al., 2006; Lever et al., 2015). Another example is the species *Sphingomonas alaskensis*, which

136 also undergoes morphological changes in response to the fluctuations in nutrients availability.

137 In its natural pelagic environment its body size is quite small (diameter 0.2-0.5 µm; length 0.5-

138 3 µm) yet when grown on nutrient rich trypticase soy agar medium it increases in both diameter

139 and length (diameter 0.8; length 2-3 µm) (Vancanneyt et al., 2001; Lever et al., 2015).

140

141 **2.2   Lifestyle: free-living vs symbionts**

142 Nano-sized organisms are thought to contain genomes coding for a very limited number of

143 functions and pathways, which is a characteristic commonly associated with symbionts,

144 however, nano-sized organisms do also exist in a free-living state. Generally, symbionts do not

145 have the means for their existence without relying on essential metabolites provided by the

146 host. However, these organisms do thrive probably due to their highly specialized and unique

147 functions which allows the host to be more competitive (McCutcheon and Moran, 2011). For

148 instance, TM7 ("*Ca*. Saccharibacteria") bacteria isolated from the human oral mucosa can

149 effectively conceal its host, *Actinomyces odontolyticus* subsp. *actinosynbacter* XH001, from

150 the human immune system response (He et al, 2015; further discussion in the section " TM7

151 bacteria or '*Candidiatus* Saccharibacteria').

152

153 **2.3   Oligotrophy and Copiotrophy**

154 Oligotrophs also known as K-strategists, are organisms that prefer low-nutrient environments

155 (Schut et al., 1997; Panikov, 2005; Torsvik and Øvreås, 2008). One of the most well-

156 characterized oligotrophic environments is the open ocean, which encompasses 90% of the

157 biosphere (i.e. the sum of all the ecosystems) (Schut et al., 1997; Hansell et al., 2009). In this

158 environment, many essential nutrients are only present in very low concentrations: iron at 0.2-

159   1.38 nmol kg^{-1}, nitrate at 1.04 µmol kg^{-1}, phosphate at 0.074 µmol kg^{-1}, silicate at 3.2 µmol

160   kg^{-1}, dissolved inorganic carbon at 11 µmol kg^{-1}, and dissolved organic carbon at 40-80 µmol

161   kg^{-1} (Johnson et al., 1997; Roshan and DeVries, 2017; Sauzède et al., 2017; Tagliabue et al.,

162   2017), which makes it difficult to mimic such conditions and obtain a detectable growth of

163   these microorganisms *in vitro*. At such low concentrations of nutrients microorganisms lower

164   their metabolic rates and become less capable of forming aggregates (i.e. colonies), as seen in

165   many pelagic organisms, such as SUP05 group bacteria and in "*Ca*. Pelagibacter ubique" (see

166   references below in the sections 'SUP05 group' and "*Ca*. Pelagibacter ubique"). Overall,

167   oligotrophs are characterized by small cell sizes, which are more advantageous in low nutrients

168   conditions. The correlation between oligotrophy and diminutive size appears almost

169   ubiquitously (Giovannoni et al., 2014), however, few studies have detected ultra-small-sized

170   microorganisms in high-nutrient systems, such as eutrophic aquifers or the human oral cavity

171   (Luef et al., 2015; He et al., 2015).

172   Copiotrophs or R-strategists, are active, fast-growing with larger cell body sizes, usually

173   motile organisms well-suited to nutrient-rich environments; they represent the majority of

174   bacteria and archaea cultured up to date (Dang and Lovell, 2016; Giovannoni, 2017). Despite

175   being easy to culture, copitotrophs appear as rarer taxa in natural environments. They take

176   advantage of sporadic high nutrients concentrations which in turn may transiently cause a rapid

177   population growth (Vergin et al., 2013; Dang and Lovell, 2016). It is thought that copiotrophs

178   are not nano-sized organisms as an increased surface area-to-volume ratio is not necessarily

179   advantageous in nutrient-rich environments (Martínez-Cano et al., 2015). However,

180   copiotrophic bacteria also tend to reduce their sizes as a response to starvation conditions in an

181   attempt to increase their surface area-to-volume ratio, as in the case of *S. aureus* (40% reduction

182   in size) and *P. syringae* (50% reduction in size) (Watson et al., 1998; Monier and Lindow,

183   2003).

184

185 **3   Characterization**

186 Due to the constraints in accurately mimicking environmental settings *in vitro,* the cultivation

187 of small organisms is often problematic and represents a main bottleneck in the process of their

188 phenotypic characterization. In order to predict functional traits of nano-sized microorganisms

189 as a part of the microbial community, culture-independent techniques are currently employed

190 as primary approaches, as stand-alone or combinations of approaches: metagenome

191 sequencing, flow-cytometry and fluorescence microscopy. Below is a brief overview of some

192 culture-independent techniques and the challenges that arise when attempting to isolate nano-

193 sized microorganisms.

194

195 **3.1   Metagenomics**

196 As indicated above, metagenomics has played a central role in attempts to detect small-sized

197 and filterable organisms and elucidate their functions. In turn, the isolation and characterization

198 of nano-sized organisms has yielded, and to some extent, validated new genomic data (Huber

199 et al., 2002; Giovannoni et al., 2005). In many of the large-scale metagenomics studies, the

200 significant proportion of assembled genomes exhibited small sizes (Rappé et al., 2002; Venter

201 et al., 2004). In particular, an in-depth investigation of the SAR11 clade led to the discovery of

202 "*Ca*. Pelagibacter ubique*",* a ubiquitous and predominant marine bacterium (Giovannoni,

203 2017; Zhao et al., 2017) . Also, microbial communities in the deep biosphere proved to be

204 more diverse than previously anticipated, with a plethora of miniature cells with small genomes

205 (Wu et al., 2015). Finally, hypersaline lakes, a good model for extreme habitats, were found to

206 contain filterable cells, about 0.6 μm in diameter, that were termed "*Ca*. Nanohaloarchaeota"

207 (Narasingarao et al., 2012). This study was in large facilitated by a more targeted sample

208 preparation (filtration) procedure and *de novo* sequencing approach. However, we must note

209   that small genomes and the ability to pass via 0.1, 0.22, and 0.45 µm pore-size filters are not

210   necessarily the evidence of small sizes of microorganisms (i.e. filterable microorganisms), for

211   instance, the symbiont "*Ca.* Tremblya princeps*"* has an extremely reduced genome of 0.13

212   Mbp, yet, examination by microscopy showed its length to be ca. 2.3 µm (McCutcheon and

213   Moran, 2011).

214

215   **3.2   Flow cytometry and FACS cell sorting**

216   The further culture-independent techniques, flow cytometry (Gasol and Morán, 1999; Miteva

217   and Brenchley, 2005; Wang et al., 2007; Neuenschwander et al., 2015) and fluorescence in

218   situ hybridization (FISH) (Glaubitz et al., 2013; Neuenschwander et al., 2015; Munson-

219   McGee et al., 2015;) have been widely used to study microbial populations in their natural

220   environments. In combination with fluorescence probes targeting SSU rRNA or

221   immunolabelling cellular proteins, this approach allows quantification of a certain taxonomic

222   group of microorganisms (Neuenschwander et al., 2015). Combining FISH/CARD-FISH

223   (Fluorescence In Situ Hybridization/Catalyzed Reporter Deposition-Fluorescence In Situ

224   Hybridization) and flow cytometry (also known as 2C-FISH) allowed for sorting and

225   obtaining relatively pure populations of microorganisms, as it was the case of LD12 clade of

226   ultramicrobacteria from freshwater. These ultramicrobacteria were known to be very difficult

227   to isolate and characterize due to their small genomes and hence limited metabolic

228   repertoires, cell sorting was therefore the crucial starting point for their subsequent genomic

229   studies (Salcher et al., 2013; Neuenschwander et al., 2015). Although improvements in

230   individual techniques were achieved in this study, the methodology of sample preparation is

231   still tedious and time-consuming with relatively limited yields of cells (Neuenschwander et

232   al., 2015). Whatever the case, the applications of cell sorting have been successful in

233   resolving a number of "single-cell-genomes" (Ishoey et al., 2008; Probst et al., 2018).

234

235   **3.3   Isolation of nano-sized microorganisms**

236   Although isolation is an essential step in characterizing organisms, it is often overlooked and

237   traditional approaches to culture them frequently prove unsuccessful. Many of the studies

238   presented in this review employed filtering through 0.1-1.2 μm pore size filters to facilitate

239   enrichment and isolation (Table 1). The exception to the filtration methodology was

240   *Nanoarchaeum equitans*, which was co-cultured with the host, *Ignicoccus hospitalis,* and then

241   separated out via centrifugation (Huber et al., 2002; Waters et al., 2003). Conversely, while the

242   target microorganisms may be small enough to pass through the membrane, certain larger

243   organisms can squeeze through pores, due to a lack of rigidity of their cells. Another example

244   of organisms squeezing through small-sized pores are archaea of families *Ferroplasmaceae*

245   (0.2-3 μm in diameter in average) and *Thermoplasmataceae* (0.5-3 μm in length and 0.2-0.5

246   μm thick), that can easily pass through a <0.45 μm pore filter due to the lack of a rigid cellular

247   envelope (Golyshina, 2014; Nagy et al., 2016) .

248   In previous studies, along with 'small-sized-organisms', many other microorganisms

249   have been co-isolated (Venter et al., 2004; Tringe et al., 2005; Garza and Dutilh, 2015). An

250   extra level of authentication is therefore necessary to reliably confirm the existence and

251   metabolic function of these organisms, e.g. through an improvement in isolation and culturing

252   techniques. Small cell size is the only certainty related to nano-sized organisms that belong to

253   a range of taxa and do not share a common metabolism. For their characterization, a prior

254   genomic analysis of the source community is critical. This would allow the targeting e.g.

255   organism-specific surface proteins to enable FACS- or immunoprecipitation-based techniques

256   targeted organisms of interest.

257

258   **4   Nano-sized and filterable microorganisms**

259 Though the different characterization techniques as mentioned above, the story of ultra-small

260 microorganisms and our understanding of their ecosystem functioning is rapidly evolving.

261 Here, some of the major milestones are outlined in regards to successful isolation and

262 characterization of a variety of nano-sized organisms. Further, we have summarized the data

263 on various microorganisms covered in this section in Table 1 and Figure 3.

264

265 **4.1   Rise of the very small**

266 Although ultramicrobacteria have been known for a long time (Oppenheimer, 1952), the

267 subject laid dormant for a number of years. This was in part due to the limitations in

268 microbiological techniques, and the lack of knowledge of their physiology and metabolism.

269 That changed when McKay et al. (1996) first claimed their existence in Martian rocks. Not

270 only did this imply that life may exist on exoplanets, but it also challenged the ideas on lower

271 limit of size of a lifeform (McKay et al., 1996; Gibson et al., 2001). It was suggested that the

272 smallest free living organism must be in the spherical diameter range of 250-300 nm to properly

273 contain the 250-300 proteins essential to life (including the ribosomal proteins), although it

274 was also suggested that, theoretically, a primitive organism can be as small as 50 nm (Kajander

275 and Ciftcioglu, 1998). This was similar to an earlier study by Mushegian and Koonin (1996)

276 who hypothesized that the minimal number of genes required for life ranges between ca. 250-

277 450, however, there was no consensus on the number of ribosomal proteins that were actually

278 needed. Importantly, it was never established in the McKay et al. (1996) study whether these

279 nano-scale objects were free-living organisms, nor was it confirmed that these objects were

280 living at all.

281

282 **4.2   *Nanoarchaeum equitans***

283 Huber et al. (2002) found that a new archaeal species, *Ignicoccus hospitalis,* isolated from hot

284 submarine vents, had in its culture a companion of a small cell size. The new phylum

285 *Nanoarchaeota* and corresponding species *Nanoarchaeum equitans* were described as the first

286 nano-sized archaea. The genome analysis revealed that it contained a chromosome of only 0.5

287 Mbp (Huber et al., 2002), while electron and fluorescence microscopy suggested that the cells

288 of *N. equitans* were ca. 400 nm in diameter and were attached to the cell surface of its host, *I.*

289 *hospitalis.* Further, it was shown that *N. equitans* was incapable of growing without its host,

290 which in contrary neither benefited or was impaired by *N. equitans* (Huber et al., 2002; Jahn et

291 al., 2008). The inability of *N. equitans* to survive without its host is reflected in its small

292 streamlined genome, which was a result of massive gene losses (Huber et al., 2002) including

293 those for key biosynthetic pathways for vitamins, cofactors and amino acids (Torrella and

294 Morita, 1981; Mushegian and Koonin, 1996; McCutcheon and Moran, 2011).

295

296 **4.3   "ARMAN" cells**

297 "ARMAN" (Archaeal Richmond Mine Acidophilic Nanoorganism) were first detected through

298 *de novo* shotgun sequencing of aqueous sample obtained from an acid mine drainage (AMD)

299 system and not through standard PCR-based surveys (Baker et al., 2006). Subsequent cryo-

300 TEM analysis revealed an accumulation of filterable cells that were 0.03 μm^3 in volume with

301 clearly defined cell walls (Comolli et al., 2009). "ARMAN" cells were initially considered

302 free-living, possibly slow-growing, organisms possessing some intracellular tubular structures

303 (Comolli et al., 2009), however, later on, their ability to free-living lifestyle was questioned

304 (Comolli and Banfield, 2014).

305   According to the metagenome analysis with almost fully assembled "ARMAN"

306 genomes of ca. 1 Mbp in size and proteomics, these organisms contain a rather unique set of

307 genes with 45% of the genes failing to match to a known biological function, while 63% of the

308   proteins identified could not be assigned to known archaeal protein families (Baker et al.,

309   2010). Due to the small sizes of their genomes, it was assumed that "ARMAN" cells are

310   certainly dependent on other community members, being either symbionts or commensals

311   (Baker et al., 2010).

312   Cultivation of an "ARMAN"-related organism, '*Ca.* Mancarchaeum acidiphilum'

313   Mia14 revealed that it was dependent on its host, euryarchaeon *Cuniculiplasma divulgatum*

314   (Golyshina et al., 2017). As in the above examples, Mia14 underwent streamlining of its

315   genome (0.95 Mbp) due to the massive gene loss. Similarly, it exhibits significant voids in its

316   biosynthesis of amino acids, CoA, NAD and NADP, vitamins and heme. Additionally, its

317   central metabolism lacks glycolysis and gluconeogenesis, pentose phosphate pathway and

318   tricarboxylic acid cycle (Golyshina et al., 2017). Interestingly, Mia14 cell sizes were only

319   marginally smaller than *Cuniculiplasma* cells, which were 0.1 to 2 μm in size (Golyshina et al.,

320   2016).

321

322   **4.4   Other *Archaea***

323   *"Candidatus* Nanobsidianus stetteri" Nst1, a member of phylum *Nanoarchaeota* was first

324   reported after the single-cell isolation alongside its host from the order *Sulfolobales* (phylum

325   *Crenarchaeota*) by Podar et al. (2013). Unlike *N. equitans*, which is associated with a single

326   host species, *I. hospitalis*, "*Ca.* N. stetteri" can use a multitude of *Sulfolobales* species as hosts.

327   Its genome was ca. 20% larger than that of *N. equitans* and possessed a complete

328   gluconeogenesis pathway (Podar et al., 2013; Munson-McGee et al., 2015). The genome

329   analysis also indicated that "*Ca*. N. stetteri" genome coded for cellular functions previously

330   not associated with the *Nanoarchaeota* taxon; the study concluded that these archaea share a

331   common ancestor with *N. equitans* (Podar et al., 2013; Munson-McGee et al., 2015). Another

332   study (Munson-McGee et al., 2015) has partially resolved two further single-cell genomes of

333   "Nanobsidianus"-related archaea from Yellowstone hot springs and suggested their close

334   relatedness with "*Ca.* N. stetteri" Nst1, but pointed at their association with archaea of

335   "Acidicryptum spp." of *Sulfolobales*. "*Ca.* Nanopusillus acidilobi" is another success story,

336   where this small-sized, reduced-genome archaeon was co-cultured with its host, *Acidilobus* sp.

337   A7 by Wurch et al. (2016). "*Ca.* Nanopusillus acidilobi" is a thermophilic ectosymbiont, much

338   like *N. equitans* and "*Ca*. Nanobsidianus stetteri". This particular species is only marginally

339   smaller in body size than *N. equitans* (approximately 100-300 nm in diameter), both share

340   approximately 80% SSU rRNA gene sequence identity (and 97-98% with '*Ca*. Nanobsidianus

341   stetteri'), and exhibit much of the same functions as judged from genomic data (Wurch et al.,

342   2016). "*Ca.* Nanopusillus acidilobi" genome possesses no genes related to respiration, ATP

343   synthesis and cannot produce its own amino acids, lipids, nucleic acids, and co-factors.

344   Genomic data suggests that, like in its relative, "*Ca.* N. stetteri", glycogen may serve as a

345   storage compound and facilitate its short-term energetic independence from the host (Wurch et

346   al., 2016). A high density of "*Ca.* Nanopusillus acidilobi" on the surface of its host *Acidilobus*

347   sp. 7A, deficiency of its genome in genes for central metabolic, biosynthetic and energy-

348   generating pathways suggest a commensal or ectoparasitic lifestyle of these nanoarchaea

349   (Wurch et al., 2016). Expression of flagellar proteins reported in proteomic data further

350   suggests that "*Ca.* Nanopusillus acidilobi" has the ability to migrate from one host to another

351   (Wurch et al., 2016).

352

353   **4.5   "*Ca*. Pelagibacter ubique"**

354   While the existence of oceanic ultramicrobacteria has been well documented, obtaining them

355   in a pure culture remained difficult. Earlier studies (Rappé et al., 2002; Morris et al., 2002)

356   revealed a very abundant clade of *Alphaproteobacteria*, SAR11, which makes up to 25% of

357   plankton in the open ocean and is represented by small-sized, simple-metabolism bacteria

358   (Giovannoni, 2017) . Initially found in pelagic water sampled from the Sargasso sea, these

359   bacteria termed "*Ca*. Pelagibacter ubique" had genomes of approximately 1.3 Mbp and are

360   considered to be one of the smallest free living cell (Giovannoni, 2017; Zhao et al., 2017).

361   Their genomes contained the necessary gene sets for producing all 20 amino acids as well as

362   other essential biosynthetic pathways (Giovannoni et al., 2005; Carini et al., 2012). Subsequent

363   studies indicated that "*Ca*. P. ubique" required an unconventional medium, which was

364   composed of methionine, glycine, pyruvate, and artificial seawater (Carini et al., 2012).

365   It was also found that "*Ca*. P. ubique" had a rather unique metabolism because of its

366   ability to use glycolate instead of glycine at low glycine concentrations. Glycolate can be used

367   in glycine biosynthesis through glyoxylate amination, with the glycine consequently being used

368   for serine biosynthesis (Carini et al., 2012; Tripp, 2013). The glycolate to serine pathways are

369   regulated by two glycine riboswitches, the first of which controlling the glyoxylate to glycine

370   biosynthesis and the second regulating the glycine to serine biosynthesis. At low glycine

371   concentrations, the first riboswitch is turned on to produce more glycine (Tripp, 2013). When

372   there are ample amounts of glycine in the cell, the first riboswitch turns off the glycine

373   biosynthesis and the second riboswitch induces the conversion of glycine to serine. The ability

374   to use glycolate instead of glycine to further create serine may be an evolutionary response to

375   relative excesses of glycolate formed by phytoplankton in carbon limited conditions (Carini et

376   al., 2012). As a free-living organism, "*Ca*. P. ubique" has the ability to adapt to changing

377   conditions fairly well despite having a streamlined genome. It also challenged the previous

378   assumption that small genome sizes were restricted to symbiotic organisms (Huber et al., 2002;

379   Giovannoni, 2017).

380

381   **4.6   SUP05 group**

382   Oxygen-depleted zone in pelagic systems with dissolved oxygen concentrations below 60

383   μmol kg^{-1} present a unique challenge to organisms moving through the transition zone from

384 high to low nutrient availability (Glaubitz et al., 2013; Rogge et al., 2017). According to cell

385 counts from flow cytometry, SUP05 bacteria are a common bacterioplankton component in

386 depleted oxygen zones (Glaubitz et al., 2013; Rogge et al., 2017). As chemolithoautotrophic

387 organisms, they metabolize sulfur compounds and play a key role in the carbon, sulfur and

388 nitrogen cycles to facilitate life in the redoxclines across the globe (Glaubitz et al., 2013;

389 Rogge et al., 2017; Shah et al., 2017). They have the ability to carry out denitrification and

390 uptake carbon dioxide in pelagic low oxygen zones, which is supported by genomic

391 predictions, radioisotopic data and cultivation attempts (Glaubitz et al., 2013; Rogge et al.,

392 2017; Shah et al., 2017). Cultivation attempts of one of the members of the SUP05 group,

393 "*Candidatus* Thioglobus autotrophicus", revealed the utilization of ammonium under

394 anaerobic conditions and nitrite production (Shah et al., 2017). Studies on the SUP05 group

395 have suggested cellular volumes ranging within 0.01-0.09 μm^3 and a genome of 1.164-1.53

396 Mbp, which indicates that these bacteria have undergone streamlining in their evolutionary

397 past, much like "*Ca*. P. ubique" (Rogge et al., 2017; Shah et al., 2017).

398

399 **4.7   Filterable forms in peatland bogs**

400 Despite the abundance of organic carbon in aquatic subsystems of peatland bogs, its

401 mineralization is very slow due to the elevated concentrations of phenolic compounds causing

402 acidification (pH 4.4-4.8), enzyme inhibition and nitrogen limitation (Fedotova et al., 2012).

403 This is the case for sphagnum peatland bogs in northern Russia, that contain a high number of

404 filterable bacteria and archaea, $1.69 \pm 0.53 \times 10^4$ and $3.16 \pm 0.43 \times 10^4$ cells/mL,

405 correspondingly (Fedotova et al., 2012). Phylogenetic analysis of 16S rRNA genes shows they

406 were derived from several phyla (Fedotova et al., 2012). One-third of the archaeal sequences

407 had a high identity (94-99%) with representatives of the orders *Methanobacteriales* and

408 *Methanosarcinales*, while the rest exhibited a distant relatedness (71-74% sequence identity)

409   to cultured methanogens and collectively belonged to the LDS (Lake Dagow sediment) cluster

410   (Glissmann et al., 2004). All detected bacterial species had high SSU rRNA gene sequence

411   identities (94-99%) to the *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*,

412   and *Actinobacteria,* which confirms that small size is an adaptation to low nutrient conditions

413   common across the broad range of higher taxa. The study also attempted to culture filterable

414   microorganisms on solid media: from the total microscopic cell count numbers, only a fraction

415   of approx. 0.5-1.2% did form colonies represented by bacterial genera *Mesorhizobium,*

416   *Bradyrhizobium, Sphingomonas* and *Agrobacterium.* A major discrepancy between the SSU

417   rRNA amplicon libraries sequences of microbial communities in those freshwater samples and

418   the taxonomy of cultured bacteria was also observed (Fedotova et al., 2012).

419

420   **4.8   Ultra-small bacteria from Greenland ice**

421   Glacial ice presents a rather unique challenge to many microbial species due to its sub-zero

422   temperatures and oligotrophic conditions and is considered a freshwater-like habitat for

423   microorganisms (Hodson et al., 2008). It has been previously noted that a number of ultrasmall

424   organisms have been detected in several ice cores (Miteva, 2008). A plethora of bacteria in

425   120,000 year-old Greenland ice, which, after melting the ice cores, passed through filters with

426   pore sizes of 0.4, 0.2 and even 0.1 μm was detected (Miteva and Brenchley, 2005). Scanning

427   electron microscopy and flow cytometry confirmed that the filtration methodology was

428   effective at removing larger cells residing in the melted ice water. The authors also stated that

429   a considerable amount of fungal colonies were also present, although these were not discussed

430   in further detail (Miteva and Brenchley, 2005), however, one can assume those were derived

431   from filterable fungal spores. It is not clear if all >1,200 cultured bacteria were ultra-small, as

432   there was evidence of larger organisms (e.g. spores of fungi and of *Firmicutes*), which possibly

433   were cultured due to the non-uniform sizes of filter pores, over-pressurizing filtration units or

434 non-rigid cell envelops of microorganisms that allowed them passing through filters (Wang et

435 al., 2007, 2008). Whatever the case, the study of Miteva and Brenchley (2005) clearly

436 demonstrated the viability in and cultivability of very small microorganisms with

437 experimentally measured average volumes ranging between 0.043-0.1µm^3 from, a polar ice

438 environment.

439

440 **4.9   WWE3, OD11 and OP1 candidate phyla of ultra-small bacteria from groundwater**

441 Much of the bacterial species discussed so far have been identified in oligotrophic

442 environments, however, ultra-small organisms are not exclusive to these habitats. The WWE3-

443 OD11-OP1 candidate phyla of groundwater bacteria were found in an eutrophic environment

444 (Luef et al., 2015). Although these bacteria have not been cultivated, ultra-small cells have

445 been successfully imaged challenging previous ideas on possible habitats of these organisms.

446   Luef et al. (2015) described the cellular structures present within ultra-small-sized-

447 organisms: using cryo-TEM images they identified pili, cell walls, cellular division and the

448 presence of viruses. The study investigated the freshwater collected from an anoxic, organic

449 carbon rich groundwater located several meters below the surface. Until that point, small-sized

450 microorganisms were thought to be either associated with oligotrophic conditions or microbial

451 communities with a reduced diversity, e.g. AMD. Importantly, it appears that small size can

452 also be beneficial in other environments. The study was unable to successfully perform CARD-

453 FISH on the proposed ultra-small cells (Luef et al., 2015) and therefore could not confirm that

454 small cells seen were indeed of the candidate phylum that they reported on.

455   Metagenomic analyses by Wrighton et al. (2012) and Kantor et al. (2013) have revealed

456 that WWE3, OP1, OD11, TM7, and SR1 candidate phyla of bacteria possessed small genomes,

457 lacked genes for several essential metabolic processes and contained genes of both archaeal

458 and bacterial origin. The genomic predictions inferred that WWE3, OP1, and OD11 candidate

459   phyla are capable of growing in organic carbon-rich environments (Wrighton et al., 2012; Luef

460   et al. 2015; Kantor et al. 2013). The RuBisCO (type II/III ribulose-1, 5-biphosphate

461   carboxylase-oxygenase), which was predicted in these groundwater ultrasmall bacteria, is not

462   likely to be involved into the classical CBB (Calvin-Benson-Bassham) pathway, but into the

463   CO_2 fixation linked with the AMP (adenosine monophosphate) recycling for ultimate ATP

464   (adenosine triphosphate) production, similarly to the type III archaeal RuBisCo (Kantor et al.,

465   2013; Wrighton et al. 2012). The occurrence of this pathway suggests that these organisms are

466   not restricted to oligotrophic environments, but can survive with higher levels of available

467   nutrients.

468

469   **4.10   TM7 bacteria or "*Candidatus* Saccharibacteria"**

470   Recent studies have shown that nano-sized organisms can also be a component of the human

471   microbiome. A member of the bacterial candidate phylum TM7 ("*Ca*. Saccharibacteria") was

472   cultivated and co-isolated with *Actinomyces odontolyticus* subsp. *actinosynbacter* strain

473   XH001 by He et al. (2015). Having spherical cells of 200-300 nm in diameter and a genome of

474   0.705 Mbp, this bacterium of phylotype TM7 (strain TM7x) is associated with human oral

475   microflora and was found to have a rather unique lifestyle. Like many of others discussed here,

476   it is dependent on its basibiont, the host of the epibiont, an organism that resides on the surface

477   of the host, *Actinomyces odontolyticus* subsp. *actinosynbacter* XH001. Under normal

478   conditions, TM7x is an obligate epibiont, but during starvation it changes its lifestyle to

479   parasitic, which eventually kills its own host and which is not usual for oral microorganisms

480   (He et al., 2015; McLean et al., 2016). Additionally, TM7x lacks the ability to produce its own

481   amino acids which further suggests its dependence on *A. odontolyticus* subsp. *actinosynbacter*

482   XH001 (He et al., 2015). Its relationship with the host is thought to exacerbate oral mucosal

483   diseases by concealing host immune responses by inhibiting *A. odontolyticus* XH001-induced

484   TNF-α mRNA expression in macrophages (He et al., 2015). However, not all *Candidate*

485   phylum TM7 members reside in the oral mucosa like TM7x: for example, RAAC3 with a small

486   (0.845 Mbp) genome was originally found in a sediment obtained from an acetate-stimulated

487   aquifer (Kantor et al., 2013). Another representative of TM7 group, "*Candidatus*

488   Saccharimonas aalborgensis", with the genome of 1.0 Mbp was obtained from the activated

489   sludge bioreactor (Albertsen et al., 2013; He et al., 2015). It remains unclear why TM7x has a

490   more streamlined genome than the other phylotypes, a possible explanation of this adaptation

491   is its specific human microbiome habitat and its complete dependency on its actinomycete host.

492

493   **5   Selective pressures for small size**

494   An important conclusion that can be made from the aforementioned studies on small-size

495   microorganisms is that their sizes and distribution are a direct consequence of nutrient

496   availability. As mentioned previously, increasing the surface area-to-volume ratio, which is an

497   attribute of smaller cells, provides microorganisms with the ability to take up nutrients more

498   efficiently (Giovannoni et al., 2014). Both symbiotic and free-living organisms seem to have

499   benefited from this change. The results from existing studies suggest that in environments with

500   high nutrient concentrations, a nano-sized organism will likely be a symbiont (or epibiont) with

501   a decreased cell size being a result of limited metabolic capabilities with complete metabolic

502   dependence on a host (Martínez-Cano et al., 2015). *Nanoarchaeum equitans* is a good example

503   of this, as hydrothermal vents are relatively nutrient-rich, but these archaea are completely

504   dependent on *Ignococcous hospitalis* (Giannone et al., 2014). As nutrients become less

505   available, the more likely the small-sized organism will be free-living because an increased

506   surface-area-to-volume ratio is incredibly advantageous under such conditions (Martínez-Cano

507   et al., 2015). The species "*Ca*. Pelagibacter ubique" is a good illustration of this scenario.

508   Residing in the nutrient-depleted open ocean, it needs to produce its own essential amino acids,

509   vitamins, etc. to survive (Carini et al., 2012). This raises the question, as to why this typical

510   adaptation (small size and limited metabolic capabilities) does also exist in relatively stable

511   nutrient-rich habitats. One possibility is that there may be selective pressures coming from

512   predatory species, especially in aquatic systems (Pernthaler et al., 2001; Simon et al., 2002;

513   Pernthaler, 2017). In the study of Pernthaler et al. (2001), the presence of the protozoan,

514   *Ochromonas* sp.*,* resulted in an increasing population of members of *Actinobacteria* cluster

515   Ac1. When an alternate protozoan predator, *Cyclidium glaucoma*, was introduced, no increase

516   in population densities of Ac1 bacteria was observed (Pernthaler et al., 2001). Apparently,

517   *Ochromonas* sp. prefers preys that are 0.8 to 4 µm in size, while *C. glaucoma* prefers those

518   smaller than 0.8 µm. Since the Ac1 are smaller than 0.8µm, the presence of only *Ochromonas*

519   sp. allowed them to proliferate (Pernthaler et al., 2001). It was later found that some isolates of

520   Ac1 were in fact ultramicro-sized (less than 0.1 μm^3 volume) and this small size prevented

521   them from predation by *Ochromonas* sp. strain DS (Hahn et al., 2003). Hence, large

522   populations of small organisms may also be a response to, or the result of, protozoan grazing

523   (Salcher, 2014).

524   Another driver of selection of particular organisms in the environment are viruses and

525   phages. Phages are host-specific and in most cases infect highly populous and dense bacterial

526   subpopulations, which allows for less competitive (e.g. slow-growing) cells to proliferate (

527   Winter et al., 2010; Salcher, 2014). Lysis of infected cells releases nutrients into the

528   environment and makes them available to other community members allowing for overall

529   microbial population growth (Weinbauer, 2004; Salcher, 2014). Viruses, similarly to predators,

530   act as population control by culling overpopulated microorganisms ("killing the winner") while

531   providing nutrients in the form of lysed cells to other species in the community (Weinbauer,

532   2004; Winter et al., 2010; Salcher, 2014).

533

6   Functional role of small-sized organisms

As documented here, small-sized organisms are not characterized by any specific type of metabolism or taxonomic affiliation. Therefore, we assume that their functional role is not restricted and may highly vary depending on the environment and actual physio-chemical conditions. Aquatic systems are incredibly complex, as fluctuations between high and low nutrient availability are common. In marine systems, the addition of nutrients e.g. in the form of nitrogen-rich fertilizers from agricultural runoffs, can greatly change the once oligotrophic environment into a copitrophic one, leading to harmful large scale phytoplanktonic blooms (Beman et al., 2005). Depending on concentrations of nutrients, populations of free-living small-celled microorganisms can either be enriched in R-strategists, or in K-strategists playing distinct roles in the community. K-strategists, e.g. SUP05 clade and "*Ca*. P. ubique", are heavily involved with carbon and nitrogen cycling in oligotrophic areas (such as the open ocean and oxygen-depleted zones) (Giovannoni, 2017; Rogge et al., 2017). They are slow-growing and are widely dispersed, and rarely form colonies (Roshan and DeVries, 2017; Dang and Lovell, 2016; Giovannoni, 2017). R-strategists, e.g. Marine *Roseobacter* Clade (MRC) members and *Bacteroidetes,* are widely distributed and typically reside in nutrient-rich systems, e.g. in coastal systems (Dang and Lovell, 2016). These free-living organisms under favorable conditions grow quickly and may form large densely packed colonies and biofilms (Dang and Lovell, 2016). MRC bacteria can produce auxins and vitamins that are beneficial for algae (Dang and Lovell, 2016), whereas catabolically versatile *Bacteriodetes* play key roles in degrading high molecular weight dissolved organic matter (DOM) and biopolymers (Dang and Lovell, 2016).

In vertebrate systems, the role of these organisms appears variable. As seen in the case of TM7x, it may be beneficial or harmful to the host. *Actinomyces* strain XH001 normally elicits an immune response but TM7x modulates this response by either suppressing TNF-α

559   gene expression in macrophages or "masking" it from macrophage detection altogether.

560   However, under extended starvation conditions, TM7x can turn parasite, which leads to the

561   host's demise (He et al., 2015).

562   Much of the literature discussed in this review has focused on a few species, however,

563   the concerted effect of the entire ultra-small-sized microbial community in ecosystem

564   functioning remains unknown. As discussed earlier, filtration through <0.45 μm pore size

565   filters, is a common method to isolate small cells from aqueous samples. Interestingly,

566   ultrafiltration was considered a method of choice to preserve freshwater samples during their

567   storage and prior the hydrochemistry analysis (Brailsford et al., 2017). 0.22 μm pore size filters

568   were considered as a safe tool for sterilization and for effective removal of microorganisms.

569   However, a recent study, which monitored the depletion of ^{14}C-glucose, ^{14}C-amino acid

570   mixture, and ^{33}P-orthophosphate in filtered and unfiltered freshwater samples showed

571   significant activity and utilization of substrates by organisms capable of passing this barrier

572   (Brailsford et al., 2017). The previous studies clearly support this claim, as a number of the

573   species were able to pass through ultrafiltration membranes (e.g. Wang et al., 2008). The great

574   abundance of small-sized organisms in aqueous environments may also be attributed to

575   selective pressures of predator-prey-viral interactions (Salcher, 2014). As discussed, protists

576   feed on bacterioplankton and select prey based on cell size (Pernthaler et al., 2001; Salcher et

577   al., 2013; Pernthaler, 2017). Conversely, viruses select for high-density preys and promote

578   generation of DOM from lysed cells (Salcher, 2014), which can then be utilized by nano-sized

579   microorganisms.

580   Nutrient cycling by ultra-small-sized organisms is not restricted to aquatic

581   environments. A number of studies have shown an active population of ultramicrobacteria

582   within a wide range of soil types (Soina et al., 2012; Lysak et al., 2013; Dobrovol'skaya et al.,

583   2015). It was previously thought that soil pores <1 μm would be inaccessible to cells, leading

584   to physical protection of organic carbon in soil. However, the potential of small-sized

585   organisms to occupy this void space alongside their functional significance in soil remain

586   unknown.

587

588   **7   Conclusions and outlook**

589   Discovery of small cells in the environment has reshaped our understanding of the microbial

590   world and life on this planet. Using culture-independent tools first insights into the

591   functionality of these organisms and a precise definition of the minimal sizes of living forms

592   have been gained. Hence, it is reasonable to think that small-sized organisms may play a

593   significant role in many environments. Many studies performed to date, however, have not

594   considered the functionality of these organisms. Future studies should therefore shift their focus

595   to understanding their physiology and function. As more ecosystems are explored and as

596   techniques are improved, the possibility of finding small-sized organisms is increasing.

597   Culture- independent analysis will remain a critical tool for modelling and predicting

598   functionalities and abundance of these organisms, however, the functional analysis of their

599   activities remains essential to validate genome-based predictions.

600

601   **8   Author contributions**

602   The review was conceived by all the authors. L.J.G searched the literature, synthesized the data

603   and wrote the manuscript. D.L.J, P.N.G, and O.V.G provided significant revisions to the

604   manuscript including data interpretation and wrote parts of the manuscript. All authors read the

605   final manuscript.

606

607   **9   Acknowledgements**

615
616   **10 References**
617

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937

938   **11 Table captions**

939   Table 1- An overview of small-sized and filterable organisms denoting average cell size,

940   average genome size, environment, separation technique (filter pore sizes), cultivability,

941   affiliation to a confirmed species, lifestyle (free living or host-dependent), and corresponding

942   references. NA denotes information not available within the respective source. Some studies

943   showed that the results were inconclusive meaning that there were conflicting conclusions in

944   the literature. *Colonies were slow-growing, taking up to a few months to become visible.

945   **Proposed Candidatus status. †Parasitic ultramicrobacteria discussed in Duda et al. (2012)

946   review.

947

948   **12 Figure captions**

949   Figure 1- Summary of definitions used to describe nano-sized organisms. References are the

950   following: [1] Duda et al. (2012) [2] Verlmirov et al. (2001, [3] Panikov (2005), [4] Shut et

951   al. (1995), [5] Miteva and Brenchley (2005), [6] Luef et al. (2015), [7] Huber et al. (2002),

952   [8], Rogge et al. (2017), [9] Giovannoni (2017), [10] Kajander and Ciftcioglu (1998), [11]

953   Fedotova et al. (2012)

954   Figure 2- Surface area (SA) and volume (V) ratios in three selected species of different sizes:

955   *Escherichia coli*, "*Candidatus* Pelagibacter ubique", and *Nanoarchaeum equitans*. The

956   microorganism with the smallest dimensions ("*Ca.* P. ubique") had the largest ratio at 22. The

957   habitat of "*Ca.* P. ubique" is the open ocean (oligotrophic environment) and hence its high

958   SA/V ratio is advantageous to living in low nutrient conditions. The total protein numbers in

959   encoded by genomes of *E. coli* (NCBI Reference Sequence: NC_000913.3), "*Ca.* P. ubique"

960   (GenBank: CP000084.1), and *N. equitans* (GenBank: AE017199.1) are given and related with

961   the proteins with membrane-spanning domains. For prediction of transmembrane helices in

962   proteins,   above   genomes   were   analyzed   using   TMMHMM   2.0   Server   at

963   http://www.cbs.dtu.dk/services/TMHMM/ (Krogh et al., 2001; Möller et al., 2001).

964   Notes: * Dimensions and calculations of surface area and volume were obtained from Young, 2006.

965   ** The diameter was obtained from Huber et al. (2002), the equations for the surface area (SA= $4\pi r^2$, where r is the radius)

966   and volume (V=$\frac{4}{3}\pi r^3$, where r is the radius) of a sphere.

967

968   Figure 3- Size comparison of nano-sized organisms. Each of the colored lines represents

969   relative range of sizes (in one dimension) of each individual. References and numerical ranges

970   for individuals can be found in Table 1. If size was reported with volume, the organism was

971   assumed to be spherical and then obtained the radius with the equation, V=$\frac{4}{3}\pi r^3$, where r is the

972   radius. *References for size guides: *Escherichia coli* (approximately 1 μm x 2 μm) and phage

973   T4 (approximately 90 nm x 200 nm) (Leiman et al., 2003). Note: *Ca.* Nanobsidianus stetteri'

974   has no available information concerning cellular dimensions.

975

976

977   **Table 1**

978

Small--sized organism(s)	Environment	Average genome size	Average/range cell size	Free-living?	Filter(s) pore size used	Cultured?	Validly published Species	Reference
'*Ca.* Pelagibacter ubique'	Open ocean	1.3 Mbp	0.01 μm³ (volume)	Yes	0.2 μm	Yes	Yes	Rappè, et al. (2002), Giovannoni et al. (2005), Carini et al (2012), Zhao et al. (2017), Giovannoni (2017)
Nanoarchaeum equitans	Submarine hot vent	0.5 Mbp	0.4 μm (diameter)	No	None	Yes	Yes	Huber et al. (2002) Waters, et al. (2003), Jahn et al (2008)
Ultrasmall Microorganisms	120,000 year old Greenland ice core	NA	<0.10 μm³ (volume)	NA	0.4 μm, 0.2 μm, and 0.1 μm	Yes*	No	Miteva and Brenchley (2005)
ARMAN cells	Acid mine drainage biofilm	1 Mbp	0.03 μm³ (volume)?	Inconclusive	0.45 μm	No	No	Comolli et al. (2009), Comolli and Banfield, (2014), Baker, et al. (2010), Baker, et al. (2006)

'Ca. Nanobsidianus. stetteri'	Obsidian Pool, Yellowstone National Park	0.651 Mbp	NA	No	0.4 µm	No	No	Podar, et al (2013), Munson-McGee, et al. (2015)
Oral TM7 *'Ca.* Saccharibacteria'	Human oral cavity	0.705 Mbp	200-300 nm (diameter)	No	0.22 µm	Yes	No	He, et al. (2015)
"*Ca.* 'Nanopusillus acidilobi'	Cistern Spring, Yellowstone National Park	0.605 Mbp	100-300 nm (diameter)	No	0.1 µm	Yes	No**	Wurch, et al. (2016)
WWE3/OP11/OD1 groundwater ultra-small bacteria	Anoxic aquifer	0.878 Mbp (WWE3) 0.694 Mbp (OD1) 0.820 to 1.050 Mbp (OP11)	0.009 µm³ (volume)	No	1.2 µm, 0.2 µm, and 0.1 µm	No	No	Luef, et al. (2015), Wrighton, et al. (2012), Kantor, et al. (2013)
'Nanobacterium sanguineum'	Human and bovine blood	NA	50 nm (diameter)	NA	0.1 µm	Inconclusive	No	Kajander and Ciftcioglu (1998), Kajander and Ciftcioglu (2003), Cisar et al (2000),
Fossil remains	Meteorite ALH84001	NA	10-200 nm (length)	NA	NA	NA	No	McKay et al. (1996), McKay et al. (2001)
SUP05 Bacteria	Pelagic redox zones	1.164 Mbp to 1.53 Mbp	0.01-0.09 µm³ (volume)	Yes	0.2 µm	No	No	Rogge et al. (2017), Glaubitz et al. (2013), Shah et al. (2017)
Filterable forms	Lake Motykino and Lake Dubrovskoe (Peatland bog)	NA	0.3-0.5 µm (rod diameter)	NA	0.22 µm	No	No	Fedotova et al. (2012)
Aurantimicrobium minutum Str. KNCT	River water	1.62 Mbp	0.04-0.05 µm³ (volume)	Yes	0.22 µm	Yes	Yes	Nakai et al. (2016)
Curvibacter sp. Str. PAE-UM	River sediment	3.28 Mbp	<0.05 µm³ (volume)	Yes	NA	Yes	Yes	Ma et al. (2016)
Free-living Ultramicroscopic bacteria	Natural biotopes (i.e., permafrost, oil slime, soil, lake silt, thermal swamp moss, *Xenopus laevis*, skin)	1.5-2.4 Mbp	0.02-1.3 µm³ (volume)	Yes	NA	No	No	Suzina et al. (2015)
Bdellovibrio spp.†	NA	3.78 Mbp	0.13 µm³ (volume)	No	NA	Yes	Yes	Duda et al. (2012)
Micavibrio admiranndus†	NA	NA	0.05 µm³ (volume)	No	NA	Yes	Yes	Duda et al. (2012)
Vampirovibrio chlorellavorus†	Reservoir water	NA	0.3-0.6 µm (diameter)	No	NA	Yes	Yes	Duda et al. (2012)
Kaistia adipata, str. NF1, NF3†	Soil and lake sediment	2.4 Mbp	0.1-0.5 µm³ (volume)	No	0.22 µm	Yes	Yes	Duda et al. (2012)
Chryseobacterium solincola, str. NF4, NF5†	Soil and lake sediment	1.7 Mbp	<0.1 µm³	No	0.22 µm	Yes	Yes	Duda et al. (2012)

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981   **Figure 1**

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Figure 1 panels:

A — Miniaturization due to external factors such as: fluctuations in nutrient avaliability and advanced age.

Terms: dwarf cells, midget cells, ultra-small, ultramicro,, ultramircocells

References: [1-3]

B — Size remains unchanged regardless of external factors. Cell volumes are 0.1 μm^3 or less (i.e. Cell diameter range of 0.05-0.40 μm) and genome sizes are between 0.58-3.2 Mbp.

Terms: ultra-small, ultramicro, nanoarchaea, nanoorganisms, nanbacteria, nanoforms

References: [1-11]

C — Large microorganisms (cell volume greater than 0.1 μm^3) pass thrriough small pore membrane filters (less than 0.45 μm) due to absent rigid cell walls.

Terms: filterable microorganisms

References: [1]

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985   **Figure 2**

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990   **Figure 3**

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