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## Water and microbial monitoring technologies towards the near future space exploration

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1 **Water and microbial monitoring technologies towards the near future space exploration**

2

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22

23 **Abstract**

24 Space exploration is demanding longer lasting human missions and water resupply from  
25 Earth will become increasingly unrealistic. In a near future, the spacecraft water monitoring  
26 systems will require technological advances to promptly identify and counteract contingent  
27 events of waterborne microbial contamination, posing health risks to astronauts with lowered  
28 immune responsiveness. The search for bio-analytical approaches, alternative to those applied  
29 on Earth by cultivation-dependent methods, is pushed by the compelling need to limit waste  
30 disposal and avoid microbial regrowth from analytical carryovers. Prospective technologies  
31 will be selected only if first validated in a flight-like environment, by following basic  
32 principles, advantages, and limitations beyond their current applications on Earth. Starting  
33 from the water monitoring activities applied on the International Space Station, we provide a  
34 critical overview of the nucleic acid amplification-based approaches (i.e., loop-mediated  
35 isothermal amplification, quantitative PCR, and high-throughput sequencing) and early-  
36 warning methods for total microbial load assessments (i.e., ATP-metry, flow cytometry),  
37 already used at a high readiness level aboard crewed space vehicles. Our findings suggest that  
38 the forthcoming space applications of mature technologies will be necessarily bounded by a  
39 compromise between analytical performances (e.g., speed to results, identification depth,  
40 reproducibility, multiparametricity) and detrimental technical requirements (e.g., reagent  
41 usage, waste production, operator skills, crew time). As space exploration progresses toward  
42 extended missions to Moon and Mars, miniaturized systems that also minimize crew  
43 involvement in their end-to-end operation are likely applicable on the long-term and suitable  
44 for the in-flight water and microbiological research.

45

46 **Keywords:**

47 International Space Station; space missions; biomonitoring; water biological contamination

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65

## 66 **1 Introduction**

67 Liquid water is essential for all known Earth-derived life forms living in space conditions,  
68 including microbes (McKay, 2014). The on-going space exploration has not yet demonstrated  
69 whether the presence of extra-terrestrial water could indicate itself the occurrence of  
70 microbial life, but the prevailing paradigm is that living microorganisms necessitate temporal  
71 and spatial proximity with aqueous solutions for their metabolism (Martín-Torres et al., 2015;  
72 Merino et al., 2019; Stevenson et al., 2015). In turn, the microbial contamination cannot be  
73 thoroughly eliminated from Earth’s waters but only controlled and attenuated on the long-  
74 term time scale (Lopez et al., 2019; Rettberg et al., 2019). Since space exploration has been  
75 demanding longer lasting missions, the analysis of waterborne microorganisms turned out of  
76 utmost importance for future human spaceflights, planetary outposts, and life-support systems  
77 (Horneck et al., 2010).

78 The prevention of crew infectious waterborne diseases is retained among the highest  
79 priorities particularly for long duration missions (Ott et al., 2014), since emergency resupply  
80 is unrealistic and recycled water could represent the only suitable source for the on-board  
81 activities. Most of the aquatic microorganisms found aboard the International Space Station  
82 (ISS) do not generally constitute a severe hazard for human health (Blaustein et al., 2019;  
83 Checinska Sielaff et al., 2019; Sobisch et al., 2019). However, they may threat astronauts  
84 with reduced immune response, mostly following the microgravity stress conditions (Garrett-  
85 Bakelman et al., 2019; Ott et al., 2016). Other concerning issues arise from microbial  
86 influences on spacecraft integrity and function, owing to the potential corrosion and  
87 degradation of stainless steel and other materials associated with the electronic equipment  
88 and life support systems (Horneck et al., 2010; Yang et al., 2018; Zea et al., 2018). Therefore,  
89 there is an increasing interest to improve the spacecraft water monitoring systems to identify  
90 and possibly counteract contingent events of microbial contamination (Van Houdt et al.,  
91 2012; Yamaguchi et al., 2014).

92 The definition, identification, and test of the microbial monitoring approaches suitable for the  
93 on-board water quality control are challenged by several technical constraints (e.g., material  
94 safety compatibility, resistance to launch vibration) and a minimal availability for managing  
95 excess power, storage, volume, mass, and crew time (Allen et al., 2018). Moreover, selected  
96 devices and their supporting reagents must remain viable for years, while operating safely  
97 and reliably in extreme conditions (e.g., in the absence of gravity). Technology flexibility is  
98 also critical, since monitoring systems should be able to detect different microbial targets  
99 (e.g., fungi, protists, prokaryotes, viruses) and to accept samples of various origin, spanning  
100 from biomedical (e.g., blood, urine, saliva samples, routine chemistry, cell cultures) to water  
101 and environmental samples (Nelson, 2011).

102 In the next years, the Chinese modular space station, built on the experience gained from its  
103 precursors Tiangong-1 and Tiangong-2, will be placed and operating in the Low Earth orbit  
104 (Gibney, 2019). The sustainable human exploration of the Moon is programmed in the  
105 meanwhile (El-Jaby et al., 2019; Pittman et al., 2016), whereas the human missions to the  
106 surface of Mars are envisioned before 2040 (ISECG, 2018). Therefore, it is hypothesized that  
107 only prospective methodological applications at a high technology readiness level (i.e., at  
108 least validated in a flight-like environment; Straub, 2015) will be selected by following their  
109 basic principles and current uses in Earth and space-analogue settings.

110 A number of review papers has recently emphasized the need for high-throughput  
111 technologies to timely monitor and achieve the stringent microbial quality requirements of  
112 future crewed space habitats (De Middleleer et al., 2019; Karouia et al., 2017; Liu, 2017;  
113 Moissl-Eichinger et al., 2016; Mora et al., 2016; Yamaguchi et al., 2014).

114 In this article, we narrow the focus on promising bio-analytic technologies for quality  
115 assessments of waters in space, with the aim to explore advantages and limitations beyond  
116 their current applications on Earth. Starting from the rigorous housekeeping program and the  
117 consolidated results of water monitoring activity on the ISS (Duncan et al., 2008; Limero and  
118 Wallace, 2017), we provide a critical overview of the microbial monitoring approaches,  
119 based on flexible technologies for the identification of microbial components and a total  
120 contamination assessment (total microbial burden) in waters circulating on crewed space  
121 vehicles.

122

## 123 **2 Water recycle and microbial monitoring aboard the International Space Station**

### 124 **2.1 The ISS water cycle**

125 The water recycling system innovations required to support ISS activities have been listed  
126 among the major benefits for humanity (Detsis and Detsis, 2013; NASA et al., 2019). The

127 ISS is provided with potable water from different suppliers, coordinated by the space  
128 agencies of United States (National Aeronautics and Space Administration - NASA), Russia  
129 (Russian Federal Space Agency - Roscosmos), Europe (European Space Agency - ESA), and  
130 Japan (Japanese Aerospace Exploration Agency - JAXA) (Bruce et al., 2005; Van Houdt et  
131 al., 2012). All the possible necessary precautions to prevent external contamination are  
132 applied throughout water transferring and loading steps over the entire treatment period  
133 before the liftoff of supply modules. For instance, the American and Russian waters are  
134 produced in conditioned and limited-access areas and preparation facilities, with no risk of  
135 accidental water quality modifications during the production process. At the research center  
136 of the Italian Società Metropolitana Acque Torino (SMAT), purified waters for space travels  
137 are also processed upon selecting well and spring waters that most closely meet the physical,  
138 chemical, and bacteriological quality standards for astronauts (Lobascio et al., 2004).  
139 Currently on ISS, waters for direct human consumption are regularly delivered and recovered  
140 in order to guarantee approximately 4 L per person per day (Figure 1). A reserve of potable  
141 water (up to approx. 2000 L) is stored in contingency containers to maintain ISS operations in  
142 response to emergency scenarios (Carter et al., 2018). Although routinely monitored and kept  
143 constant, the overall water mass balance represents a recurrent major challenge owing to the  
144 various ISS water needs (Pickett et al., 2020).  
145 Beside the on-demand crew consumption, on-board waters are distributed for different  
146 purposes, comprising hygiene and cleaning practices, urinal flushing, oxygen generation via  
147 electrolysis, life-support systems, and flexible water-based experimental activities (e.g.,  
148 vegetable and food production systems, animal physiology and behavioral adaptation tests)  
149 (Baiocco et al., 2018; Chatani et al., 2015; Massa et al., 2016; Niederwieser et al., 2018;  
150 Ronca et al., 2019; Wolff et al., 2018). Wastewaters are continuously collected and recycled  
151 at high efficiency level (Pickett et al., 2020). In the US segment, the Water Recovery and

152 Management System was reported to recuperate up to 85% from crew urine and flush water,  
153 along with the water content from liquid wastes and humidity condensate from the cabin.  
154 Various containers, reservoirs, tanks and bellows are also necessary to maintain water  
155 pressure and circulation through the distribution network (Carter et al., 2018).  
156 Since microbial growth is unavoidable in persistent stagnation zones and at varying residence  
157 times along the water distribution network (Lautenschlager et al., 2010; Ling et al., 2018), the  
158 pre- and in-flight addition of biocides is used for residual microbial control. Molecular iodine  
159 is applied in the U.S. segment, while the ionic silver level is amended in Russian waters, both  
160 at low concentrations (i.e., not detrimental for human health) (Artemyeva, 2016; Lobascio et  
161 al., 2004). Moreover, high temperature in the catalytic reactor, multifiltration beds within the  
162 Water Processor Assembly, UV-C LEDs within the CO<sub>2</sub> Concentration Assembly of the  
163 Advanced Closed Loop System, and novel antimicrobial coatings on various ISS surfaces  
164 were proven effective against potential microbial biomass growth (Bockstahler et al., 2017;  
165 Carter et al., 2018; Perrin et al., 2018; Petala et al., 2020; Roman et al., 2006; Sobisch et al.,  
166 2019). Finally, the ISS is maintained at pressure and oxygen levels very close to those at sea  
167 level on Earth, with a cabin temperature of about 22°C and a relative humidity of about 60%,  
168 in order to minimize detrimental growth of microbial biofilms on cabin surfaces (Pierson et  
169 al., 2013).

170

## 171 **2.2 On-board water monitoring and microbial contamination**

172 The achievement and maintenance of water quality standards are evaluated by systematic  
173 monitoring procedures (Limero and Wallace, 2017). Major water physical-chemical  
174 parameters including conductivity, pH, total organic, total inorganic and total carbon, nitrate,  
175 potassium, chloride and ammonium are monitored in-flight, together with iodine and silver  
176 levels (Artemyeva, 2016). Moreover, a robust monitoring program was implemented to verify



177 that risks of microbial contamination were within acceptable limits in samples collected from  
178 different sites of the Russian and US segments, respectively once every three months and  
179 each month (Pierson et al., 2013; Van Houdt and Leys, 2012). Crewmembers use handheld  
180 equipment to monitor ISS waters and humidity condensate from surfaces. Chemical and  
181 biological samples are taken concurrently and at a frequency that may change due to real-  
182 time flight necessities (Pierson et al., 2013).

183 Water samples can be processed on-board by cultivation-based methods using the US-  
184 supplied Water Microbiology Kit for the quantification of total heterotrophic bacteria and  
185 coliforms (Bruce et al., 2005). The maximum total number of aerobic heterotrophic viable  
186 bacterial cells, counted as colony forming units on a rich agar medium, was internationally  
187 defined according to the concentration levels that are achievable with the current prevention  
188 and monitoring technologies available and applicable for space (i.e., HPC  $\leq$  50 CFU/ml).

189 Microbial quality standards are also set for ISS internal surfaces, from which humidity  
190 condensate is collected (maximum bacterial load = 10000 CFU/100 cm<sup>2</sup>; maximum fungal  
191 load = 100 CFU/100 cm<sup>2</sup>) (Van Houdt and Leys, 2012). Along with the on-board monitoring  
192 activities, archival water samples are regularly collected in teflon bags (Figure 1), preserved,  
193 and returned to Earth approximately every three months for post-flight analyses (Limero and  
194 Wallace, 2017).

195 The microbial contamination level was above the former acceptability limits several times  
196 during previous in-flight monitoring surveys. Events of microbial biofilm growth within  
197 space vehicles were mainly associated with the water layer covering internal surfaces and life  
198 support systems (La Duc et al., 2004; Novikova et al., 2006; Roman et al., 2006). Thus,  
199 waters collected from humidity condensate were retained among the major sources of  
200 microbiological hazard for potable water quality deterioration (Horneck et al., 2010).

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**Figure 1.** A water drop floating on the ISS (upper left). The Italian astronaut Samantha Cristoforetti and the Russian cosmonaut Salizhan Sharipov showing the Teflon bag (upper right), the water tank (lower left) and removal system (lower right), used for drinking purposes and water storage on the ISS (Credits: ESA and NASA).

Several cultivable microbial isolates obtained from spaceflights were mainly affiliated to Bacteria and Fungi (Bruce et al., 2005; Coil et al., 2016; Novikova et al., 2006). For the purposes of this review, it is worth noting that the analysis of the microbial cultivable fraction were likely to provide only a limited snapshot of the highly diverse community found on the ISS by cultivation-independent methods (Checinska Sielaff et al., 2019; Coil et al., 2016; De Middelmeer et al., 2019; Ichijo et al., 2016; Lang et al., 2017; Mora et al., 2019; Morris et al., 2012). Despite major advantages arise from target-specific isolation and characterization of different types of waterborne microorganisms and pathogens in pure culture, a number of technical and logistic disadvantages characterizes the space application of cultivation-based methods. The microgravity conditions provide conflicting results on microbial growth and

218 virulence of opportunistic human pathogens (Morrison et al., 2017; Zhang et al., 2019), while  
219 the analysis of archival samples generates a detrimental time gap for a flight-supportive result  
220 interpretation (Huang et al., 2018; Novikova et al., 2006). HPC was proven to be also  
221 affected by carbon sources in cultivation media, the incubation time, and the initial microbial  
222 load level (Amalfitano et al., 2018b). Finally, the prompt development and requirement  
223 update of alternate bio-analytical technologies for microbial monitoring during space  
224 exploration is pushed fundamentally by the need to avoid microbial regrowth from analytical  
225 wastes (Wong et al., 2017).

226

### 227 **3 Nucleic acid amplification-based methods for microbial identification in space** 228 **waters**

229 Methods based on nucleic acid amplification offer the advantage of specific and fast  
230 detection, easy automatization and standardization. Many of the limitations of cultivation-  
231 based techniques adopted for bacterial detection and water quality monitoring are  
232 overwhelmed by largely reducing the time required for the microbial identification including  
233 also viable but not cultivable bacteria and un-cultivable pathogens.

234

#### 235 **3.1 Sample pre-treatments and nucleic acid extraction in space**

236 Sample pre-treatment and nucleic acid extraction are key starting points for most of the  
237 molecular approaches and product developments dedicated to microbial water quality  
238 monitoring. Nucleic acids extracted from water samples are amplified to specific markers.  
239 The extraction and purification of nucleic acids rely on cell lysis and the selective binding of  
240 cellular DNA and RNA to solid surfaces through filtration and column-based protocols.  
241 A fast biomolecular analysis can be conducted in space settings with the in-line  
242 implementation of optimized protocols for fast DNA and RNA extraction and sample

243 preparation for sequencing. Published studies reported new promising field-deployable  
244 amplification devices and approaches for on board applications (Boguraev et al., 2017;  
245 Montague et al., 2018) (Figure 2). Simplified, sample processing and DNA purification,  
246 strategies have currently been tested aboard the International Space Station (ISS). The  
247 Wetlab-2 project has already developed and tested on the ISS a Sample Preparation Module  
248 (SPM) to lyse cells, and to provide high quality extraction of nucleic acids extract, by  
249 circumventing operational issues related to microgravity, surface tension alteration, reduced  
250 operational space and handling expertise (Parra et al., 2017).

251 Numerous commercialized kits for solid-phase extraction allow handy and rapid nucleic acid  
252 purification procedures, in which potential contaminants are removed through sequential  
253 washing steps based on centrifugation or DNA separation by paramagnetic beads (Tan and  
254 Yiap, 2009). Methods for the direct PCR amplification without DNA extraction were also  
255 developed (Williams et al., 2017). Recent papers reported simplified methods for nucleic acid  
256 purification using filtration membranes and DNA amplification from the nucleic acid directly  
257 on filters (Kaliyappan et al., 2012; Rodriguez et al., 2016). Notably, a cellulose-paper-based  
258 dipstick was used to efficiently bind, wash, and elute purified nucleic acids from different  
259 matrices without any pipetting or electrical equipment (Zou et al., 2017).

260 Nevertheless, a major bottleneck for the in-flight application of amplification based  
261 approaches still lies on the need for time-consuming and waste-producing sample  
262 concentration and DNA extraction (Girones et al., 2010). In particular, the method sensibility  
263 is lowered when amplifying targets present at low levels over the total extracted DNA  
264 (Brandt and Albertsen, 2018). Suboptimal DNA extraction and purification are known to  
265 affect results of biomolecular analysis, mainly owing to uncomplete lysis of more resistant  
266 bacterial populations and poor removal of DNA polymerase inhibitors (Albertsen et al., 2015;  
267 Girones et al., 2010). DNA purification requires effective cell disruption, inactivation of

268 nucleases, and purification of DNA from contaminants that might interfere with the  
269 amplification efficiency. Residual extracellular DNA could not be easily discriminated from  
270 that of viable dangerous microorganisms, thus leading to an overestimation of risk for human  
271 health (Girones et al., 2010). Moreover, waterborne substances can concentrate together with  
272 DNA during sample processing and inhibit polymerase enzymes, thus influencing the  
273 sensitivity and reliability of the PCR-based microbial detection. Overall, the relative accuracy  
274 and precision (i.e., repeatability and reproducibility) of nucleic acid amplification based  
275 methods are affected by sample type and pre-treatment methods (concentration and DNA  
276 extraction), thus requiring to be evaluated across the whole sample processing (Hospodsky et  
277 al., 2010; Kralik and Ricchi, 2017).

278

### 279 **3.2 Target-based techniques for detection and quantification of nucleic acids**

280 The polymerase chain reaction (PCR) is widely used for detecting genes and microorganisms  
281 of health concern in water (Ramírez-Castillo et al., 2015). Among these target-based  
282 detection approaches, the loop-mediated isothermal amplification (LAMP) of nucleic acids  
283 was retained as a rapid and sensible option (Zhao et al., 2015), with minimal requirements for  
284 the in-flight water quality monitoring and pathogen detection (Ott et al., 2014). The  
285 amplification takes place at isothermal temperature (60-65°C) and positive reactions can be  
286 visualised by naked eye (i.e., without post-amplification steps) following the increase of  
287 sample turbidity or colour owing to the addition of fluorescent dyes (Notomi et al., 2015).  
288 Being less sensible than PCR to inhibition and not significantly influenced by non-target  
289 DNA, direct LAMP assays are currently used with good analytical performances (Etchebarne  
290 et al., 2017; Samhan et al., 2017). Low amount of DNA can be amplified up to generating  $10^9$   
291 copies within 1 h and producing as final amplification product a complex stem-loop DNA,  
292 with several inverted repeats of the target and cauliflower-like structures. Commercial kits for

293 the rapid on-site detection of water pathogens are already available for terrestrial  
294 applications, also comprising quantitative real-time LAMP, reverse transcription RT-LAMP,  
295 *in situ* LAMP, and viable LAMP (Notomi et al., 2015). For the scopes of this review, it is  
296 worth noting that a rapid method for detecting approximately 1 CFU/100 ml of *Legionella*  
297 *pneumophila* in tap water was developed and efficiently applied on field in less than 2  
298 working hours through a direct on-filter LAMP amplification with live/dead propidium  
299 monoazide (PMA) differentiation (Samhan et al., 2017).

300 Among the target-based quantification approaches, the quantitative PCR (qPCR) represents a  
301 popular technique for the in-flight water quality monitoring (Oubre et al., 2013). The  
302 quantification of target sequences is based on the development of a fluorescent signal  
303 proportional to the amount of amplified product obtained during the PCR thermal cycles.  
304 Multiplex qPCR can be used for the simultaneous detection and quantification of multiple  
305 pathogens, consistently reducing analytical time and costs (Ibekwe et al., 2002; LaGier et al.,  
306 2004). With low detection limits (<400 cells per sample) and volume requirements (<100 µl  
307 of sample), qPCR assays are routinely applied in monitoring plans for the detection and  
308 quantification of waterborne pathogens (Girones et al., 2010; Ramírez-Castillo et al., 2015).  
309 Good repeatability and reproducibility of qPCR outcomes were reported for gene targets of  
310 fecal origin and a standardized workflow achieved consistent results, with low intra- and  
311 inter-laboratory coefficients of variation (median CV = 0.1-3.3% and 1.9-7.1%, respectively)  
312 (Ebentier et al., 2013; Orin et al., 2012). A large body of the scientific literature has  
313 documented the development and use of qPCR for pathogenic viruses, bacteria, protozoa, and  
314 fungi (Kralik and Ricchi, 2017; Ramírez-Castillo et al., 2015). Through a pre-treatment with  
315 cell membrane impermeant DNA intercalating dyes, the so-called Viable qPCR was applied  
316 for discriminating between viable (with intact membrane) and dead (with damaged  
317 membrane) bacteria, allowing the quantification of water- and food-borne pathogens such as

318 *Campylobacter*, *E. coli* O157:H7, *Legionella pneumophila*, *Salmonella*, *Cryptosporidium*  
319 (Banihashemi et al., 2012; Brescia et al., 2009; Delgado-Viscogliosi et al., 2009).

320 Given the versatile applications of the numerous available assays, both LAMP and qPCR  
321 have been retained as suitable water monitoring methods for long-term exploration missions.  
322 Owing to the high sensitivity, specificity, and simple post-amplification steps to detect the  
323 amplified targets, LAMP was proposed by the Japanese Aerospace Exploration Agency as  
324 alternative microbial contamination monitoring system for the ISS (Ott et al., 2014). For the  
325 analysis of crew health related genetic modifications, qPCR technologies have been  
326 successfully tested on-board the ISS within the projects Gene in Space and Wet-lab2, devoted  
327 to definition of a robust, user-friendly nucleic acid extraction and sequencing approach  
328 aboard ISS (Boguraev et al 2017; <https://www.genesinspace.org/>;  
329 [https://www.nasa.gov/mission\\_pages/station/research/experiments/1913.html](https://www.nasa.gov/mission_pages/station/research/experiments/1913.html);  
330 <https://www.nasa.gov/ames/research/space-biosciences/wetlab-2>). Moreover, the RAZOR EX  
331 PCR, launched on Space-X 9 (July 2016) within Water Monitoring Suite project will allow  
332 performing direct PCR amplification from water samples.

333 In all current space applications, however, the selected target-based detection and  
334 quantification approaches require the use of disposable materials and labour intense  
335 protocols, which will inevitably reduce their long-term applicability in space conditions. The  
336 general precautions used on Earth to limit contamination risks, including the most stringent  
337 procedures applied in clean rooms (Rettberg et al., 2019), might also represent a limiting  
338 practical issue in the small close spacecraft environment, since the high sensibility of PCR-  
339 based detection increases the chance of amplifying carry over contamination, with the  
340 consequent production of false positive results. This is crucial for LAMP owing to the limited  
341 accessibility to degradation of DNA products, while a major limitation of qPCR is also the  
342 occurrence of inhibitors that can be co-concentrated or extracted along with nucleic acids

343 from the target microorganisms (Gibson et al., 2012). The presence of qPCR inhibitors  
344 introduces a number of problems, ranging from low amplification efficiency and reduced  
345 assay sensitivity to complete reaction failure and false negative results (Radstrom et al.,  
346 2008).

347

### 348 **3.3 Sequencing-based “-omics” approaches for microbial community characterization**

349 The High-Throughput Sequencing (HTS) encloses a popular suite of technologies,  
350 methodological approaches, and data elaboration workflows used to characterize the  
351 phylogenetic composition of the total microbial community in different aquatic matrices. The  
352 taxonomic classification is based on the huge amount of sequences generated by either a  
353 portion of the cellular nucleic acid content or the whole genome, through the so-called  
354 amplicon and shotgun sequencing approaches, respectively (Peabody et al., 2015).

355 The amplicon analysis represents the extension of sequencing based methods of organisms’  
356 classification, defined by specific protocols under the general name of “genetic” or  
357 “molecular barcoding” (Hebert and Gregory, 2005), which is based on the information  
358 carried by a single conventional marker, such as the 16S rRNA for microorganisms.

359 The shotgun sequencing consists in generating millions of DNA fragments of different  
360 lengths from a starting pool of genomes. These fragments cover a quote of the original  
361 genomes inversely proportional to the genome lengths, and taxonomic assignment may be  
362 quantitative according to the proportion of fragments classified for each taxon. The amplified  
363 products are sequenced, controlled for quality, and assembled in longer contigs. The obtained  
364 sequences are classified at different taxonomic levels, according to the best match with a  
365 reference sequence database and following different similarity criteria (Segata et al., 2012).

366 Currently, the most used sequencing platforms are characterized by different technical  
367 principles and include Roche 454 GS-FLX (pyrosequencing), Illumina MiSeq and HiSeq



368 (reversible terminator sequencing by synthesis), Ion PGM (semiconductor based sequencing  
369 by synthesis), and the nanopore GridION and MinION™ (Check Hayden, 2015; Clooney et  
370 al., 2016; Ghanbari et al., 2015; Glenn, 2011).

371 Performance evaluation and standardization of HTS workflows are still limited for water  
372 microbial community characterization. The DNA extraction procedures and primers sets were  
373 reported to influence the assessment of the bacterial community composition in drinking  
374 waters, with a generally effective representation of the core abundant taxa at total cell  
375 abundance levels of  $10^3$ - $10^5$  cells/ml (Brandt and Albertsen, 2018). Moreover, the method  
376 sensitivity was detrimentally affected owing to the occurrence of contaminating bacteria in  
377 extraction kits and laboratory reagents (Salter et al., 2014).

378 HTS-based technologies have been already tested under microgravity conditions and directly  
379 on the ISS (Carr et al., 2020; Castro-Wallace et al., 2017; McIntyre et al., 2016). The most  
380 suitable candidate technology to be transported and mounted on the ISS was the MinION  
381 pocket-size device (Figure 2), which can provide rapid identification up to 300 kb with single  
382 strand and 60 kb with double strands reads (Jain et al., 2016). The integrity and activity of  
383 nanopores, in which DNA/RNA molecules pass through during base reading, were not  
384 adversely affected by storage conditions, launch, cosmic radiations or handling in  
385 microgravity, and reusability of flow cells was warranted. Specific indications to avoid air  
386 bubbles interference at the nanopores were implemented (Castro-Wallace et al., 2017;  
387 Rizzardi et al., 2016). Moreover, MinION performances in sequencing a mixture of genomic  
388 DNA from a virus, a bacterium, and of mammal mtDNA, were comparable to those of  
389 MinION, Illumina MiSeq and PacBio RSII run on ground. Accuracy respect to MiSeq and  
390 RSII was slightly inferior (89% identity to the reference genome, respect to > 99%), but  
391 sufficient for sequence analysis. Sequencing reads were successfully assigned to the correct  
392 reference sequences in 90% of cases. Likely, with the novel improved versions of flow cells,

393 the molecular detection of aquatic microorganisms will improve notably (Kilianski et al.,  
394 2015). Tests also demonstrated that real-time metagenomics analysis was also possible by  
395 using a laptop device that can be integrated in the ISS hardware. Considering genomic data  
396 analysis, one of the least demanding platforms has been tested successfully simulating flight  
397 conditions and hardware availability onboard of the ISS, demonstrating its realistic  
398 applicability even on a laptop base (Castro-Wallace et al., 2017). Provided the adaptation of  
399 hardware and instruments to the spatial and logistic limitations onboard, both the shotgun and  
400 amplicon approaches, can be considered as suitable tools for microbial community  
401 characterization and pathogen detection on the ISS.

402 The amplicon approach requires straightforward PCR amplification protocols, downstream  
403 bioinformatics, and database searches. Nevertheless, this technique is prone to fail to detect  
404 unknown or highly divergent 16S rRNA gene sequences, which may be associated to novel  
405 or previously undetected pathogens. Moreover, taxonomic assignment is highly sensitive to  
406 the gene region selected as a marker and to the assignment method (Claesson et al., 2010; Liu  
407 et al., 2008; Tremblay et al., 2015), while accuracy of quantification can be affected by  
408 the variability in the number of gene copies shown by microbial taxon and even between  
409 individual cells of the same species (Větrovský and Baldrian, 2013). Finally, a common  
410 scientific view of suitable markers for viruses is still missing (Chakraborty et al., 2014).

411 In comparison to amplicon analysis, the shotgun sequencing offers a wide genome coverage  
412 of organisms, allowing the detection of unknown contaminants and the possibility to screen  
413 for both bacteria and viruses. In the field of water quality monitoring, the shotgun sequencing  
414 was used, for instance, as the benchmark method to assess the occurrence of potential  
415 multiple waterborne pathogens, novel indicators for human sewage contamination, and  
416 microbial safety of drinking water in relation to the efficiency of reclamation treatments  
417 (Chao et al., 2013; Lu et al., 2015; McLellan and Eren, 2014; Newton et al., 2015). Although

418 the possibility to obtain up to a few thousands of base pairs for each read improved the  
419 microbial identification specificity, non-trivial disadvantages include the initial skimming of  
420 the huge number of produced reads, their quality check and assemblage in contigs useful for  
421 classification, which require high computational effort and time (Tan et al., 2015).  
422



423  
424 **Figure 2.** The NASA astronaut Peggy Whitson performing the Genes in Space investigation  
425 on the ISS using the miniPCR and MinION. Credits: NASA  
426

#### 427 **4 Real-time technologies for early warning microbial monitoring**

428 The total contamination assessment (total microbial burden) through the accurate  
429 quantification of microbial cell abundance and viability in waters circulating is a recognized  
430 necessity on space crewed vehicles (Morris et al., 2012). Different early-warning real-time  
431 methods that target parameters at the single-cell level (e.g., cellular biomolecules, membrane  
432 integrity, enzyme activity, substrate uptake) have been developed for water monitoring, but  
433 only few can efficiently operate in flight-like and space settings.  
434

#### 435 **4.1 ATP-metry**

436 An option for real-time monitoring of biological contamination in water samples is the  
437 analysis of adenosine triphosphate (ATP) cell content. The analysis is carried out through the  
438 chemical and/or enzymatic extraction of this molecule from microbial cells, followed by the  
439 measurement of light emission derived when the dissolved ATP, in presence of magnesium,  
440 reacts with the luciferine (substrate) - luciferase (enzyme) complex. The emitted light  
441 intensity is linearly related to the ATP concentration, easily measurable using a luminometer,  
442 and gives virtually instant information (within minutes) of the metabolically active microbial  
443 population. This peculiarity makes it suitable as an early-warning approach for measuring  
444 bacterial contamination and monitoring water treatment efficacy in near real-time (Hammes  
445 et al., 2010). The method is robust, easy to perform, and suitable to detect both cultivable and  
446 uncultivable cells, with better estimates of total active microorganisms compared to  
447 heterotrophic plate counts. The concomitant use of traditional cultivation-based approaches  
448 showed levels of cultivable cells order of magnitude lower than those estimated by ATP  
449 (Siebel et al., 2008; Zhang et al., 2019).

450 The ATP analysis was performed on pre-flight and post-flight ISS water samples and  
451 revealed a biological contamination ranging between 0 (drinking water) and  $4.9 \times 10^4$  cells/ml  
452 (humidity condensate) (Bacci et al., 2019; La Duc et al., 2004). Recently, the viable microbial  
453 contamination on-board ISS was reliably monitored on surface samples by intracellular ATP  
454 measurement (Perrin et al., 2018; Venkateswaran et al., 2003). ATP assays were also proven  
455 effective in monitoring microbial contamination on surfaces from an inflated lunar/Mars  
456 analogous habitat during long-term human occupation (Mayer et al., 2016). The total (ATP  
457 content from both dead and live microbes) and viable microorganisms (intracellular ATP  
458 content) were in the range of  $10^5$ - $10^6$  relative luminescence unit (RLU)/m<sup>2</sup>. These results  
459 were in line with ATP data measured directly on-board within the experiment T2 carried out

460 during Euromir-95 mission (Guarnieri et al., 1997). Recently, the ATP-metry was selected for  
461 the real-time monitoring of the biological contamination on board ISS within the H2020  
462 European project BLOWYSE (Biocontamination integrated control of wet systems for space  
463 exploration, <http://biowyse.eu>), aimed at developing a compact, automatic, and microgravity-  
464 compatible on-board systems for the prevention, monitoring and control of microbial load in  
465 waters and on wet surfaces (Figure 3). A humid area sampler was recently developed and  
466 patented (ref. 102018000009137, dated 03/10/2018) (Detsis et al., 2018; Guarnieri et al.,  
467 2019).

468 Today, commercial kits for quantitative ATP-metry are available to monitor the microbial  
469 biomass level in water and to validate cleaning and disinfection procedures, with a wide  
470 number of bulk and intracellular ATP measurements on microbial communities reported from  
471 natural and engineered aquatic environments (Abushaban et al., 2019; Fillinger et al., 2019;  
472 van der Wielen and van der Kooij, 2010; Vang et al., 2014). Although the apparent  
473 advantages, major technical limitations are related to the low sensitivity and result  
474 reproducibility at low cell concentration and sample volume (< 100 µl), along with the  
475 susceptibility to environmental conditions (e.g., pH, temperature, occurrence of enzyme  
476 inhibitors). Some drawbacks of the method are partly circumvented by using external and  
477 internal standards and by operating under controlled reproducible settings. Therefore, the  
478 correlation between intracellular ATP content and microbial cell counts will rely on a robust  
479 cross-calibration with results from other reference methods (Amalfitano et al., 2018b;  
480 Hammes et al., 2010). It has been calculated that the average ATP-per-cell content is  
481 approximately  $1.75 \times 10^{-10}$  nmol/cell or  $6.87 \times 10^{-17}$  g ATP/cell (Zhang et al., 2019).  
482 However, the amount of per-cell ATP can be significantly influenced by the phylogenetic  
483 affiliation and the cellular physiological status, with diverse ATP-content reported between

484 either eukaryotic or prokaryotic cells from the same cultures (Bajerski et al., 2018; Yaginuma  
485 et al., 2015).

486

## 487 **4.2 Flow cytometry**

488 Ubiquitously applied from the bio-medical research to environmental sciences, flow  
489 cytometry (FCM) is considered an unparalleled high-throughput technology for single cell  
490 analysis (Robinson and Roederer, 2015). This generic technology allows the measure (-  
491 metry) of the optical properties of cells (cyto-) transported by a liquid sheath (flow).

492 Following sample intake, a pressurized laminar flow is generated and suspended particles are  
493 individually forced to cross a light source excitation point for scanning and evaluation. Light  
494 scatter and fluorescence signals (either with or without a secondary staining with fluorescent  
495 dyes) are detected at the single-cell level and instantly converted into digital information to  
496 be shown on multidimensional plots, along with real-time data analysis and statistics  
497 (Shapiro, 2005).

498 Flow cytometry is included in the roadmaps of national space agencies worldwide and  
499 deemed as a necessary technology for defining and monitoring spaceflight-associated  
500 requirements since early 80's. Owing to the high versatility for diagnostic medicine (e.g.,  
501 hematology, immunology, and physiology), this technology was so far retained as a prime  
502 asset for health monitoring and clinical laboratory diagnostics for astronauts, in view of the  
503 upcoming deep-space exploration missions (Crucian and Sams, 2012). A flow cytometry  
504 platform was already successfully tested on-board the ISS to monitor and understand the  
505 physiological adaptations of astronauts to microgravity (Dubeau-Laramée et al., 2014; Phipps  
506 et al., 2014) (Figure 3). Advanced developments of the prototype were based on  
507 commercialized flow cytometer with significant additional engineering modifications, mostly  
508 aimed to generate laminar particle flow (distinct from the standard sheath fluid based

509 method), and to reduce the significant amount of liquid biohazardous waste and energy  
510 operating requirements. The laminar flow within the flow chamber was found to be  
511 dramatically altered by microgravity. In turn, the unavoidable elimination of the fluid  
512 mechanical setting for particle hydrodynamic focusing was proven to significantly reduce  
513 liquid waste and the total system operational load, in terms either of instrument size and  
514 weight or energy consumption (Cohen et al., 2011; Crucian and Sams, 2005).

515 Notwithstanding the recent system design improvements, peripheral blood cells has been  
516 retained as the only target for first studies in space environments (McMonigal and Crucian,  
517 2015). Since a flow cytometer can provide support to a wide range of scientific applications  
518 (e.g., biology, microbiology, and environmental science), it is needless to point out that a  
519 spaceflight compatible machine could satisfy the unmet flight requirements for water  
520 monitoring and treatment to complete future long-duration missions in closed healthy  
521 environments.

522 The high sensitivity for scanning very small objects (i.e., from virus-like-particles to  
523 prokaryotes, to pico- and micro-eukaryotes) and the wide detectable cell concentration range  
524 (generally between  $10^2$  and  $10^7$  cells ml<sup>-1</sup> without concentrating or diluting the samples)  
525 represent unmatched features of this technology for water monitoring. A broad suite of assays  
526 is available for microbial quality assessments in natural and engineered aquatic ecosystems  
527 (Amalfitano et al., 2014; Boi et al., 2016; Gasol and Morán, 2015; Van Nevel et al., 2017).

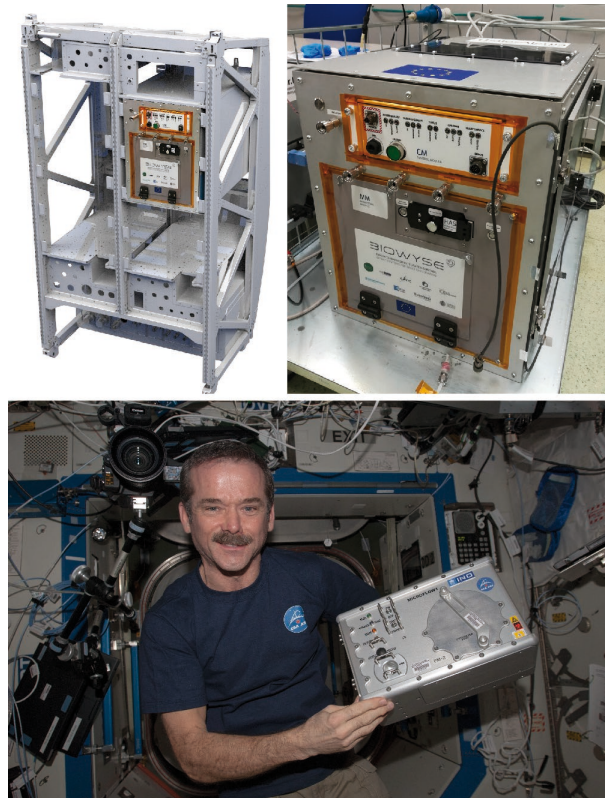
528 Successful applications are reported to provide early warning indications of unexpected water  
529 contamination events through the rapid detection of a variety of microbiological threats,  
530 including pathogenic and potentially toxic microorganisms (Vital et al., 2010; Weisse and  
531 Bergkemper, 2018; Yang et al., 2010). Moreover, flow cytometric measurements are suitable  
532 to evaluate the efficiency of various industrial microbial bioprocesses (e.g., food and  
533 pharmaceutical preparations) (Díaz et al., 2010), and the performances of engineered systems

534 for water treatment (Besmer and Hammes, 2016; Safford and Bischel, 2019). Remarkably,  
535 FCM was also useful to determine the presence of nonliving organic and inorganic  
536 substances, including nano- and micro-sized particles, suspended solids, flocs and aggregates  
537 of various origins (Aulenta et al., 2013; Casentini et al., 2016; Liu et al., 2016). On-going  
538 instrumental developments for on-site applications are directed to install flow cytometers on  
539 either mobile units (e.g., ships and vehicles) or fixed locations (e.g., treatment plants, marine  
540 buoys, off-shore platforms), with the possibility for automatic programmable staining of  
541 aquatic microorganisms and remote data transfer (Buyschaert et al., 2018; Pomati et al.,  
542 2011; Thyssen et al., 2007).

543 Leaving aside the high costs of all sophisticated systems, the need for specially trained staff is  
544 likely to represent a major drawback in the daily scheduled monitoring practices. Moreover,  
545 the reproducibility of cytometric data was reported to be adversely affected by changing cell  
546 staining protocols (e.g., fixatives, type of fluorescent dye), incubation conditions (e.g.,  
547 temperature, time to analysis), instrumental settings (e.g., fluidic and signal amplification  
548 systems), and the source water (e.g., from natural or engineered systems) (Nescerecka et al.,  
549 2016; Prest et al., 2013). Further work is also required to establish user-independent strategies  
550 for gating and data handling (Amalfitano et al., 2018a; Koch et al., 2014). One more specific  
551 challenge is the lack of phylogenetic resolution. Despite the enumeration of targeted taxa may  
552 rely on specific fluorescence staining procedures (Couradeau et al., 2019; Manti et al., 2011;  
553 Neuenschwander et al., 2015), the cytometric information characterizing different  
554 subpopulations of microbial cells is generally ataxonomic. A direct link between cytometric  
555 fingerprinting and microbial diversity, assessed by 16S rRNA gene amplicon sequencing,  
556 was demonstrated in recent studies (Props et al., 2017, 2016). However, the computational  
557 workflows are convoluted and still under development (Rubbens et al., 2019).

558





559

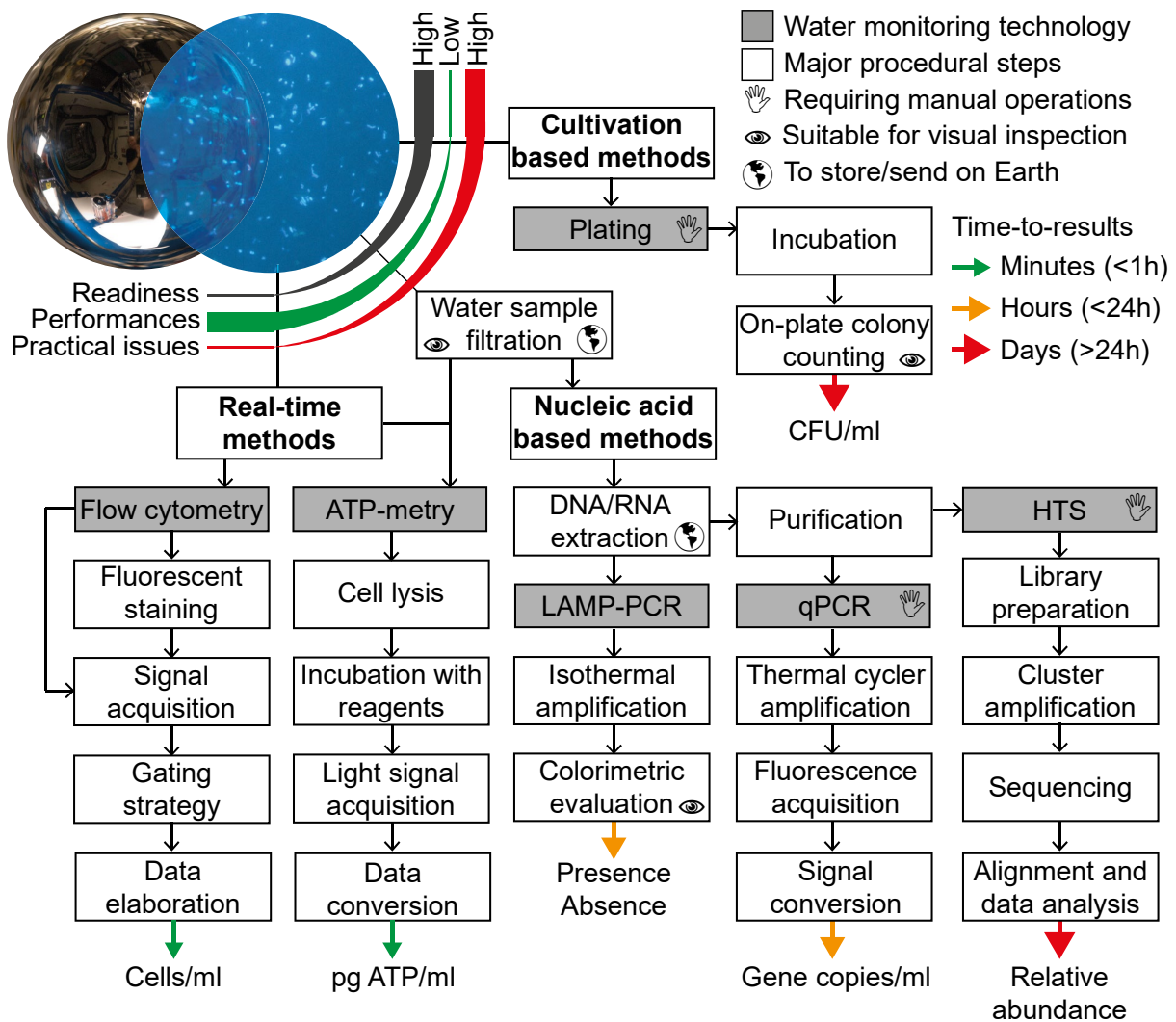
560 **Figure 3.** Integrated breadboard of the ATP-metry system, developed within the European  
561 H2020 project BIOWYSE. The hardware was designed (upper left photo) and built (upper  
562 right photo) to fly within the European Drawer Rack Mark 2. The photo below shows the  
563 Canadian astronaut Chris Hadfield holding the flow cytometry platform Microflow1,  
564 successfully deployed and tested on the ISS (Credits: BIOWYSE project consortium and  
565 NASA).

566

## 567 **5 Comparative analysis of space-relevant technological features**

568 The procedural workflows of each of the mature technologies, herein entitled for water  
569 microbial monitoring in space, comprise major steps and provide different types of results.

570 The overall time-to-results can vary from minutes to days, largely relying on user-dependent  
571 manual operations and technological solutions for automation (figure 4).



572

573 **Figure 4.** Workflows of mature technologies entitled for microbial water monitoring during  
 574 space missions. Cultivation-, nucleic acid-based, and real-time methods are suitable to  
 575 identify and quantify the waterborne microorganisms, ideally present in a drop of ISS water  
 576 and visualized by epifluorescence microscopy on a filtration membrane (upper left pictures).  
 577 Comparative levels of readiness, analytical performances, and practical issues were reported  
 578 along with major procedural steps, timing, and types of achievable results.

579

580 Both current and future applications in space will be necessarily bounded by the definition of  
 581 novel standards of microbial quality (i.e., other than those applied on Earth by cultivation-

582 dependent approaches) (Amalfitano et al., 2018b), but also by a compromise between the best  
583 analytical performances and detrimental practical issues to cope with during spaceflights.  
584 The implementation of successful on-board workflows will critically rely on the overall water  
585 cycle management (Pickett et al., 2020), while fundamental analytical aspects can be  
586 constrained by the minimal amount of water available for routine monitoring activities. For  
587 example, technological accuracy and result reproducibility are directly linked to workable  
588 water volumes and the constitutive occurrence of microbial targets in the sample (e.g.,  
589 microbial cell abundance, per-cell gene copies, cell viability). Volumes required for accurate  
590 and reproducible analyses will reasonably range between few tens of microliters (e.g., for  
591 flow cytometric assessments) and hundreds of milliliters (e.g., for cultivation- and nucleic  
592 acid-based methods), as also reported in terrestrial studies (Safford and Bischel, 2019).  
593 Notably, sample filtration, a major pre-treatment step for ATP-metry and nucleic acid-based  
594 methods, can selectively concentrate the microbial biomass and target microorganisms, while  
595 minimizing the water loss for analytical needs. The filtering surfaces are also suitable for  
596 visual inspection (e.g., by microscopy), storage, and comparative analysis (e.g., on Earth).  
597 However, filtration can influence the composition of the dissolved organic matter of the  
598 permeate water, possibly triggering microbial regrowth in the downstream distribution  
599 system (Park et al., 2018), and it will also necessitate additional crew time for manual  
600 operations (e.g., filter substitution, regeneration, disposal).  
601 Sufficient data are not yet available from real space conditions to implement protocol details  
602 within the procedural workflows. Although the selected technologies were demonstratively  
603 applied under either simulated or real microgravity conditions, their own critical advantages  
604 and limitations will require full reconsideration for flight-like and space applications.  
605 From the one hand, analytical benefits will necessarily include the time needed to achieve  
606 results (i.e., speed to results), the accuracy and flexibility in detecting specifically-selected

607 microbial targets (i.e., identification depth) with reproducible consistent results (i.e.,  
608 reproducibility), and the number and multiple type of achievable results (i.e., herein named  
609 multiparametricity). On the other hand, the operating conditions can be particularly stringent  
610 and limiting the direct applicability in space, unless addressing critical requirements such as  
611 reagent usage, waste production, operator skills, and crew time (table 1).

612 Using a simplistic pairwise comparison, we sought to emphasize that few selected  
613 technological features have to be consciously retained from the methodological proof-of-  
614 concept level up to the device deployment, instrumental demonstration, and routine use in the  
615 on-board housekeeping program. Although the comparative scores were assigned  
616 subjectively (table S1), it is likely evident that the stringent requirements of microbial water  
617 monitoring in space cannot be met by a single technological solution.

618

619 **Table 1.** Advantages and limitations of the most promising approaches for microbial  
620 monitoring in space settings. The selected technologies are flexible (i.e., applicable to  
621 different microbial targets in samples of various origin), suitable for miniaturization and  
622 automation with limited maintenance, and already tested in flight-like conditions. A  
623 comparative score was arbitrarily assigned through a pairwise comparison matrix for each of  
624 selected space-relevant technological features, including major analytical performances (i.e.,  
625 speed to results, identification depth, reproducibility, multiparametricity – green marks) and  
626 practical issues to cope with in space (i.e., reagent usage, waste production, operator skills,  
627 crew time – red marks).

628

Technology	Advantages	Limitations	Analytical performances/ Practical issues	Comparative Scores	Applications in space
<b>Plate Cultivation</b>	<ul style="list-style-type: none"> <li>– No pretreatment</li> <li>– Detection limit 1 cell/100 ml</li> <li>– Low equipment requirements</li> </ul>	<ul style="list-style-type: none"> <li>– No direct quantification</li> <li>– Risks of contamination</li> <li>– Reliant on cultivation conditions</li> </ul>	Speed to results	■ ■	La Duc et al., 2004 Morrison et al., 2017 Pierson et al., 2013
			Identification depth	■ ■ ■ ■	
			Reproducibility	■	
			Multiparametricity	■ ■	
			Reagent usage	■ ■ ■	
			Waste production	■ ■ ■ ■ ■ ■	
			Operator skills	■ ■ ■	
			Crew time	■ ■ ■ ■ ■	
<b>LAMP</b>	<ul style="list-style-type: none"> <li>– Detection limit <math>\sim 10^2</math> gene copies/ml</li> <li>– Unaffected by template conc.</li> <li>– No effects of inhibitory compounds</li> </ul>	<ul style="list-style-type: none"> <li>– No direct quantification</li> <li>– Risks of contamination</li> </ul>	Speed to results	■ ■ ■ ■	Ott et al., 2014
			Identification depth	■ ■ ■ ■ ■ ■	
			Reproducibility	■ ■ ■ ■	
			Multiparametricity	■ ■ ■	
			Reagent usage	■ ■ ■ ■	
			Waste production	■ ■ ■	
			Operator skills	■ ■	
			Crew time	■ ■	
<b>qPCR</b>	<ul style="list-style-type: none"> <li>– Target-specific and quantitative</li> <li>– Detection limit <math>\sim 10^3</math> gene copies/ml</li> </ul>	<ul style="list-style-type: none"> <li>– Pretreatment processing</li> <li>– Affected by template and inhibitory compounds</li> <li>– Reliant on PCR related issues</li> </ul>	Speed to results	■ ■ ■	Boguraev et al., 2017 Parra et al., 2017
			Identification depth	■ ■ ■ ■ ■	
			Reproducibility	■ ■ ■ ■	
			Multiparametricity	■ ■ ■ ■ ■	
			Reagent usage	■ ■ ■ ■ ■	
			Waste production	■ ■ ■ ■ ■	
			Operator skills	■ ■ ■ ■ ■	
			Crew time	■ ■ ■ ■ ■	
<b>High Throughput Sequencing</b>	<ul style="list-style-type: none"> <li>– In-depth phylogenetic resolution</li> <li>– Specific for unknown non targeted microorganisms</li> </ul>	<ul style="list-style-type: none"> <li>– Pretreatment processing</li> <li>– Reliant on PCR related issues</li> <li>– Complex data interpretation</li> </ul>	Speed to results	■ ■	Castro-Wallace et al., 2017 McIntyre et al., 2016
			Identification depth	■ ■ ■	
			Reproducibility	■ ■	
			Multiparametricity	■ ■ ■ ■ ■ ■ ■	
			Reagent usage	■ ■ ■ ■ ■ ■ ■	
			Waste production	■ ■ ■ ■ ■ ■ ■	
			Operator skills	■ ■ ■ ■ ■ ■ ■	
			Crew time	■ ■ ■ ■ ■ ■ ■	
<b>ATP-metry</b>	<ul style="list-style-type: none"> <li>– Real-time data (&lt; 5 min)</li> <li>– Detection limit <math>\sim 0.1</math> pg/ml</li> </ul>	<ul style="list-style-type: none"> <li>– Unspecific detection</li> <li>– Destructive analysis</li> <li>– Risks of contamination</li> </ul>	Speed to results	■ ■ ■ ■ ■ ■ ■	Guarnieri et al., 1997 La Duc et al., 2004
			Identification depth	■	
			Reproducibility	■ ■ ■ ■ ■	
			Multiparametricity	■	
			Reagent usage	■ ■	
			Waste production	■ ■	
			Operator skills	■	
			Crew time	■	
<b>Flow cytometry</b>	<ul style="list-style-type: none"> <li>– No pretreatment</li> <li>– Quantitative and near-real time data (&lt; 20 min)</li> <li>– Detection limit <math>\sim 10^2</math> cells/ml</li> </ul>	<ul style="list-style-type: none"> <li>– Complex data interpretation</li> <li>– Clogging issues</li> </ul>	Speed to results	■ ■ ■ ■ ■ ■	Dubeau-Laramée et al., 2014 Phipps et al., 2014
			Identification depth	■ ■	
			Reproducibility	■ ■ ■ ■ ■ ■ ■	
			Multiparametricity	■ ■ ■ ■ ■	
			Reagent usage	■	
			Waste production	■	
			Operator skills	■ ■ ■ ■ ■ ■ ■	
			Crew time	■ ■ ■ ■ ■	

## 631 **6 System miniaturization and future challenges**

632 An important point is that the monitoring technologies can be mission-dependent, but only  
633 those instruments that minimize crew involvement in their end-to-end operation are likely to  
634 be applicable on the long-term (Karouia et al., 2017). Overall, the selected devices have to be  
635 compact, suitable for automation, low power-consuming, and virtually invisible except when  
636 needed (Limero and Wallace, 2017). The ground-based counterparts have already been  
637 miniaturized using microfluidics, but the deployment of monitoring-effective tools onboard  
638 spacecrafts will also require substantial reengineering and instrumental customization. The  
639 instruments deployed so far have not been yet permanently used in spaceflight water  
640 monitoring, yet numerous examples of successful international projects and commercial  
641 partnerships dedicated to the human space exploration let argue that critical space water-  
642 related tasks can be operatively accomplished at reasonable costs and times (< 5 years)  
643 (Karouia et al., 2017).

644 Considerable progress has been made in miniature the onboard instrumentation to assess the  
645 water microbiological contamination. This also includes microbial cultivation systems, such  
646 as the AquaPad developed by the French CNES space agency (Augelli, 2018). The  
647 microfluidic chips that allow cell isolation and incubation have been developed for ground-  
648 based applications, and appear suitable for space uses. For example, the encapsulation of  
649 single cells from a mixed microbial community into small droplet compartments of a water-  
650 in-oil emulsion can offer further opportunities for physiological studies and viability assays  
651 (e.g., metabolic by-products diffusion, resistance to toxicants, enzymatic activities) on clonal  
652 populations isolated into plugs or micro-Petri dishes (Boedicker et al., 2008; Boitard et al.,  
653 2015).

654 Considering the ability of microfluidic systems to efficiently conduct measurements on small  
655 volumes of complex fluids without the need for a skilled operator, lab-on-a-chip technologies

656 and portable diagnostic devices have gained increased popularity for sensing a wide range of  
657 water parameters and microbial pathogens even in the most remote settings (Mairhofer et al.,  
658 2009). More recently, new qPCR platforms based on microfluidic technologies have been  
659 developed allowing the simultaneous analysis of numerous genes and samples in volume  
660 chambers of few nanoliters, placed at high density on a chip (Ricchi et al., 2017). Being less  
661 sensitive to inhibitors than qPCR, the digital PCR (dPCR) is mainly applied to monitor gene  
662 transcriptions in microbial cells without the need of a standard curve for gene copy  
663 quantification (Devonshire et al., 2016).

664 The system miniaturization was also considered the most appealing trait for space  
665 applications of the nanopore DNA sequencer MinION (Castro-Wallace et al., 2017). By  
666 assembling miniaturized and lab-on-a-chip solutions, the Water Monitoring Suite developed  
667 by NASA represents so far the best performing custom-built device applied successfully on  
668 the ISS to monitor different water quality properties. Along with the HACH colorimeter and  
669 the Organic Water Module, respectively used for inorganic and organic chemical  
670 assessments, the hardware suite also includes the PCR-based instrument RAZOR EX, with  
671 customized sample pouch kits containing all pre-loaded reagents necessary for sampling,  
672 sample preparation, and real-time PCR

673 ([https://www.nasa.gov/mission\\_pages/station/research/experiments/explorer/Investigation.ht](https://www.nasa.gov/mission_pages/station/research/experiments/explorer/Investigation.html?id=1847)  
674 [ml?id=1847](https://www.nasa.gov/mission_pages/station/research/experiments/explorer/Investigation.html?id=1847)). However, as space exploration progresses toward extended missions to cis-  
675 lunar space and Mars, PCR-based and multi-omics instruments onboard spacecraft should not  
676 be considered in separation from other technologies needed for the in-flight microbiological  
677 research (Karouia et al., 2017).

678 In particular, microfluidic platforms developed for the direct cell detection showed promising  
679 perspectives because of the potential for precise and easy-to-use analytical procedures. While  
680 enhancing analytical performances, the system miniaturization presents also the advantages

681 of reduced consumption of reagents and the ability to integrate monitoring and isolation  
682 procedures within a single device (Auroux et al., 2002). A simple microfluidic system was  
683 successfully tested for rapid and semi-automated bacterial enumeration in freshwaters and  
684 promising outcomes suggested its applicability to drinking waters under both ground and  
685 space conditions (Yamaguchi et al., 2014). Moreover, both sensitivity and specificity of ATP-  
686 metry and FCM can be also improved by miniaturizing the core systems down to scales  
687 closer to the ones of microorganisms. A micro-fluorescence-activated cell sorting ( $\mu$ FACS)  
688 was used to sort out cells of interest by changing the flow direction after cell detection (Fu et  
689 al., 1999). An integrated platform that combines two different force fields in a single  
690 microfluidic device (Dielectrophoretic–Magnetic Activated Cell Sorter - iDMACS) was  
691 applied for simultaneous sorting of multiple bacterial targets (Kim and Soh, 2009). More  
692 recently, a high-throughput Raman flow cytometer was developed on a microfluidic chip for  
693 the label-free molecular fingerprinting at the single-cell level (Hiramatsu et al., 2019).  
694 The onboard laboratory miniaturization included also the fluidic components (e.g., pumps,  
695 valves, electronics), thus paving the way to the use of advanced biosensors for screening food  
696 safety and water quality in space (Roda et al., 2018). Following the proofs of concept and  
697 wearable technologies suited to monitor astronauts' health, the biosensing diagnostic  
698 instrumentation, most reasonably based on electrochemical and optical detectors, was argued  
699 as a secondary future option for in-flight water biochemical analyses (Choi et al., 2018;  
700 Limero and Wallace, 2017; Nelson, 2011). In particular, the amperometric biosensors were  
701 proven sensitive to monitor different water analytes, chemical contaminants (e.g., pesticides,  
702 organophosphates, carbamates), and numerous microbial biomarkers successfully targeted to  
703 detect the major microbiological agents, food- and water-borne pathogens (e.g., *E. coli*,  
704 *Salmonella*, *L. monocytogenes*, *C. jejuni*, *B. cereus*, *M. smegmatis*) (Grieshaber et al., 2008;  
705 Velusamy et al., 2010). However, despite providing concrete benefits for health services and



706 our life on Earth and beyond, the spectroscopic and biosensing devices are still at a low level  
707 of technological readiness for in-flight applications (García-Descalzo et al., 2019; Own et al.,  
708 2019; Roda et al., 2018), thus falling out of the scopes of this review.

709 In general, a critical aspect of all microfluidic and lab-on-a-chip solutions is bound to the  
710 very limited system reusability. Most miniaturized devices for terrestrial applications are of  
711 single-use and, in many cases, based on disposable cartridges that cannot be safely stowed  
712 onboard during long-term space missions. When cells are delivered to different system  
713 compartments for collection, there is no clear approach for removal the analyzed samples  
714 without compromising the system functioning and risking contamination. Clogging issues  
715 may also interfere with the analyses due to the processing of large sample volumes. Reusable  
716 systems have been tested, but their applications in space are likely constrained by the risk of  
717 sample carryover, reduced analytical performance upon extended reuse, and the resources  
718 required for cleaning and reactivation procedures. In this regard, the technological  
719 development is highly demanding and still far from being accomplished in a near future.

720

## 721 **7 Conclusions**

- 722 • Current technologies for water microbial monitoring can satisfy the needs of long-term  
723 space exploration missions at reasonable costs and times, although substantial  
724 instrumental reengineering has to be considered.
- 725 • The suitable methodological applications at a high technology readiness level (i.e., at  
726 least validated in a flight-like environment) will require reduced space to be allocated and  
727 can potentially provide rapid and specific responses regarding the in-flight occurrence of  
728 the microbiological contamination.
- 729 • The advanced biomolecular characterization of water samples from the ISS is promoting  
730 a better understanding of the onboard levels and patterns of microbial contamination, thus

731 contributing to the development of space bound technologies for the rapid and specific  
732 identification of microorganisms of health concerns.

- 733 • As space exploration progresses toward longer missions, PCR-based and multi-omics  
734 approaches can be complemented by real-time technologies needed for the in-flight  
735 microbiological research and suitable for the early-warning microbial monitoring of space  
736 waters.

737

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742

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