Water and microbial monitoring technologies towards the near future space exploration

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Abstract

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

Keywords:

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

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1 Introduction

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

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

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and possibly counteract contingent events of microbial contamination (Van Houdt et al., 2012; Yamaguchi et al., 2014).

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

In early 2020s, the Chinese modular space station, built on the experience gained from its precursors Tiangong-1 and Tiangong-2, will be placed and operating in the Low Earth orbit (Gibney, 2019). The sustainable human exploration of the Moon is programmed in the meanwhile (El-Jaby et al., 2019; Pittman et al., 2016), whereas the human missions to the surface of Mars are envisioned before 2040 (ISECG, 2018). Therefore, it is hypothesized that only prospective methodological applications at a high technology readiness level (i.e., at least validated in a flight-like environment; Straub, 2015) will be selected by following their basic principles and current uses in Earth and space-analogue settings.

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

In this article, we narrow the focus on promising bio-analytic technologies for quality assessments of waters in space, with the aim to explore advantages and limitations beyond

 doi:10.20944/preprints201910.0005.v1

their current applications on Earth. Starting from the rigorous housekeeping program and the consolidated results of water monitoring activity on the ISS (Duncan et al., 2008; Limero and Wallace, 2017), we provide a critical overview of the microbial monitoring approaches, based on flexible technologies for the identification of microbial components and a total contamination assessment (total microbial burden) in waters circulating on crewed space vehicles.

2 Water recycle and microbial monitoring aboard the International Space Station

2.1 The ISS water cycle

The water recycling system innovations required to support ISS activities have been listed among the major benefits for humanity (Detsis and Detsis, 2013; NASA et al., 2019). The ISS is provided with potable water from different suppliers, coordinated by the space agencies of United States (National Aeronautics and Space Administration - NASA), Russia (Russian Federal Space Agency - Roscosmos), Europe (European Space Agency - ESA), and Japan (Japanese Aerospace Exploration Agency - JAXA) (Bruce et al., 2005; Van Houdt et al., 2012). All the possible necessary precautions to prevent external contamination are applied throughout water transferring and loading steps over the entire treatment period before the liftoff of supply modules. For instance, the American and Russian waters are produced in conditioned and limited-access areas and preparation facilities, with no risk of accidental water quality modifications during the production process. At the research center of the Italian Società Metropolitana Acque Torino (SMAT), purified waters for space travels are also processed upon selecting well and spring waters that most closely meet the physical, chemical, and bacteriological quality standards for astronauts (Lobascio et al., 2004). Currently on ISS, waters for direct human consumption are regularly delivered and recovered in order to guarantee approximately 4 L per person per day (Figure 1). A reserve of potable

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water (up to approx. 2000 L) is stored in contingency containers to maintain ISS operations in response to emergency scenarios (Carter et al., 2018). Although routinely monitored and kept constant, the overall water mass balance represents a recurrent major challenge owing to the various ISS water needs.

Beside the on-demand crew consumption, on-board waters are distributed for different purposes, comprising hygiene and cleaning practices, urinal flushing, oxygen generation via electrolysis, life-support systems, and flexible water-based experimental activities (e.g., vegetable and food production systems, animal physiology and behavioral adaptation tests) (Baiocco et al., 2018; Chatani et al., 2015; Massa et al., 2016; Niederwieser et al., 2018; Ronca et al., 2019; Wolff et al., 2018). Wastewaters are continuously collected and recycled at high efficiency level. In the US segment, the Water Recovery and Management System was reported to recuperate up to 85% from crew urine and flush water, along with the water content from liquid wastes and humidity condensate from the cabin. Various containers, reservoirs, tanks and bellows are also necessary to maintain water pressure and circulation through the distribution network (Carter et al., 2018).

Since microbial growth is unavoidable in persistent stagnation zones and at varying residence times along the water distribution network (Lautenschlager et al., 2010; Ling et al., 2018), the pre- and in-flight addition of biocides is used for residual microbial control. Molecular iodine is applied in the U.S. segment, while the ionic silver level is amended in Russian waters, both at low concentrations (i.e., not detrimental for human health) (Artemyeva, 2016; Lobascio et al., 2004). Moreover, high temperature in the catalytic reactor, multifiltration beds within the Water Processor Assembly, UV-C LEDs within the CO_2 Concentration Assembly of the Advanced Closed Loop System, and novel antimicrobial coatings on various ISS surfaces were proved effective against potential microbial biomass growth (Bockstahler et al., 2017; Carter et al., 2018; Perrin et al., 2018; Roman et al., 2006; Sobisch et al., 2019). Finally, the

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ISS is maintained at pressure and oxygen levels very close to those at sea level on Earth, with a cabin temperature of about 22°C and a relative humidity of about 60%, in order to minimize detrimental growth of microbial biofilms on cabin surfaces (Pierson et al., 2013).

2.2 On-board water monitoring and microbial contamination

The achievement and maintenance of water quality standards are evaluated by systematic monitoring procedures (Limero and Wallace, 2017). Major water physical-chemical parameters including conductivity, pH, total organic, total inorganic and total carbon, nitrate, potassium, chloride and ammonium are monitored in-flight, together with iodine and silver levels (Artemyeva, 2016). Moreover, a robust monitoring program was implemented to verify that risks of microbial contamination were within acceptable limits in samples collected from different sites of the Russian and US segments, respectively once every three months and each month (Pierson et al., 2013; Van Houdt and Leys, 2012). Crewmembers use handheld equipment to monitor ISS waters and humidity condensate from surfaces. Chemical and biological samples are taken concurrently and at a frequency that may change due to real-time flight necessities (Pierson et al., 2013).

Water samples can be processed on-board by cultivation-based methods using the US-supplied Water Microbiology Kit for the quantification of total heterotrophic bacteria and coliforms (Bruce et al., 2005). The maximum total number of aerobic heterotrophic viable bacterial cells, counted as colony forming units on a rich agar medium, was internationally defined according to the concentration levels that are achievable with the current prevention and monitoring technologies available and applicable for space (i.e., HPC $\leq$ 50 CFU/ml). Microbial quality standards are also set for ISS internal surfaces, from which humidity condensate is collected (maximum bacterial load = 10000 CFU/100 cm^2; maximum fungal load = 100 CFU/100 cm^2) (Van Houdt and Leys, 2012). Along with the on-board monitoring

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activities, archival water samples are regularly collected in teflon bags (Figure 1), preserved, and returned to Earth approximately every three months for post-flight analyses (Limero and Wallace, 2017).

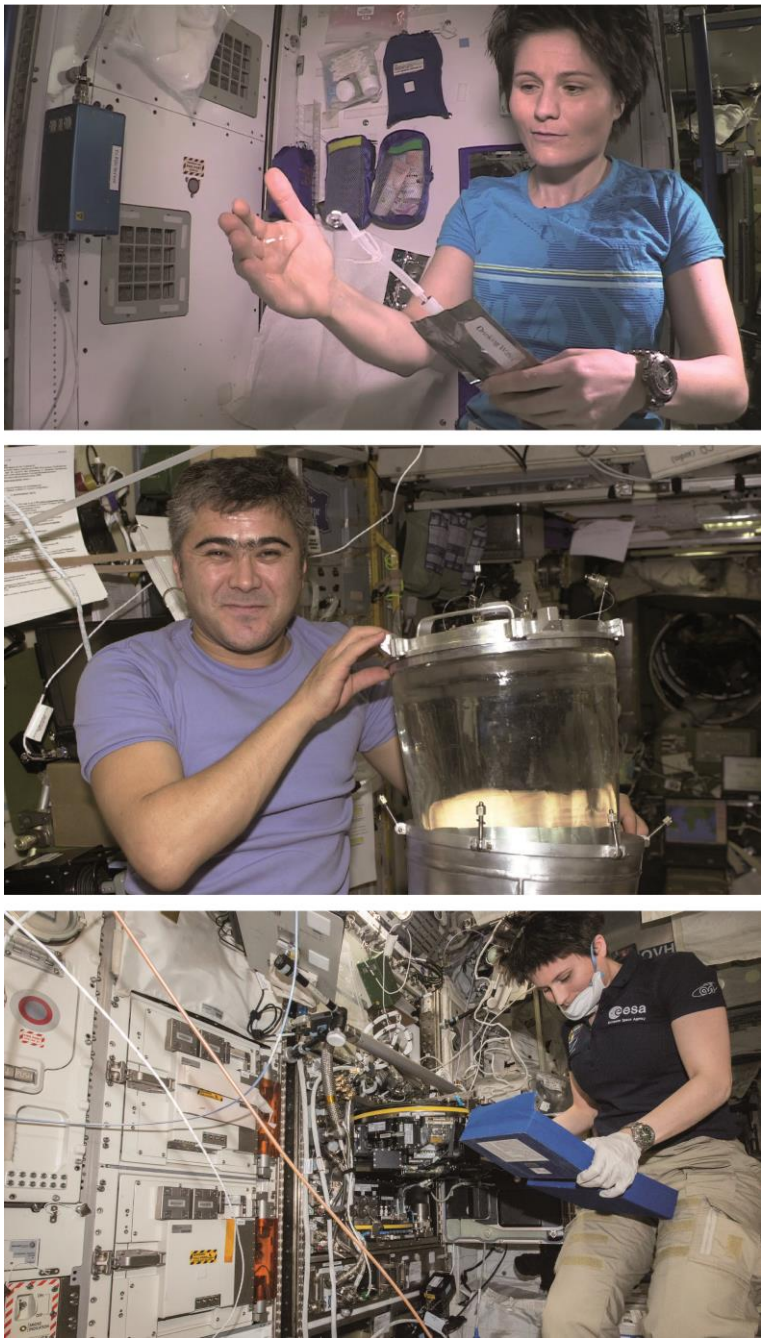

Figure 1. The Italian astronaut Samantha Cristoforetti and the Russian cosmonaut Salizhan Sharipov showing the Teflon bag, the water tank and removal system, used for drinking purposes and water storage on the ISS (Credits: ESA and NASA).

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The microbial contamination level was above the former acceptability limits several times during previous in-flight monitoring surveys. Events of microbial biofilm growth within space vehicles were mainly associated with the water layer covering internal surfaces and life support systems (La Duc et al., 2004; Novikova et al., 2006; Roman et al., 2006). Thus, waters collected from humidity condensate were retained among the major sources of microbiological hazard for potable water quality deterioration (Horneck et al., 2010). 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 Middeleer 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 water-borne 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 virulence of opportunistic human pathogens (Morrison et al., 2017; B. Zhang et al., 2019), while the analysis of archival samples generates a detrimental time gap for a flight-supportive result interpretation (Huang et al., 2018; Novikova et al., 2006). HPC was proved to be also affected by carbon sources in cultivation media, the incubation time, and the initial microbial load level (Amalfitano et al., 2018b). Finally, the prompt development and requirement update of alternate bio-analytical technologies for microbial monitoring during space

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exploration is pushed fundamentally by the need to avoid microbial regrowth from analytical wastes (Wong et al., 2017).

3 Nucleic acid amplification-based methods for microbial identification in space waters

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

3.1 Sample pre-treatments and nucleic acid extraction in space

Sample pre-treatment and nucleic acid extraction are systematic procedural steps and key starting points for most of the downstream molecular approaches and product developments for monitoring the water microbial quality. Nucleic acids extracted from unknown water samples are amplified, either entirely or partly to specific portions conventionally chosen as markers. The extraction and purification of nucleic acids from aquatic microorganisms rely on cell lysis and the selective binding of cellular DNA and RNA to solid surfaces through column-based protocols.

A fast independent biomolecular analysis can be entirely conducted in space settings with the in-line implementation of optimized protocols for fast DNA and RNA extraction in microgravity and sample preparation for sequencing. Published studies reported new promising field-deployable amplification devices and approaches for on board applications (Boguraev et al., 2017; Montague et al., 2018) (Figure 2). Simplified, sample processing and DNA purification, strategies have currently been tested aboard the International Space Station

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(ISS). The Wetlab-2 project has already developed and tested on the ISS a Sample Preparation Module (SPM) to lyse cells, and to extract and purify RNA, by circumventing all the issues related to microgravity, surface tension alteration, reduced operational space and handling expertise, providing high quality extraction of nucleic acids (Parra et al., 2017).

A large research effort on Earth is devoted to address the demand for simpler and more rapid nucleic acid-based detection methods to be applied outside the modern laboratory environment. Numerous commercialized kits for solid-phase extraction offer the possibility for handy and rapid nucleic acid purification procedures, in which potential contaminants are removed through sequential washing steps based on centrifugation or DNA separation by paramagnetic beads (Tan and Yiap, 2009). Methods for the direct PCR amplification without DNA extraction were also developed, although mainly applied to pure cultures or matrices without significant amount of inhibitors (Williams et al., 2017). Recent papers reported simplified methods for nucleic acid purification using different types of membranes, followed by the direct DNA amplification form the nucleic acid on the membranes (Kaliyappan et al., 2012; Rodriguez et al., 2016). Notably, a cellulose-paper-based dipstick was used to efficiently bind, wash, and elute purified nucleic acids from different matrices (e.g., plant, animal, and microbial biomass) without requiring any pipetting or electrical equipment (Zou et al., 2017).

Nevertheless, a major bottleneck for the in-flight application of amplification based approaches still lies on the need for sample concentration and DNA extraction, which are relatively time consuming procedures and increase the level of produced solid and liquid waste (Girones et al., 2010). Suboptimal DNA extraction and purification are known to largely affect results of biomolecular analysis, mainly owing to uncomplete lysis of more resistant bacterial populations and poor removal of DNA polymerase inhibitors (Albertsen et al., 2015; Girones et al., 2010). DNA purification requires effective cell disruption,

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inactivation of nucleases, and purification of DNA from contaminants (e.g., protein, carbohydrate, lipids, or other nucleic acid) that might interfere with the amplification efficiency. Residual extracellular DNA could not be easily discriminated from that of viable dangerous microorganisms, thus leading to an overestimation of risk for human health when applied to pathogen detection (Girones et al., 2010). Moreover, waterborne substances can concentrate together with DNA during sample processing and inhibit polymerase enzymes. Therefore, the source and quality of the water sample can directly influence the sensitivity and reliability of the PCR-based microbial detection.

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

The polymerase chain reaction (PCR) is widely used for detecting genes and microorganisms of health concern in water environmental hygiene studies (Ramírez-Castillo et al., 2015). In PCR-based approaches, specific nucleic acids sequences, recognized by the use of primers complementary to the targeted base pair sequence, are exponentially amplified by sequential thermal cycles that enable DNA denaturation and polymerase enzyme activity. Positive PCR amplification of a DNA fragment at the expected size is successively visualized by gel electrophoresis. Isothermal amplification methods for nucleic acid detection and quantification were also successfully tested in flight-like settings.

Among these target-based detection approaches, the loop-mediated isothermal amplification (LAMP) of nucleic acids was retained as a rapid and sensible option (Zhao et al., 2015), with minimal requirements for the in-flight water quality monitoring and pathogen detection (Ott et al., 2014). The amplification takes place at isothermal temperature (60-65°C) through the use of DNA-polymerase enzymes (*Bst* polymerase from *Bacillus staerothermophilus* and *Bsm* polymerase from *Bacillus smithii*), with high strand displacement activity in order to avoid the denaturation step. LAMP reaction involves the use of 4 to 6 primers, which provide

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high specificity and ultimately accelerate the reaction. Positive reactions can be visualised by naked eye (i.e., without post-amplification steps) following the increase of sample turbidity or colour owing to the addition of fluorescent dyes (Notomi et al., 2015). Being less sensible than PCR to inhibition and not significantly influenced by non-target DNA, direct LAMP assays are currently used for point of use diagnostic in clinical and environmental settings, with good analytical performances on moderately pre-treated samples and in a range of temperature, pH and elongation times (Etchebarne et al., 2017; Samhan et al., 2017). Low amount of DNA can be amplified up to generating 10^9 copies within 1 h and producing as final amplification product a complex stem-loop DNA, with several inverted repeats of the target and cauliflower-like structures. Commercial kit for the rapid on-site detection of water pathogens are already available for terrestrial applications, also comprising quantitative real-time LAMP, reverse transcription RT-LAMP, *in situ* LAMP, and viable LAMP (Notomi et al., 2015). For the scopes of this review, it is worth noting that a rapid method for detecting approximately 1 CFU/100 ml of *Legionella pneumophila* in tap waters was developed and efficiently applied on field in less than 2 working hours through a direct on-filter LAMP amplification with live/dead propidium monoazide (PMA) differentiation (Samhan et al., 2017).

Among the target-based quantification approaches, the quantitative PCR (qPCR) represents a popular technique for the in-flight water quality monitoring (Oubre et al., 2013). The quantification of target sequences is based on the development of a fluorescent signal proportional to the amount of amplified produced during the PCR thermal cycles. Using either SYBR-based or probe-based chemistry (e.g. TaqMan probe), DNA amplification can be followed as an exponential increase of fluorescent intensity during the reaction. The quantification is based on the number of amplification cycles (Cq quantification cycle or Ct threshold cycle) that produce a fluorescence signal above the reaction background

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fluorescence (threshold line). By the use of TaqMan probes labelled with different fluorophores in a single reaction, the Multiplex qPCR is also suitable for the simultaneous detection and quantification of multiple pathogens, consistently reducing analytical time and costs (Ibekwe et al., 2002; LaGier et al., 2004). With low detection limits (<400 cells per sample) and volume requirements (<100 µl of sample), qPCR assays are routinely applied and approved by national and international agencies in monitoring plans for the detection and quantification of waterborne pathogens (Girones et al., 2010; Ramírez-Castillo et al., 2015). A large body of the scientific literature has documented the development and use of qPCR for pathogenic viruses, bacteria, protozoa, and fungi (Kralik and Ricchi, 2017; Ramírez-Castillo et al., 2015). Through a pre-treatment with cell membrane impermeant DNA intercalating dyes, the so-called Viable qPCR was applied for discriminating between viable (with intact membrane) and dead (with damaged membrane) bacteria, allowing the quantification of water- and food-borne pathogens such as *Campylobacter*, *E. coli* O157:H7, *Legionella pneumophila*, *Salmonella*, *Cryptosporidium* (Banihashemi et al., 2012; Brescia et al., 2009; Delgado-Viscogliosi et al., 2009).

Given the versatile applications of the numerous available assays, both LAMP and qPCR have been retained as suitable water monitoring methods for long-term exploration missions. Owing to the high sensitivity, specificity, and simple post-amplification steps to detect the amplified targets, LAMP was proposed by the Japan Aerospace Exploration Agency as alternative microbial contamination monitoring system for the ISS (Ott et al., 2014). For the analysis of crew health related genetic modifications, qPCR technologies have been successfully tested on-board the ISS within the projects Gene in Space and Wet-lab2, devoted to definition of a robust, user-friendly nucleic acid extraction and sequencing approach aboard ISS by the use of spaceflight tested tools (Boguraev et al 2017; https://www.genesinspace.org/;

https://www.nasa.gov/mission_pages/station/research/experiments/1913.html; https://www.nasa.gov/ames/research/space-biosciences/wetlab-2). Moreover, the RAZOR EX PCR, launched on Space-X 9 (July 2016) within Water Monitoring Suite project will allow performing direct PCR amplification from water samples.

In all current space applications, however, the selected target-based detection and quantification approaches require the use of disposable materials and labour intense protocols, which will inevitably reduce their long-term applicability in space conditions. The general precautions used on Earth to limit contamination risks, including the most stringent procedures applied in clean rooms (Rettberg et al., 2019), might also represent a limiting practical issue in the small close spacecraft environment, since the high sensibility of PCR-based detection increase the chance of amplifying carry over contamination, with the consequent production of false positive results. This is crucial for LAMP owing to the limited accessibility to degradation of DNA products, while a major limitation of qPCR is also the occurrence of inhibitors that can be co-concentrated or extracted along with nucleic acids from the target microorganisms (Gibson et al., 2012). The presence of qPCR inhibitors introduces a number of problems, ranging from low amplification efficiency and reduced assay sensitivity to complete reaction failure and false negative results (Radstrom et al., 2008).

3.3 Sequencing-based "-omics" approaches for microbial community characterization

The High-Throughput Sequencing (HTS) encloses a popular suite of technologies, methodological approaches, and data elaboration workflows used to characterize the phylogenetic composition of the total microbial community in water samples and different aquatic matrices. The taxonomic classification is based on the huge amount of sequences generated by either a portion of the cellular nucleic acid content or the whole genome,

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354 through the so-called amplicon and shotgun sequencing approaches, respectively (Peabody et
355 al., 2015).
356 The amplicon analysis represents the extension of sequencing based methods of organisms'
357 classification, defined by specific protocols under the general name of "genetic" or
358 "molecular barcoding" (Hebert and Gregory, 2005). A barcode marker is a conserved DNA
359 sequence, with some variation at interspecific level, such as the 16S rRNA for
360 microorganisms, mtDNA CoxI gene for most animals, or many chloroplast genes for plants
361 (Chakraborty et al., 2014; Valentini et al., 2009). A wide set of algorithms and pipelines have
362 been developed to compare an unknown barcode sequence with reference sequences of
363 known species stored in specific database, allowing samples classification with acceptable
364 sensitivity and specificity (Escobar-Zepeda et al., 2015; Gonzalez et al., 2019).
365 The shotgun sequencing consists in generating millions of DNA fragments of different
366 lengths from a starting pool of genomes. These fragments cover a quote of the original
367 genomes inversely proportional to the genome lengths, and taxonomic assignment may be
368 quantitative according to the proportion of fragments classified for each taxon. The amplified
369 products are sequenced, controlled for quality, and assembled in longer contigs. The obtained
370 sequences are classified at different taxonomic levels, according to the best match with a
371 reference sequence databases and following different similarity criteria (Segata et al., 2012).
372 Currently, the most used sequencing platforms are characterized by different technical
373 principles and include Roche 454 GS-FLX (pyrosequencing), Illumina MiSeq and HiSeq
374 (reversible terminator sequencing by synthesis), Ion PGM (semiconductor based sequencing
375 by synthesis), and the nanopore GridION and MinION™ (Check Hayden, 2015; Clooney et
376 al., 2016; Ghanbari et al., 2015; Glenn, 2011).
377 HTS-based technologies have been already tested under microgravity conditions and directly
378 on the ISS (Castro-Wallace et al., 2017; McIntyre et al., 2016). First studies entailed to test

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the feasibility and performance of sequencing protocols, and to verify the potential application of real time sequencing, genome assembly for metagenomics, infectious disease diagnosis, and gene expression. In particular, the MinION pocket-size device was retained as the most suitable candidate technology to be transported and mounted on the ISS (Figure 2). Specific changes in base sequences are detected when DNA/RNA molecules pass through nanopores (protein) embedded in membranes. Depending on the sequencing of single (1D) or double strands (2D), the approach can provide real time identification up to 300 kb with 1D and 60 kb with 2D reads (Jain et al., 2016). The integrity and activity of nanopores were not adversely affected by storage conditions, launch, cosmic radiations or handling in microgravity, and reusability of flow cells was warranted. Specific indications to avoid air bubbles interference at the nanopores were implemented (Castro-Wallace et al., 2017; Rizzardi et al., 2016). MinION performances were also evaluated in sequencing a mixture of genomic DNA from a virus, a bacterium, and of mammal mtDNA, in comparison with MinION, Illumina MiSeq and PacBio RSII sequencing runs made on ground. Accuracy in base-calling on-board of the ISS was not significantly different respect to the runs obtained on the ground, while accuracy respect to MiSeq and RSII was slightly inferior (89% identity to the reference genome, respect to > 99%), but sufficient for sequence analysis. Sequencing reads were successfully assigned to the correct reference sequences in 90% of cases, and 10% of cases remained unassigned. Single-read error rates of 7-20%, intrinsic of the flow cell, were likely caused by unidentified assignments. It is predictable that, with novel improved versions of flow cells, the gap with other platforms will progressively become irrelevant. Moreover, tests demonstrated that real-time metagenomics analysis, from read processing to microbial identification and finally to genome assembly was also possible by using a laptop device that can be integrated in the ISS hardware. Considering genomic data analysis, one of the least demanding platform has been tested successfully simulating flight conditions and

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hardware availability onboard of the ISS, demonstrating its realistic applicability even on a laptop base (Castro-Wallace et al., 2017). Provided the adaptation of hardware and instruments to the spatial and logistic limitations onboard, both the shotgun and amplicon approaches with their intrinsic advantages and drawbacks, can be considered as suitable tools for microbial community characterization and pathogen detection on the ISS.

In terrestrial applications, the use of HTS-based techniques for microbiological quality assessments has been continuously growing (Tan et al., 2015), and the molecular detection of aquatic microorganisms improved notably since the first implementations of the MinION nanopore sequencing-based approach (Kilianski et al., 2015).

The amplicon approach requires straightforward PCR amplification protocols, downstream bioinformatics, and database searches. Nevertheless, this technique is prone to fail to detect unknown or highly divergent 16S rRNA gene sequences, which may be associated to novel or previously undetected pathogens. A specific evaluation of 16Sr RNA gene resolution highlighted that taxonomic assignment is highly sensitive to the gene region selected as a marker and to the assignment method. Sequences of at least 250 bp in the variable regions V2-V3 and V4-V5 allowed a correct taxonomic assignment nearly at the same level of full-length sequences (Claesson et al., 2010; Liu et al., 2008; Tremblay et al., 2015). Another known potential bias of 16S rRNA gene based detection is related to variability in the number of gene copies between microbial taxon and even between individual cells of the same species or strain, which may inflates and affect the accuracy of quantification (Větrovský and Baldrian, 2013). A further important limitations of amplicon analysis and barcoding approach is the lack of a common scientific view of suitable markers for viruses and, consequently, the absence of a standardized and exhaustive reference database (Chakraborty et al., 2014).

In comparison to amplicon analysis, the shotgun sequencing offers a wide genome coverage of organisms, without any selective constraints posed by marker choice, thus allowing the

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detection of unknown contaminants and the possibility to screen for both bacteria and viruses. In the field of water quality monitoring, the shotgun sequencing was used as the benchmark method to assess the occurrence of potential multiple water-borne pathogens, novel indicators for human sewage contamination, the relationships between the presence of fecal indicator bacteria and pathogen-like sequences, and microbial safety of drinking waters in relation to the efficiency of reclamation treatments (Chao et al., 2013; Lu et al., 2015; McLellan and Eren, 2014; Newton et al., 2015). Although the possibility to obtain up to a few thousands of base pairs for each read improved the microbial identification specificity, non-trivial disadvantages include the initial skimming of the huge number of produced reads, their quality check and assemblage in contigs useful for classification, which require high computational effort and time (Tan et al., 2015).

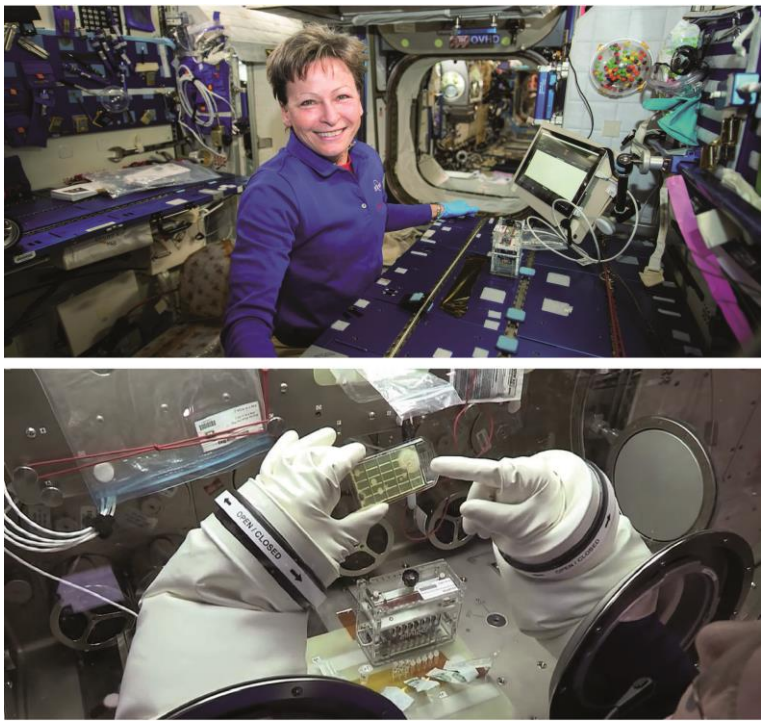

Figure 2. The NASA astronaut Peggy Whitson performed the Genes in Space investigation aboard the space station using the miniPCR and MinION. Credits: NASA

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4 Real-time technologies for early warning microbial monitoring

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

4.1 ATP-metry

An option for real-time monitoring of biological contamination in water samples is the analysis of adenosine triphosphate (ATP) content. ATP represents the universal energy source for the main cellular functions and plays a key role in energy exchange in every type of cell by linking catabolic and anabolic processes. The analysis is carried out through the chemical and/or enzymatic extraction of this molecule from microbial cells, followed by the measurement of light emission derived when the dissolved ATP reacts with the luciferine (substrate) - luciferase (enzyme) complex. In the presence of ATP and magnesium, the enzyme catalyzes the oxidation of the substrate, which is associated with light emission. The emitted light intensity is linearly related to the ATP concentration and easily measurable using a luminometer. Measurement of bioluminescence gives virtually instant information (within few minutes) of the metabolically active microbial population. This peculiarity makes it suitable as an early-warning approach for the study of living processes and for measuring bacterial contamination and monitoring water treatment efficacy in near real-time (Hammes et al., 2010). The method is robust, easy to perform, and suitable to detect both cultivable and uncultivable cells, with better estimates of total active microorganisms compared to heterotrophic plate counts. The concomitant use of traditional cultivation-based approaches

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showed levels of cultivable cells order of magnitude lower that those estimated by ATP (Siebel et al., 2008; K. Zhang et al., 2019).

The ATP analysis was performed on pre-flight and post-flight ISS water samples and revealed a biological contamination ranging between 0 (drinking water) and $4.9x10^4$ cells/ml (humidity condensate) (Bacci et al., 2019; La Duc et al., 2004). Recently, the viable microbial contamination on-board ISS was reliably monitored on surface samples by intracellular ATP measurement (Perrin et al., 2018; Venkateswaran et al., 2003). ATP assays were also proved effective in monitoring microbial contamination on surfaces from an inflated lunar/Mars analogous habitat during long-term human occupation (Mayer et al., 2016). The total (ATP content from both dead and live microbes) and viable microorganisms (intracellular ATP content) were in the range of 10^5-10^6 relative luminescence unit (RLU)/m^2. These results were in line with ATP data measured directly on-board within the experiment T2 carried out during Euromir-95 mission (Guarnieri et al., 1997). Recently, the ATP-metry was selected for the real-time monitoring of the biological contamination on board ISS within the H2020 European project BIOWYSE (Biocontamination integrated control of wet systems for space exploration, http://biowyse.eu), aimed at developing a compact, automatic, and microgravity-compatible on-board systems for the prevention, monitoring and control of microbial load in waters and on wet surfaces (Figure 3). A humid area sampler was recently developed and patented (ref. 102018000009137, dated 03/10/2018) (Detsis et al., 2018; Guarnieri et al., 2019).

Today, commercial kits for quantitative ATP-metry are available to monitor the microbial biomass level in water and to validate cleaning and disinfection procedures, with a wide number of bulk and intracellular ATP measurements on microbial communities reported from natural and engineered aquatic environments (Abushaban et al., 2019; Fillinger et al., 2019; van der Wielen and van der Kooij, 2010; Vang et al., 2014). However, most applications in

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water monitoring on Earth have not been used as frequently as expected. Although the apparent advantages, major technical limitations are related to the low sensitivity and result reproducibility at low cell concentration and sample volume (< 100 µl), along with the susceptibility to environmental conditions (e.g., pH, temperature, occurrence of enzyme inhibitors). Some drawbacks of the method are partly circumvented by using external and internal standards and by operating under controlled reproducible settings. Therefore, the correlation between intracellular ATP content and microbial cell counts will rely on a robust cross-calibration with results from other reference methods (Amalfitano et al., 2018b; Hammes et al., 2010). It has been calculated that the average ATP-per-cell content is approximately 1.75 x 10^{-10} nmol/cell or 6.87 x 10^{-17} g ATP/cell (K. Zhang et al., 2019). However, the amount of per-cell ATP can be significantly influenced by the phylogenetic affiliation and the cellular physiological status, with diverse ATP-content reported between either eukaryotic or prokaryotic cells from the same cultures (Bajerski et al., 2018; Yaginuma et al., 2015).

4.2 Flow cytometry

Ubiquitously applied from the bio-medical research to environmental sciences, flow cytometry (FCM) is considered an unparalleled high-throughput technology for single cell analysis (Robinson and Roederer, 2015). This generic technology allows the measure (-metry) of the optical properties of cells (cyto-) transported by a liquid sheath (flow). Following sample intake, a pressurized laminar flow is generated by the fluidic system of the instrument. The suspended particles are individually forced to cross a light source excitation point for scanning and evaluation. At the interrogation point, any fluorophore on or in the cell will absorb the laser beam energy and emit photons in a range of wavelengths, proportionally to the amount of incident light and the number of fluorescent molecules. Light scatter and

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fluorescence signals are detected at the single-cell level and instantly converted into digital information to be shown on multidimensional plots, along with real-time data analysis and statistics. The power of the technique comes also from the variety of laser light sources and commercialized fluorophores that can be used for sample staining and illumination (Shapiro, 2005).

Flow cytometry is included in the roadmaps of national space agencies worldwide and deemed as a necessary technology for defining and monitoring spaceflight-associated requirements since early 80's. Owing to the high versatility for diagnostic medicine (e.g., hematology, immunology, and physiology), this technology was so far retained as a prime asset for health monitoring and clinical laboratory diagnostics for astronauts, in view of the upcoming deep-space exploration missions (Crucian and Sams, 2012). A flow cytometry platform was already successfully tested on-board the ISS to monitor and understand the physiological adaptations of astronauts to microgravity (Dubeau-Laramée et al., 2014; Phipps et al., 2014) (Figure 3). Advanced developments of the prototype were based on commercialized flow cytometer with significant additional engineering modifications, mostly aimed to generate laminar particle flow (distinct from the standard sheath fluid based method), and to reduce the significant amount of liquid biohazardous waste and energy operating requirements. The laminar flow within the flow chamber was found to be dramatically altered by microgravity. In turn, the unavoidable elimination of the fluid mechanical setting for particle hydrodynamic focusing was proved to significantly reduced liquid waste and the total system operational load, in terms either of instrument size and weight or energy consumption (Cohen et al., 2011; Crucian and Sams, 2005).

Notwithstanding the recent system design improvements, peripheral blood cells has been retained as the only target for first studies in space environments (McMonigal and Crucian, 2015). Since a flow cytometer can provide support to a wide range of scientific applications

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(e.g., biology, microbiology, and environmental science), it is needless to point out that a spaceflight compatible machine could satisfy the unmet flight requirements for water monitoring and treatment to complete future long-duration missions in closed healthy environments.

The high sensitivity for scanning very small objects (i.e., from virus-like-particles to prokaryotes, to pico- and micro-eukaryotes) and the wide detectable cell concentration range (generally between 10^2 and 10^7 cells ml^{-1} without concentrating or diluting the samples) represent unmatched features of this technology for water monitoring. A broad suite of assays is available for microbial quality assessments in natural and engineered aquatic ecosystems (Amalfitano et al., 2014; Boi et al., 2016; Gasol and Morán, 2015; Van Nevel et al., 2017). Successful applications are reported to provide early warning indications of unexpected water contamination events through the rapid detection of a variety of microbiological threats, including pathogenic and potentially toxic microorganisms (Vital et al., 2010; Weisse and Bergkemper, 2018; Yang et al., 2010). Moreover, flow cytometric measurements are suitable to evaluate the efficiency of various industrial microbial bioprocesses (e.g., food and pharmaceutical preparations) (Díaz et al., 2010), and the performances of engineered systems for water treatment (Besmer and Hammes, 2016; Safford and Bischel, 2019). Remarkably, FCM was also useful to determine the presence of nonliving organic and inorganic substances, including nano- and micro-sized particles, suspended solids, flocs and aggregates of various origins (Aulenta et al., 2013; Casentini et al., 2016; Liu et al., 2016). On-going instrumental developments for on-site applications are directed to install flow cytometers on either mobile units (e.g., ships and vehicles) or fixed locations (e.g., treatment plants, marine buoys, off-shore platforms), with the possibility for automatic programmable staining of aquatic microorganisms and remote data transfer (Buysschaert et al., 2018; Pomati et al., 2011; Thyssen et al., 2007).

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570 Leaving aside the high costs of all sophisticated systems, the need for specially trained staff is
571 likely to represent a major drawback in the daily scheduled monitoring practices. Moreover,
572 the reproducibility of cytometric data was reported to be adversely affected by changing cell
573 staining protocols (e.g., fixatives, type of fluorescent dye), incubation conditions (e.g.,
574 temperature, time to analysis), instrumental settings (e.g., fluidic and signal amplification
575 systems), and the source water (e.g., from natural or engineered systems) (Nescerecka et al.,
576 2016; Prest et al., 2013). Further work is also required to establish user-independent strategies
577 for gating and data handling (Amalfitano et al., 2018a; Koch et al., 2014). One more specific
578 challenge is the lack of phylogenetic resolution. Despite the enumeration of targeted taxa may
579 rely on specific fluorescence staining procedures (Couradeau et al., 2019; Manti et al., 2011;
580 Neuenschwander et al., 2015), the cytometric information characterizing different
581 subpopulations of microbial cells is generally ataxonomic. A direct link between cytometric
582 fingerprinting and microbial diversity, assessed by 16S rRNA gene amplicon sequencing,
583 was demonstrated in recent studies (Props et al., 2017, 2016). However, the computational
584 workflows are convoluted and still under development (Rubbens et al., 2019).
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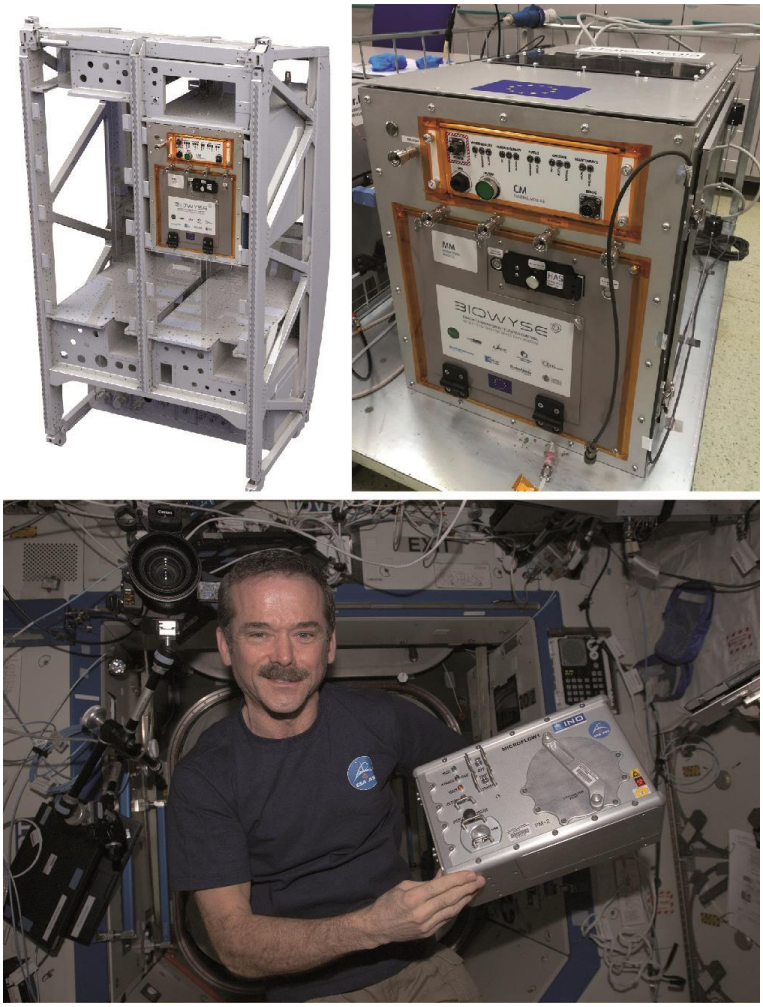

Figure 3. Integrated breadboard of the ATP-metry system, developed within the European H2020 project BIOWYSE. The hardware was designed (upper left photo) and built (upper right photo) to fly within the European Drawer Rack Mark 2. The photo below shows the Canadian astronaut Chris Hadfield holding the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

5 Comparative analysis of space-relevant technological features

The mature technologies, herein entitled for water microbial monitoring in space, showed their own critical advantages and limitations (table 1). Both current and future applications in space will be necessarily bounded by the definition of novel standards of microbial quality

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(i.e., other than those applied on Earth by cultivation-dependent approaches) (Amalfitano et al., 2018b), but also by a compromise between the best analytical performances and detrimental technical issues to cope with during spaceflights.

From the one hand, analytical benefits for space applications will necessarily include the time needed to achieve results (i.e., speed to results), the accuracy in detecting specifically-selected microbial targets (i.e., identification depth) with reproducible consistent results (i.e., reproducibility), and the number and multiple type of achievable results (i.e., herein named multiparametricity). On the other hand, the operating conditions can be particularly stringent and limiting the direct applicability in space, unless addressing critical requirements such as reagent usage, waste production, operator skills, and crew time. Using a simplistic pairwise comparison, we sought to emphasize that few selected technological features have to be consciously retained from the methodological proof-of-concept level up to the device deployment, instrumental demonstration, and routine use in the on-board housekeeping program. Although the comparative scores were assigned subjectively, it is likely evident that the stringent requirements of microbial water monitoring in space cannot be met by a single technological solution.

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

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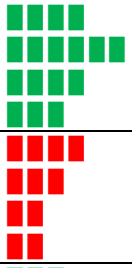
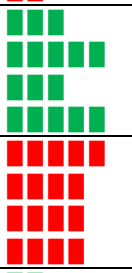
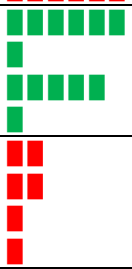
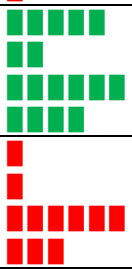
Technology	Advantages	Limitations	Analytical performances/ Technical issues	Comparative scores	Applications in space
Plate Cultivation	– No pretreatment – Detection limit 1 cell/100 ml – Low equipment requirements	– No direct quantification – Risks of contamination – Reliant on cultivation conditions	Speed to results Identification depth Reproducibility Multiparametricity Reagent usage Waste production Operator skills Crew time		La Duc et al., 2004 Morrison et al., 2017 Pierson et al., 2013
LAMP	– Detection limit ~10^2 gene copies/ml – Unaffected by template conc. – No effects of inhibitory compounds	– No direct quantification – Risks of contamination	Speed to results Identification depth Reproducibility Multiparametricity Reagent usage Waste production Operator skills Crew time		Ott et al., 2014
qPCR	– Target-specific and quantitative – Detection limit ~10^3 gene copies/ml	– Pretreatment processing – Affected by template and inhibitory compounds – Reliant on PCR related issues	Speed to results Identification depth Reproducibility Multiparametricity Reagent usage Waste production Operator skills Crew time		Boguraev et al., 2017 Parra et al., 2017
High Throughput Sequencing	– In-depth phylogenetic resolution – Specific for unknown non targeted microorganisms	– Pretreatment processing – Reliant on PCR related issues – Complex data interpretation	Speed to results Identification depth Reproducibility Multiparametricity Reagent usage Waste production Operator skills Crew time		Castro-Wallace et al., 2017 McIntyre et al., 2016
ATP-metry	– Real-time data (< 5 min) – Detection limit ~0.1 pg/ml	– Unspecific detection – Destructive analysis – Risks of contamination	Speed to results Identification depth Reproducibility Multiparametricity Reagent usage Waste production Operator skills Crew time		Guarnieri et al., 1997 La Duc et al., 2004
Flow cytometry	– No pretreatment – Quantitative and near-real time data (< 20 min) – Detection limit ~10^2 cells/ml	– Complex data interpretation – Clogging issues	Speed to results Identification depth Reproducibility Multiparametricity Reagent usage Waste production Operator skills Crew time		Dubeau-Laramée et al., 2014 Phipps et al., 2014

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6 System miniaturization and future challenges

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

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

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

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

The system miniaturization was also considered the most appealing trait for space applications of the nanopore DNA sequencer MinION (Castro-Wallace et al., 2017). By assembling miniaturized and lab-on-a-chip solutions, the Water Monitoring Suite developed by NASA represents so far the best performing custom-built device applied successfully on the ISS to monitor different water quality properties. Along with the HACH colorimeter and the Organic Water Module, respectively used for inorganic and organic chemical assessments, the hardware suite also includes the PCR-based instrument RAZOR EX, with customized sample pouch kits containing all pre-loaded reagents necessary for sampling, sample preparation, and real-time PCR (https://www.nasa.gov/mission_pages/station/research/experiments/explorer/Investigation.html?#id=1847). However, as space exploration progresses toward extended missions to cis-lunar space and Mars, PCR-based and multi-omics instruments onboard spacecraft should not be considered in separation from other technologies needed for the in-flight microbiological research (Karouia et al., 2017).

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

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of reduced consumption of reagents and the ability to integrate monitoring and isolation procedures within a single device (Auroux et al., 2002). A simple microfluidic system was successfully tested for rapid and semi-automated bacterial enumeration in freshwaters and promising outcomes suggested its applicability to drinking waters under both ground and space conditions (Yamaguchi et al., 2014). Moreover, both sensitivity and specificity of ATP-metry and FCM can be also improved by miniaturizing the core systems down to scales closer to the ones of microorganisms. A micro-fluorescence-activated cell sorting (μFACS) was used to sort out cells of interest by changing the flow direction after cell detection (Fu et al., 1999). An integrated platform that combines two different force fields in a single microfluidic device (Dielectrophoretic–Magnetic Activated Cell Sorter - iDMACS) was applied for simultaneous sorting of multiple bacterial targets (Kim and Soh, 2009). More recently, a high-throughput Raman flow cytometer was developed on a microfluidic chip for the label-free molecular fingerprinting at the single-cell level (Hiramatsu et al., 2019).

The onboard laboratory miniaturization included also the fluidic components (e.g., pumps, valves, electronics), thus paving the way to the use of advanced biosensors for screening food safety and water quality in space (Roda et al., 2018). Following the proofs of concept and wearable technologies suited to monitor astronauts' health, the biosensing diagnostic instrumentation, most reasonably based on electrochemical and optical detectors, was argued as a secondary future option for in-flight water biochemical analyses (Choi et al., 2018; Limero and Wallace, 2017; Nelson, 2011). In particular, the amperometric biosensors were proved sensitive to monitor different water analytes, chemical contaminants (e.g., pesticides, organophosphates, carbamates), and numerous microbial biomarkers successfully targeted to detect the major microbiological agents, food- and water-borne pathogens (e.g., *E. coli*, *Salmonella*, *L. monocytogenes*, *C. jejuni*, *B. cereus*, *M. smegmatis*) (Grieshaber et al., 2008; Velusamy et al., 2010). However, despite providing concrete benefits for health services and

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

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

7 Conclusions

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

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

Acknowledgements

Authors wish to acknowledge H2020 Project called BIOWYSE "Biocontamination Integrated cOntrol of Wet sYstems for Space Exploration" (http://biowyse.eu). Grant Agreement number: 687447.

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