Potential biofilm control strategies for extended spaceflight missions

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ABSTRACT

Biofilms, surface-adherent microbial communities, are associated with microbial fouling and corrosion in terrestrial water-distribution systems. Biofilms are also present in human spaceflight, particularly in the Water Recovery System (WRS) on the International Space Station (ISS). The WRS is comprised of the Urine Processor Assembly (UPA) and the Water Processor Assembly (WPA) which together recycle wastewater from human urine and recovered humidity from the ISS atmosphere. These wastewaters and various process streams are continually inoculated with microorganisms primarily arising from the space crew microbiome. Biofilm-related fouling has been encountered and addressed in spacecraft in low Earth orbit, including ISS and the Russian Mir Space Station. However, planned future missions beyond low Earth orbit to the Moon and Mars present additional challenges, as resupplying spare parts or support materials would be impractical and the mission timeline would be in the order of years in the case of a mission to Mars. In addition, future missions are expected to include a period of dormancy in which the WRS would be unused for an extended duration. The concepts developed in this review arose from a workshop including NASA personnel and representatives with biofilm expertise from a wide range of industrial and academic backgrounds. Here, we address current strategies that are employed on Earth for biofilm control, including antifouling coatings and biocides and mechanisms for mitigating biofilm growth and damage. These ideas are presented in the context of their applicability to spaceflight and identify proposed new topics of biofilm control that need to be addressed in order to facilitate future extended, crewed, spaceflight missions.

1. Introduction. Biofilms and spacecraft Environmental Control and Life Support Systems (ECLSS)

Biofilms are surface-adherent accumulations of microorganisms in an extracellular polymeric substance (EPS) matrix. The EPS is mostly composed of polysaccharides, proteins, lipids, and nucleic acids that provide them with a scaffold to form a three-dimensional structure and which enables them to adhere to surfaces. Biofilms associated with microbial corrosion often have a variety of minerals present in the matrix [1]. This extracellular biofilm matrix improves cell-to-cell communication and can protect the microbes from mechanical stresses, biocides, antimicrobials, and ultraviolet radiation, among other types of stresses [2,3]. Biofilms have an important role on multiple types of infections in humans, including medical device-associated infections, dental caries, cystitis, pulmonary infections associated with cystic fibrosis and endocarditis [4]. They can also degrade the surface upon which they grow, including corrosion of metals and mineralization and weakening of polymers [5]. Furthermore, they can accumulate to the point of causing structural and/or functional damage to mechanical parts (biofouling). The first investigations regarding controlled biofilm growth in microgravity (with Burholderia cepacia and Pseudomonas aeruginosa) were first reported in 1999 and 2001, respectively [6,7], although there were...
certainly earlier indications of biofouling problems in spacecraft. For example, the Soviet Salyut 6 and 7 and the Mir space stations experienced problems derived from microbial contamination on piping, behind panels, water recycling systems, electrical connectors, radiators, air conditioning, oxygen electrolysis block, a navigation window, an extra-vehicular activity (EVA) suit’s headphone, and thermal control system [8]. Similarly, the International Space Station (ISS) has had challenges arise from microbial contamination and biofilm formation, notably in the wastewater collection reservoir component of the Water Recovery System (WRS), which is a part of the Environmental Control and Life Support System (ECLSS) [9]. As seen in Table 1, some of the most common microbial organisms isolated from the WRS (namely on the filter immediately downstream of the WPA wastewater tank), are *Ralstonia pickettii*, *Bulkmoholderia sp.* and *Cupriavidus metallidurans* [10]. More recently several metagenomic studies have been performed in the ISS (e.g. Ref. [11,12]). In the case of the US-segment of the ISS, the WPA’s wastewater tank – the component that has shown the most problems related to biofilm – receives crew urine (treated with an oxidizer and an inorganic acid) distillate, cabin humidity condensate, and water produced from CO₂ and H₂ by the Sabatier reactor (when in operation). The WPA processes the contents of the wastewater tank into potable water for the crew and multiple other systems [9]. Biofilm formation can be problematic in any spacecraft system, however, it is of particular importance when it occurs in the ECLSS, and the WRS in particular, given that this key life-support system serves to provide the crew and other critical systems with potable water [13]. To maintain this critical function, an improved method for biofilm control must be developed and implemented on future missions. Based on experiences to date with the WRS in the ISS [9] and similar biofilm occurrence seen with municipal drinking water distribution systems [14], and systems involving greywater recycling [15]; total biofilm eradication in the WRS of spacecraft does not appear feasible. The working mitigation strategy is to control and not eradicate biofilm growth, since the latter is likely not feasible particularly in the context of an extended mission beyond low Earth orbit. The ultimate goal is to simply prevent biofilm growth from impacting the mechanical functionality of the system via corrosion, fouling, or some component organisms bypassing the disinfection processes and affecting the potable water. Consistent with this strategy is the requirement to maintain biofilm control in the WRS with initial system operation, rather than attempting to regain control of biofilm growth by using methods to destroy and/or detach an existing biofilm. Detecting and monitoring bacterial and biofilm levels will be necessary in key ECLSS components, so that appropriate life support functions are maintained. While the bulk of the identified biofilm issues in spacecraft relate to the WRS component of ECLSS [10], one might also anticipate microbial growth on surfaces prone to water condensation or absorption. Volatile organic compound condensation onto surfaces has been proposed as a strategy for extracting airborne compounds during analytical chemical approaches [16] and so one might assume that a similar process along with associated biofilm formation would occur on surfaces or

| Microbial species                       | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 |
|----------------------------------------|------|------|------|------|------|------|------|------|------|------|------|
| *Acidovorax temperans*                 |      |      |      |      | x    | x    | x    | x    |      |      |      |
| *Burkholderia multivorus*              | x    | x    | x    | x    | x    | x    | x    | x    |      |      |      |
| *Burkholderia species*                 |      |      |      |      | x    | x    | x    | x    |      |      |      |
| *Cupriavidus basilensis*               | x    |      |      |      |      | x    | x    | x    | x    | x    | x    |
| *Cupriavidus metallidurans*            | x    | x    | x    | x    | x    | x    |      |      |      |      |      |
| *Curvibacter lanceolatus*              |      |      |      |      |      | x    | x    | x    | x    | x    | x    |
| *Flexibacter species*                  |      |      |      |      |      |      |      |      | x    | x    |      |
| *Lectyophora species*                  |      |      |      |      |      |      | x    |      |      |      |      |
| *Lectyophora mutabilis*                |      |      |      |      |      |      | x    |      |      |      |      |
| *Microbacterium laevaniformans*        |      |      |      |      |      |      |      |      | x    |      |      |
| *Novosphingibium species*              |      |      |      |      |      |      |      |      |      |      |      |
| *Paeleimyces species*                  |      |      |      |      |      |      |      |      |      |      | x    |
| *Ralstonia insidiosa*                  | x    |      |      |      | x    | x    | x    | x    | x    |      |      |
| *Ralstonia pickettii*                  | x    | x    | x    | x    | x    |      |      |      |      |      |      |
| *Shingobium xenokuyae*                 |      |      |      |      |      |      |      |      |      |      | x    |
| *Shingobium yanoikuyae*                |      |      |      |      |      |      |      |      |      |      |      |
| *Unidentified Gram-negative rod*        |      |      |      |      |      |      |      |      |      |      |      |
| *Acinetobacter species*                |      |      |      |      |      | x    |      |      |      |      |      |
| *Ajfia species*                        |      |      |      |      |      |      |      |      |      |      | x    |
| *Braulihiolesia species*               |      |      |      |      |      |      |      |      |      |      |      |
| *Burkholderia kururiensis*             |      |      |      |      |      |      |      |      |      |      | x    |
| *Burkholderia kururiensis*             |      |      |      |      |      |      |      |      |      |      |      |
| *Caulobacter vibrioidei*               |      |      |      |      |      |      |      |      |      |      |      |
| *Chitinophaga arvensicola*              |      |      |      |      |      |      |      |      |      |      | x    |
| *Chitinophaga species*                 |      |      |      |      |      |      |      |      |      |      |      |
| *Chryseobacterium gleum*               |      |      |      |      |      |      |      |      |      |      | x    |
| *Cryptococcus laurentii*               | x    | x    |      |      |      |      |      |      |      |      |      |
| *Curvibacter lanceolatus*              |      |      |      |      |      |      |      |      |      |      |      |
| *Leptonia species*                     |      |      |      |      |      |      |      |      |      |      | x    |
| *Mesorhabdus species*                  |      |      |      |      |      |      |      |      |      |      |      |
| *Methylobacterium species*             | x    | x    |      |      |      |      |      |      |      |      |      |
| *Microbacterium species*               |      |      |      |      |      |      |      |      |      |      |      |
| *Pelomonas species*                    |      |      |      |      |      |      |      |      |      |      |      |
| *Phyllobacterium myrmecearum*          |      |      |      |      |      |      |      |      |      |      | x    |
| *Rhodopseudomonas species*             |      |      |      |      |      |      |      |      |      |      |      |
| *Shingobium xenokuyae*                 |      |      |      |      |      |      |      |      |      |      | x    |
| *Sphingomonas asaccharolytica*          |      |      |      |      |      |      |      |      |      |      |      |
| *Sphingomonas capsulata*               |      |      |      |      |      |      |      |      |      |      | x    |
| *Sphingomonas paucimobilis*            |      |      |      |      |      |      |      |      |      |      |      |
| *Sphingomonas sanguinis*               |      |      |      |      |      |      |      |      |      |      |      |
| *Staphylococcus epidermidis*           |      |      |      |      |      |      |      |      |      |      | x    |
| *Wautersia metallidurans*              |      |      |      |      |      |      |      |      |      |      |      |

Table 1: Microbial isolates collected from the wastewater tank (WW), portable water bus (PWB), or condensate.
materials prone to water accumulation on spacecraft. In non-ECLSS situations, prevention or removal of moisture accumulation represents a feasible strategy for biofilm control.

Furthermore, the problems resulting from biofilm formation on future spacecraft may be exacerbated by observed changes on bacterial phenotype and gene expression when grown in microgravity [17, 18]. For example, an in-vitro investigation performed in space using Burkholderia cepacia resulted in (i) larger cell counts of bacterial biofilms grown in stainless steel submerged in water and a (ii) decreased sensitivity to iodine (which is commonly used as potable water disinfectant [19]), with respect to matched Earth controls [7]. Another investigation that used Pseudomonas aeruginosa cultured in modified artificial urine medium (mAUM) in space, and with respect to matched Earth controls, showed an increase in (i) number of viable cells, (ii) biomass, and (iii) mean biofilm thickness, and (iv) a ‘column-and-canopy’ biofilm structure, unlike the flat mats observed on the ground samples [20] (reviewed in Refs. [21, 22]).

One notable condition that is encountered during spaceflight is microgravity. Although short duration microgravity conditions can be encountered by parabolic aircraft missions or drop tower experiments, prolonged microgravity studies need to be performed during spaceflight including the International Space Station. Flight opportunities are rare and so there has been the development of a number of microgravity simulation approaches, most notably random positioning (RP) devices [23, 24] and clinostat technology involving rotating wall vessels (RWVs) [25]. Microgravity analog devices such as RWV and RP devices do mimic many but not all aspects of spaceflight (see reviews by Refs. [21, 23, 25–27]). The main advantage of analog experiments is one of accessibility and relatively low cost when compared to space flight. In spite of limitations, microgravity analog approaches represent an important, accessory to space biology research.

2. Moon and Mars crewed missions

While spare parts can and are sent to ISS for system maintenance, this approach will be more complicated on missions to the Moon and prohibitive on a mission to Mars. Hence the importance of proactively mitigating the risks derived from biofilm formation on future spacecraft, namely on key components of the WRS. The magnitude of this challenge is different between missions to the Moon and Mars and it heavily depends on two things: orbital mechanics and mission architecture.

There are three major categories of trajectories that can be taken for Earth-Moon and Earth-Mars missions: (i) ballistic, (ii) low-thrust, and (iii) cyclers. Given that the second and third options take longer for the spacecraft to arrive to its destination, ballistic trajectories are the preferred option for crewed-mission planners. In the case of lunar missions, a ballistic trajectory requires a minimum 3.125 km/s velocity to leave Earth, and under these circumstances, it takes about 5 days (~120 h) to reach the Moon [28]. In fact, Apollo missions took between 66 and 90 h for the crew to arrive to lunar orbit. These short-duration flights enable the implementation of relatively low-complexity ECLSS, for example, dumping the urine outside the spacecraft instead of recycling it. Ability for Mars missions compared with those staying in low Earth orbit (LEO).

Regardless of the mission architecture chosen for a human mission to Mars, the ECLSS will need to be operable for at least ~30 months to sustain the crew. How long a given spacecraft’s ECLSS needs to remain fully functional depends on the mission architecture, however. For example, a single-spacecraft approach for Earth-Mars transit, stay on Mars, and Mars-Earth transit, as proposed in Ref. [32], means that this spacecraft would need to remain completely viable for ~30 months. An architecture based on the use of a Mars transit vehicle to get to Martian orbit, a lander to descend to the surface, stay on the Martian surface in a habitat, return to Martian orbit on the same lander, and return to Earth on the same transit vehicle has its own, unique, ECLSS requirements. As currently envisioned at the time of writing this paper, the Mars transit vehicle needs to have an operable ECLSS for seven months during transit from Earth to Mars, be able to remain viable during a 17-month partial or total dormancy period and be active again for another six months during the return flight to Earth. Similarly, to the lunar lander, the Martian lander would have relatively-low complexity ECLSS requirements as it would not have to be closed-loop. The Martian habitat’s ECLSS, however, shall remain fully functional for at least 17 months. All of this would be further exacerbated if these systems would need to be able to support more than one fast-transit mission – thus instead of being ~30 months, they would need to be operable for more than five years – and/or be reusable. We present a series of potential biofilm mitigation approaches that can be implemented on future interplanetary-transit spacecraft ECLSS – namely on the WRS due to the inherent sensitivity with biofilm growth, with a focus on the most stringent requirements from the previously described microgravity scenarios: Gateway and the Martian transit vehicle. The basis of this work comes from a NASA-Montana State University joint biofilm workshop, which took place in Bozeman, MT on July 18, 2019, and subsequent research into each topic discussed. This workshop provided a unique opportunity whereby representatives from NASA, the academic community, and industries with an impressive background in biofilm research could provide input. Potential control strategies for extended spaceflight missions were discussed, which were grouped under six different categories: (a) Biofilm mitigation approaches including combining physical and chemical treatment or equipment replacement that might be considered. In the following sections, we will explore some of these concepts.
3. Biofilm control strategies

3.1. Biocides

There are two applications for biocides in the WRS, including the potable water and the wastewater. Each application has a fundamentally different role for the biocide function. In WPA product water, microbial growth is primarily maintained by the sterilization process in the WPA’s catalytic reactor, the low organic content (typically less than 0.5 mg/L), and the stringent processing requirements implemented during assembly. As such, biofilm growth has not been an issue for the potable water plumbing. The function of the biocide is to provide residual microbial control in response to any atypical microbial presence. The ISS has historically used iodine as the potable water bus biocide via an iodinated resin initially developed by the Umpqua Research Company for use on NASA Space Shuttle program. This technology currently disburses 1–4 mg/L of iodine into the WPA product water [33]. New options such as silver are being considered as replacements for potable water [34]. While Ag nanoparticles have shown promise against biofilms in some tests (e.g. Ref. [35]), other investigators have shown that Ag nanoparticles can induce a change in P. aeruginosa biofilms to a non-culturable but metabolically active state, which reduces effectiveness [36]. Certainly, other biocides (shown in Table 2) could be considered, however the effectiveness in spacecraft as well as a low potential for volatile (potentially harmful) byproducts does need to be considered. In contrast, no biocide is currently used in the WPA wastewater (urine distillate and humidity condensate). During the July 2019 workshop at Montana State University, industrial and academic experts agreed that a biocide would be essential in maintaining biofilm control in the WRS of future NASA missions, though likely in conjunction with another method. Many variables must be understood before selecting a biocide for the wastewater application, such as material compatibility, effective concentration, shelf life, and kill spectrum. These variables are currently being reviewed on multiple biocides under consideration for use in the WPA wastewater tank (summarized in Table 2). Biocide impact on biofilm formation as a byproduct of planktonic cell growth disruption is the center for future technology analysis.

As Li et al. [79] explain in their literature review, silver biocides are broad spectrum and would require very little maintenance in long-duration missions. Aside from this, it is pointed out that combined physical and chemical methods, such as sonication and a biocide, would be sufficient to inhibit biofilm growth. Separately, chlorine and bromine have been biocidal options for the potable water bus and may also have application for the wastewater. However, iodine’s lower vapor pressure and chlorine and bromine’s ability to form byproducts are the main reasons for which iodine was chosen for the potable water bus. Nevertheless, chlorine and bromine remain common disinfectants used in industrial private and public water systems as regulated by the Environmental Protection Agency (EPA) [77,80,81]. Rodriguez et al. [82], tested multiple metallic materials involved in the water processor assembly, such as corrosion resistant steel, titanium, and hastelloy, and some non-metallic polymer materials. The materials were tested against multiple biocides, some of them especially popular in the industrial use of clean rooms, mainly against spore-forming bacteria, such as peracetic acid, hydrogen peroxide, and sodium hypochlorite.

One of the most common biocidal treatments of water for microbial control is the use of oxidizing chemicals, which can be categorized as either halogenated or non-halogenated. The most typical halogenated oxidizing biocides employ chlorine or bromine. The addition of a chlorinated biocide to water creates a mixture of hypochlorous acid and hypochlorite ions where the disinfecting properties of the mixture are attributed to the hypochlorous acid portion. Biocides which depend on hypochlorous acid for disinfecting properties are most effective within pH ranges of 6.0–7.5. Since the stability of hypochlorous acid is pH dependent, the disinfecting properties are quickly lost at ranges of 8.0 and higher. Consideration should also be given to the residual organic

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Table 2

Some of the biocides being considered for wastewater tank biofilm mitigation. Concentrations, kill-time, use and effectiveness spectrum usually change by organism type in the literature.

| Biocide                        | Concentration Effectivity Spectrum Common Use                                                                 |
|--------------------------------|---------------------------------------------------------------------------------------------------------------|
| Lysozymes and proteases [37, 38] | Lysozyme is usually used in a concentration of 10mg/mL in 10mM Tris-Cl (pH 8.0). Stability of the aqueous solution is a problem. 10mg/ml ~ 10,000 ppm. Vegetative bacterial cells and some potency against non-vegetative cells. Lysozyme is used by academia to degrade bacterial cell wall peptidoglycan and spore cell walls prior to DNA purification and as a food cleaning product. Protease is used for lytic purposes in research, laundry detergents etc. Chelating agent in cosmetics and personal care products as well as medical and veterinary equipment. Commercial and residential disinfection/sanitization products; Deodorant active ingredient for personal care products; Antimicrobial active/preservative for personal care products; pharmaceutical; agriculture; industrial; biofilm control. |
| Tetrasodium EDTA [39]          | 4% of total volume Bacterial cell wall, removes Mg2+ from gram negative outer membranes. | | |
| Silver dihydrogen citrate [40,41] | 1:80 dilution (30 ppm ionic silver) | Bacteria, fungi, and viruses | | |
| Colloidal silver and AgF [34, 42] | Maximum concentration of 400 ppm used for water disinfection in Russian module of ISS | Bacteria, fungi, and viruses | Commercial and residential disinfection/sanitization products; Deodorant active ingredient for personal care products; Antimicrobial active/preservative for personal care products; pharmaceutical; agriculture; industrial; biofilm control. |
| Peracetic Acid [43]            | Around 0.2% of total volume | Bacteria, fungi, bacterial spores, and viruses | Mostly surface disinfections in the medical industry. |
| Hydrogen Peroxide [44, 45]     | Around 3% of total volume. A mixture of hydrogen peroxide and peracetic acid can be effective at 22% hydrogen peroxide and 4.5% peracetic acid ‘about 10 to about 90% by weight of sodium chloride; about 10 to about 90% by weight of sodium bromide; and about 5 to about 90% | Bacteria, fungi, bacterial spores, and viruses | Mostly surface disinfections in the medical industry. |

(continued on next page)
content within the water, as hypochlorous acid will react with the organic content and deplete the disinfecting potential. Various forms of brominated biocides, such as bromine monochloride (BrCl), hypobromous acid (HOBr-), and bromodimethylhydantoin, have been applied as microbial control agents to treat water. Similar to chlorinated water treatments, a brominated biocide creates a mixture of hypobromous acid and hypobromite with the disinfecting portion being attributed to hypobromous acid, which is slightly more stable at increasing alkalinity, but begins to lose meaningful disinfectant efficacy at pH of 9.0 and higher. Hydrolysis of an activated bromide salt or bromine chloride will produce a mixture of hypobromous acid and hydrochloric acid with sodium chloride which can be utilized for water disinfection. Another chlorinated biocide which has been effectively employed to control microbial growth within water is chlorine dioxide (ClO₂). The major advantages of the use of chlorine dioxide include biocidal efficacy at broader pH ranges, improved stability within the presence of residual organic compounds, and efficacy at relatively low concentrations (e.g. <1 ppm ClO₂). Combining hydrochloric acid with a mixture of hypochlorite and sodium chloride or mixing a sodium chloride with a strong chlorine solution will produce chlorine dioxide in situ. Since ClO₂ gas can be explosive, the appropriate safety precautions should be considered when applying ClO₂ for microbial control of water. Ozone is another strong oxidizer employed to treat water systems for the purposes of microbial control. A major benefit to the use of ozone as a microbicidal treatment of water systems is the lower potential of corrosivity compared to other oxidative chemistries. The factors which can negatively impact the disinfecting potential of ozone are pH, temperature, and organic content. Increasing levels of any of these factors may deplete the microbicidal effectiveness of ozone within water treatment applications.

Biocides are a commonly used strategy to combat biofouling. However, in the context of spaceflight several issues must be addressed. Mass restrictions would require biocides to be effective at low concentrations, to reduce payload mass. Alternatively, biocides, notably ozone, could be generated in situ, although equipment reliability would need to be addressed. Gaseous and volatile compounds would represent a potential safety risk to crew members in the event of an accidental release. This would be relevant to the original biocide as well as any chemicals that may result from biocide interactions with microorganisms or other compounds in the water [83]. At least one study has illustrated the impact of disinfectant exposure to promoting asthma exacerbation in susceptible health care workers [84]. Material compatibility and corrosion risks are described above. While biofilms are inherently tolerant to many antimicrobials, the potential for resistance to a single compound can be mitigated by the use of strategies employing multiple compounds [83].

### 3.2. Coatings

The utilization of surface coatings to prevent biofilm formation has been widely studied across a number of application areas. A review of literature from 1968 to 2010 using the quid software package (https://quid.com/) revealed 301 publications dealing with biofilm control and/or prevention on surfaces and representative technologies are summarized in Table 3. The areas of focus included fungal control of...
Table 3

Listing of representative antibiofouling coating technologies in the literature. Strategies described include coatings used; potential for rechargeable coatings (to address need to regenerate anti-fouling surfaces); and incorporation of other strategies with antibiofouling coatings.

| Coating Characteristics | Effectiveness | Common Use |
|-------------------------|---------------|------------|
| **Rechargeable coatings** |               |            |
| Metal ions (Ag⁺, Cu²⁺, tributyl tin) [87] | Release of Ag⁺ or Cu²⁺ ions, or organic tin; and potential generation of reactive oxygen species. | Ag is used widely in biomedical and other applications. Cu is traditionally used in plumbing but susceptible to biofilm corrosion. Organic tin is an effective marine antifouling compound | Ag used widely in biomedical and other applications. Copper-containing biocides used in ship coatings. Tributyl tin is used in ship antifouling paint but has toxicity concerns. |
| Titanium alloys and mixtures [88, 89] | Couples strength and corrosion resistance of Ti with antimicrobial properties of associated metals (e.g. Ag) and other compounds. Strategy is to inhibit surface adsorption of soluble proteins and other organic molecules onto surfaces (i.e. conditioning film prevention). Conditioning films normally promote biofilms. | Antimicrobial and biofilm prevention mainly due to materials added to Ti. | Used in medical and dental implants due to bone integration (osseointegration) corrosion resistance and low toxicity of Ti. PEG polymers used in a number of clinical trials. Other polymers including glycoproteins being investigated for biocompatibility and longevity. |
| Various synthetic polymers (e.g. polyethylene glycol (PEG), poly N-vinylpyrrolidone (PVP), zwitterionic materials [90]) | Quorum signal disrupting chemicals and enzymes [91,92] | Common strategy by some organisms in nature, but larger scale studies needed to assess longevity, effectiveness, in different chemical conditions. | Some promising early results in experimental trials. Would need validation for use in long term spaceflight. |
| **Surface modification by altering hydrophobicity [93]** | Interferes with chemical interactions associated with initial bacterial adhesion. | Works well in lab situations with monocultures and defined bacterial strains. In complex chemical environments with mixed populations, not as effective. | May work in association with other technologies. |
| Silicone coatings [94] | Interferes with chemical reactions associated with initial bacterial adhesion. | Promising test results in food applications, although longevity after repeated use is not as apparent. | Promising initial trials, long term use is not apparent. |
| Slippage coatings (Lubricant-Impregnated Surfaces) [95-97] | Strategy is to reduce strength of adhesion of microorganisms to surfaces to promote detachment. | Promising in initial trials with marine systems (ships) and long-term seawater immersion. | Mechanical durability concerns as materials can be prone to shear forces. |
| Alterations of surface topography [98,99] | Several microscale alterations of surface topography (including shark skin similarities). May interfere with available adhesion points or interfere with surface mobility and bacterial aggregation. | Promising in some biomedically relevant trials with defined bacterial strains and culture conditions. | Technology has not been investigated in wastewater situation. |
| **Rechargeable coatings** | Antimicrobial characteristic capable of being regenerated by in situ chemical or physical treatment | Lab-based tests against model organisms show promise. | Based on literature, still in development stage, but notable potential |
| N-halamine [100,101] | Compounds contain nitrogen-halogen covalent bonds. Often used to coat polyurethane and other polymers. | Similar disinfecting characteristics to hypochlorite. Regenerated by exposure to hypochlorite. | Experimental trials being conducted with various polymers. Safety concerns during spaceflight with potential C₂ gas generation. Regeneration accomplished by cleaning surface with nitric acid then using AgNO₃ to regenerate silver nanoparticles [104]. |
| Silver nanoparticles [102-104] | Release of Ag⁺ ions and potential generation of reactive oxygen species. | Used widely in biomedical and other applications. | |
| **Coating coupled with other technology** | | | |
| Copper and gromming [105] | Process whereby copper-based antifouling paint used on ships and is periodically cleaned using brushing or some other mechanical treatment. | Useful in control of macrofouling (i.e. barnacles) although microbial colonization can occur | Requires access to surfaces prone to biofouling, so likely not practical for spaceflight. |
| Aeration (bubble formation) and antifouling coating [106] | Aeration provides shear forces that mechanically remove loosely adherent biofilms. | Shows promise in controlling macrofouling. Used in ships and also membrane bioreactors. | Phase separation requirements (air removal) not practical in microgravity. |
| Bioelectric or ultrasound augmentation of antifouling treatments [107,108] | Several mechanisms proposed including enhancement of biocide entry into biofilms, generation of reactive oxygen species or other electrochemically generated ions. Low intensity ultrasound enhances antimicrobial penetration, but in one study does not alter structure. | Mixed results, depending on experimental conditions. Tests include attempts to prevent initial biofilm attachment, or removal of pre-existing biofilms. | Lack of conclusive support would not merit investigations of this strategy during long-term spaceflight. |

surfaces, efficacy of surface treatments on biofilm control, prevention of bacterial adhesion to surfaces, use of anti-fouling coatings to control biofilm within marine applications, biofilm control on medical devices, prevention of bio-influenced corrosion, and biofilm control within the paper industry, heat exchangers and within spaceflight systems. Fig. 1, analyzed by quid software (https://quid.com/) illustrates the relative density of publications within each of these applications of research over the past 51 years, as well as how closely these areas of biofilm control align with each other. Interestingly the cluster analysis of this literature record reveals the studies of coatings for biofilm control within spaceflight systems are not strongly aligned with other studies in this area. Fig. 2 illustrates how the number of studies related to biofilm control through the utilization of surface coatings has rapidly increased since the mid-1990s. The publications related to biofilm control within spaceflight systems were published between 1998 and 2010 [5,19,85,86]. Interestingly, while the studies into biofilm control within spaceflight systems fell off, an increasing number of studies focused on how surface coatings might play a role in controlling bacterial adhesion to surfaces, the efficacy of surface treatments, control of bio-fouling within marine environments, the impact of surface coatings to control biofilms on medical devices and the role of coatings to reduce bio-corrosion of surfaces. Investigations of biofilm control in other environments via improved surface-coating technology may help foster new research into the control of biofilms within spaceflight systems.
Lubricant-impregnated surfaces (LIS), also known as slippage coatings, use the concept of the biofilm-resistant surfaces of the Nepenthes pitcher plant [96]. LIS incorporate both modifications of surface fine structure and the incorporation of a lubricant, so as to reduce initial adhesion of microorganisms and interfere with surface motility. In this technology a surface is roughened so that it can promote adhesion to a lubricating fluid. The lubricating fluid typically is immiscible with the liquid containing the microorganisms [97] so that it remains associated with the surface. LIS have shown promise in biofilm prevention in *P. aeruginosa* during lab culture [96,109] and the prevention of

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Fig. 1. Cluster analysis of publications on biofilm control and/or prevention on surfaces by quid software (https://quid.com/) from 1968 to 2019, showing the relative density of publications per application. This analysis indicates that studies of coatings for biofilm control within spaceflight systems are not strongly aligned to other applications.

Fig. 2. Number of studies on the use of surface coatings to control biofilms showing a rapid increase in publications in the last two decades. Safeflight systems-related studies were published between 1998 and 2010.
biofilm-associated mineral deposition [110]. Recent investigations by Goodband et al. [97] showed that smoothing of the textured surface and loss of the oil lubricant diminished the effectiveness of LIS during prolonged use. There is an ongoing study currently underway on the ISS to investigate biofilm formation on different materials including LIS under microgravity conditions [22]. Another long-term experiment, conducted on the ISS from 2011 to 2016, investigated the relative susceptibility of various treated textile and metallic materials to biofilm formation [111]. Included in these tests were materials pre-treated with rhamnolipid biosurfactants, hydrogen peroxide, or silica and silver; as well as untreated materials (controls). Bacterial exposure resulted from the materials being exposed to cabin air followed by space crew members periodically touching or breathing on the various materials. Bacterial contamination was assessed by measurements of ATP levels, qPCR, and the composition of the microorganisms determined by 16S rRNA sequencing. These authors found low levels of organisms to be present and the organisms identified included the orders Actinomycetales, Bacillales, Enterobacteriales and Lactobacillales which agrees with previous studies of ISS flora [112–114]. Interestingly, pre-treatment of the materials did not have a significant benefit in terms of microbial load, however there were modest differences in microbial communities present.

One issue that is a concern with antibiofilm coatings is one of longevity. In the case of antimicrobial coatings that release active materials, there would be a finite time of effectiveness until the concentration of the inhibitory compound dropped beneath an effective level (reviewed in Refs. [115,116]). Another issue relates to the long-term mechanical and chemical stability of coatings. Certainly, a number of coatings give extremely promising results in the short term (e.g. Ref. [115]). However, the chemical and physical stability of prospective coatings may change over a prolonged period of time and diminish effectiveness [97]. Marine fouling is a global concern for shipping, and antifouling longevity and control mechanisms historically employed a variety of toxic, biocide-based antibiofouling compounds including tributyl tin and more recently copper- and zinc-based coatings [87]. Some of these biocide compounds, notably tributyl tin, have adverse environmental impacts; and due to toxicity considerations would be unsuitable for purification of drinking water ultimately intended for human use. Fouling release coatings including silicone and fluoropolymer coatings are being explored as an alternative marine biofouling control measure. The concept behind this approach is that modifications to surface chemistry or topography (i.e. sub-micrometer-scale patterns resembling shark skin or another pattern) either inhibit bacterial surface motility (aggregation into microcolonies) or reduce strength of adhesion [93,94,98,99]. In marine applications, fouling release coatings are typically employed along with physical approaches (removing loosely adherent microorganisms with brushing or some other mechanical approach). Biofilms are a major problem in the biomedical field being associated with medical device-associated infections including catheter-associated infections. Here, toxicity considerations for the human patient as well as effectiveness against biofilms are major considerations for the use [115]. Antimicrobial coatings are used in some cases, e.g. silver-containing urinary catheters [116], although the long-term effectiveness would be reduced due to silver leaching from the catheter. There have also been developments in the use of bacterial signal disrupting molecules (including furanones, nitric oxide, and other small molecules) (reviewed in Ref. [117]). Signal disruption does show considerable promise in biofilm prevention, but current technology in this area relies on the release of inhibitory compounds from coating materials and would have associated longevity concerns. In summary, the major issues confronting the use of coating technology involve the need for effectiveness over the anticipated length of the mission (3–5 years) as well as toxicity issues of released compounds in potable water, and chemical compatibility of the technology with other components of the WRS.

3.3. Ionizing radiation

Ionizing radiation, primarily ultraviolet (UV) light, has been used for some time to control microorganisms in wastewater [118]. With increasing drought and human population, a number of regions are now beginning to employ wastewater recycling as a key component of municipal drinking water. Aside from being used for non-potable uses such as crop and parkland irrigation, some recycled water is being used for potable water [119]. As well, UV light is used in many broad-distribution and also point of use systems [120]. UV light is now frequently produced by UV-light-emitting diodes [120,121] and the most effective wavelengths ranging between 200 and 300 nm [120,122]. Nucleic acids absorb UV light around 260 nm [123] and one mechanism of cellular damage is the formation of cyclobutane pyrimidine dimers, notably thymine dimers that form between adjacent thymine residues on a single strand of DNA. Other UV-induced photoproducts also include binding of adjacent thymine and cytosine residues [124], as well as the formation of reactive oxygen species which can damage other cellular components and even result in small numbers of double-strand DNA breaks [125]. While bacteria do possess mechanisms for DNA repair, notably photoreactivation [124] and other repair mechanisms that do not require light but may be error-prone [125]; excess damage to nucleic acids and other key cellular components is lethal.

Higher energy ionizing radiation, notably gamma radiation, is used commercially as a sterilant, although considerable shielding is needed to protect humans working in the vicinity [126]. The higher energy of gamma radiation induces double-strand DNA breaks, damage to key proteins and lipids and generation of reactive oxygen species, all of which contributes to lethality [127]. A radiation dose of 25 kGy (2.5 Mrad) is used as a representative sterilization dose for materials to be used in a number of medical applications [128].

A number of factors influence the ability of ionizing radiation to reduce microbial populations. Turbidity certainly interferes with UV light penetration [118]. Organisms also vary in their capacity to repair radiation-induced DNA damage [127], so in that context it is not surprising that Hu et al. [129] observed a population shift in wastewater that was disinfected with UV. During spaceflight above the protective ozone layer of the Earth’s atmosphere, solar radiation would be present, and may represent a natural source for disinfection. Table 4 describes series of experiments performed on the International Space Station (ISS) between 2008 and 2016 which examined the ability of various microorganisms to survive extraterrestrial UV radiation (summarized in Ref. [130]). During these experiments, organisms previously shown to be radiation resistant were exposed to solar radiation in low Earth orbit for 469 days (reviewed in Ref. [130]) and then returned to Earth for analysis. During the duration of the experiment, the UV flux (λ = 100 nm in one condition, and λ = 200–400 nm in a second condition) was estimated between 4.58–4.92 x10^2 kJ/m² and 0.5 Gy of cosmic radiation [131]. In the first experimental condition, the organisms were exposed to space vacuum. In the second condition, organisms were exposed to simulated Mars light and atmospheric conditions. While biofilms did offer additional protection, the mechanism of the protection is not fully understood and may be due to altered pigmentation, matrix composition, or some unidentified mechanism [130]. Finally, in contrast to many biocides, UV does not have a residual effect in wastewater, meaning that biofilm growth is expected to continue in any region not directly exposed to the UV light.

In summary, ionizing radiation represents a potential mechanism whereby microbial populations could be reduced. However, a number of organisms have been shown to be resistant to UV-flux even under exposure to solar radiation (Table 4). There is at least one report showing sublethal doses of UVA (λ 365 nm, 25 W m^−2) enhanced biofilm formation under some culture conditions in P. aeruginosa PAO1 [134]. As a result, radiation if considered, would likely need to be combined with another approach such as biocide application for biofilm control.
biofilm detachment

During the formation of biofilms, bacteria go through several developmental stages (reversible and irreversible adhesion, aggregation and maturation, and finally dispersion) (reviewed in Refs. [135–137]) and there is evidence that biofilm-associated antimicrobial tolerance occurs at an early stage of biofilm development [138,139]. Biocontrol strategies typically address the first stages of biofilm formation (i.e. interfering with adhesion and aggregation) or involve various antimicrobial compounds (biocides and antibiotics) to combat established biofilms (Tables 2 and 3, addressed above). Adhesion interference strategies involve surface modification to reduce bacterial adhesion and coalescence into aggregates (biofilm microcolonies), or the development and testing of various antimicrobial compounds to combat established biofilms (addressed previously). One new approach that is being explored for biofilm control is an approach geared towards inducing biofilm detachment (final stage of the biofilm life cycle) [136,140–142]. When organisms leave biofilms and reenter the planktonic growth phase, antimicrobial susceptibility returns, although the rate of decline in biofilm-derived tolerance depends on the individual organisms as well as the process by which organisms leave biofilms (e.g. sloughing of cell populations, fragmentation of biofilms into individual cells, etc.) (reviewed in Refs. [136,143]). Both nutrient-based detachment stimuli [144–146] and specific detachment signals [147–149] have been proposed. While supplementation of some nutrients (e.g. succinate) have stimulated detachment in Pseudomonas aeruginosa [150], of more relevance to the proposed space mission is the potential role of starvation as several studies show that starvation induces detachment of biofilms (reviewed in Refs. [136,145]).

Biofilm detachment signaling is different from other types of signaling, notably quorum signaling (which is associated with biofilm formation) [136,151]. A key issue in biofilm physiology and associated genetic regulation is that the role of a second signaling system, bis-(3’-5’)-cyclic dimeric GMP (c-di-GMP) [152]. During biofilm formation c-di-GMP levels become elevated approximately 3–4 fold when compared to planktonic cells due to an increase in diguanylate cyclase activity [153,154]. Among other things elevated c-di-GMP is associated with antimicrobial tolerance in biofilms and a loss of flagella. During detachment c-di-GMP levels are reduced by the activity of phosphodiesterase and as a consequence, bacteria within biofilms begin to lose antimicrobial tolerance, form flagella, and degrade the biofilm matrix prior to reentering the planktonic population. Most of these experiments have been performed under laboratory conditions and often employ monocultures. The advantage of a detachment-based strategy is the reduction of biofilm-mediated tolerance, while enabling current antimicrobial therapy. To the knowledge of the authors, no detachment experiments have been conducted during spaceflight (microgravity) or in microgravity analog experiments. Given the early stage of detachment research, it is likely premature to consider deliberate promotion of detachment as a biofouling control mechanism for extended spaceflight.

The most probable issue of biofilm detachment and population change is likely to occur during the dormancy phase. During the dormancy phase of a proposed spaceflight mission, exogenous nutrient input from space crew wastewater would cease, and the microbial community would initially rely on endogenous nutrients (use of metabolites and dead microorganisms) along with altered physiology, and then enter a period of starvation [155]. One would also anticipate that the population composition and physiology of both the biofilm and planktonic communities would change due to the stagnant water present during dormancy. Aside from nutrient limitation, oxygen consumption by microbial communities would potentially generate an anaerobic environment and alter microbial community composition, as has been shown in a domestic drinking water environment [156]. Detached biofilms may represent a potential clogging concern when ECLSS is reactivated following dormancy.

3.5. Biocontrol of biofilms

Control of microorganisms in anthropogenic water handling systems has been conventionally performed by the application of chemical biocides. However, with increased restrictions coming into place on the use of biocides and preservatives for industrial applications, there is an increasing interest into looking at naturally occurring or greener biocides. Biological control is defined as the “use of a living organism to depress the population of an unwanted species or pest” has been practiced in the macro-biology world for many years [157,158]. But now there is a renewed interest in utilizing non-corrosion inducing and low slime producing microorganisms to combat the proliferation of other species that are considered harmful or damaging.

i. Predatory Bacteria. Bacterial predators such as Bdellovibrio and Ensifer have evolved a very unique survival strategy in which they obtain energy and other biosynthetic materials by taking them from other living bacteria. Sometimes described as a living antibiotic, they are considered a potentially safe alternative to antimicrobials for agriculture and water treatment applications [159,160]. There are four steps that must be completed for a bacterial cell to attack and consume another cell: (i) The predator bacteria finds its target prey, either through a chemotaxis mechanism or because the population of prey bacteria is sufficiently large so as to result in random collisions between the cells, (ii) the predator cell undergoes an irreversible interaction with the prey cell, (iii) the predator cell begins to degrade the prey cell by releasing specific macromolecules, (iv) the predator cell assimilates the released macromolecules which are used as nutrients in a specific and beneficial manner [161]. Similarly, there are different strategies for predation: (a) wolfpack or group predation, in which a number of predator cells release hydrolytic enzymes that degrade the cells of the near-by prey bacteria [162], (b) epibiotic, individual cell to cell attack in which the predator bacteria attach to the outer surface of the prey cell, which assimilates the host molecules, (iii) direct invasion, where the predator bacteria enter the prey cell cytoplasm in a process called diacytosis, and (iv) periplasmic, where the predatory bacteria invade and grow within the periplasmic space found in Gram negative cells [161,163,164].

Unlike chemical biocide programs, researchers have shown that
predatory bacteria actually target prey bacteria in biofilms. For example, in the treatment of periodontitis, predatory bacteria were found to target and remove oxygen tolerant bacteria in the superficial layers of the biofilm and in the process expose anaerobic microorganisms deeper in the biofilm, making them susceptible to predatory attack [159,164]. The high bacterial densities within biofilms represents a very rich hunting ground for predatory bacteria. It is also interesting to note that in the Silva et al. [164] study, bacteria associated with gum health remained unaffected by the treatment. An additional advantage for the use of predatory bacteria for biocontrol is that the predatory bacteria can be non-pathogenic to humans.

Though predatory bacteria are viable for specific terrestrial applications, their usefulness for the NASA water treatment system may be limited. Predatory bacteria may not be effective against the entire microbial consortium in the WPA waste tank; thus, the remaining bacterial or fungal species would likely continue biofilm growth without competition from other microorganisms. A second consideration is whether predatory bacteria would maintain a functioning population in the WPA or if these organisms would need to be replenished. A third consideration is a question of whether predatory bacteria would also establish biofilms or enhance biofilm growth by prey bacteria. These considerations do not support the concept of biological control during spaceflight by predatory bacteria.

ii. Bacteriophage. A bacteriophage (phage) is a virus that infects and kills bacteria [165,166]. The bacteriophage attaches itself to a very specific site on the target bacterial cell wall and infects the host cell by injecting its DNA [167]. In doing this, the bacteriophage hijacks the host cellular machinery forcing it to make viral components, which ultimately form new bacteriophages. In the lytic cycle, new bacteriophages then lyse the cell, burst out of the host, and infect other bacteria. In terms of biofilm control and prevention, bacteriophages are known to have three different mechanisms. The first is a process where proteins known as EPS depolymerases are produced by the bacteriophage [168,169]. These enzymes break up the biofilm matrix through a chemical disruption mechanism. The second process involves bacteriophage infection of the bacteria within the biofilm causing direct cell lysing. The third process is where the cell walls are lysed as a result of the adsorption of phage virions and the onset of the phage lytic cycle [170].

While it has been shown that bacteriophage-induced lysis of targeted bacteria has the potential to break up biofilms, there are a number of limiting factors that may hinder this technology from being viable for NASA’s biofilm control applications. Bacteriophages are generally specific to only one species of bacteria [171] so with biofilms that contain a large variety of different microorganisms a bacteriophage treatment would require a cocktail of bacteriophages to target each bacterial species in the biofilm [170]. Additionally, bacteriophage preparation requires culture of the phage with their host followed by a separation of bacterial cell remnants from the bacteriophage of interest. Phage titers can be measured using a bioassay (plaque-forming assay) or via quantitative PCR using phage-specific primers [172]. With the increasing onset of antibiotic resistance, phage therapy is being reexamined and would particularly need to be explored in a variety of environments (including wastewater), microbial growth conditions (bacteria tend to be more susceptible during active growth) and microbial populations including biofilms [173]. While on advantage of a phage is that it would replicate and so be able to reinfect their hosts, it is unclear whether resistance would develop. Resistance is certainly probable, given that bacterial biofilms and associated phage have co-evolved over several billion years [174].

iii. Amoeba and other protozoa. Amoeba are eukaryotic microorganisms whose most body most often consists of a single cell. Similar to other eukaryotic cells, their cytoplasm and cellular contents are enclosed within a cell membrane and their DNA is packaged into a central nucleus. Amoeba are known to consume bacteria and biofilm through a process known as phagocytosis. In this process receptors on the amoeba cell surface attach and bind to bacteria and are gathered and ingested within the amoeba. Larger amoeba will actually engulf their prey by gathering their pseudopods around the bacteria and ingest it in a process known as pseudopodia [175]. Other protozoa also ingest bacteria, although the specific mechanisms of ingestion may differ from those in amoeba [175,176]. As many protozoa routinely prey on bacteria, including biofilm-associated bacteria, there has been renewed interest in exploring amoeba and other protozoa as a biofilm control strategy. While this concept is certainly appealing several issues must be considered. Some bacteria, notably Legionella pneumophila and Stenotrophomonas maltophilia have evolved mechanisms to persist within amoeba and presumably other protozoa wherein they can gain access to nutrients within the amoeba cytoplasm while inhibiting the host cell enzymatic digestive processes [177,178]. Several investigators have examined biofilm susceptibility to protozoa and other bacteria-ingesting organisms in monoculture and polymicrobial settings (e.g. Ref. [175,179,180]). While there has been some promise in pure culture (monoculture) lab trials, the results under more natural conditions are not as encouraging as individual microorganisms vary as to their susceptibility to predation by protozoa and other organisms. As well, bacteria, including those associated with biofilms, have been shown to evolve quickly and so the onset to increased protozoan resistance is certainly a possibility [180,181].

iv. Tardigrades. Tardigrades, commonly known as water bears, can be found in almost every habitat on Earth [182]. They are a phylum of small invertebrates that feed on the fluids from plant cells, animal cells, and bacteria. Their small size and relative ease to culture and obtain offspring make these hardy creatures an interesting target for biocontrol, particularly as at least two studies have shown their ability to survive radiation and temperature extremes during spaceflight [182,183]. A recent study showed that some metabolically-active tardigrades are heat sensitive [184]. However, there is limited research and development in the area of biofilm control and a thorough investigation into the use of tardigrades as a viable biofilm control strategy would need to be performed.

v. Probiotics. It is estimated that about 4 million adults in the United States use probiotics each month. Probiotics live microorganisms intended to have health benefits but not induce disease, when consumed or applied to the body [185,186]. In the gastrointestinal tract, several strains of Bacillus, Bifidobacterium, Escherichia coli, Lactobacillus, and Propionibacterium have been used as probiotics (reviewed in Ref. [185]). Some probiotics, such as Lactobacilli reuteri, can produce a variety of compounds including reuterin and H2O2 that can react with biofilms, changing their structure and viability [187]. Other compounds produced by probiotics and normal flora can act as signaling molecules, which in some cases can promote biofilm growth. Examples of biofilm-promoting signals include quorum signals (QS) such as N-acylated homoserine lactones (QS in many gram negative bacteria) [188], small peptides (QS in many gram positive bacteria) [189], and autotoxins (considered to be universal in both gram positive and gram negative bacteria) [190], and polyamines [185]. However, the use of probiotics to control biofilms in water treatment system has not yet been systematically interrogated [187].

Each type of biocontrol has its limits and advantages, as summarized in Table 5. Further research will need to be done to evaluate the efficacy of these strategies and their potential for space applications. Biofilms are
now considered to be a very ancient form of life as they are associated within stromatolites from which fossils exist in the Precambrian era [191, 192]. From an evolutionary standpoint, organisms within biofilms have co-evolved with organisms (bacteriophage, other bacteria, eu-

Table 6
Examples of types of biocontrol and associated advantages and disadvantages.

| Type of Biocontrol | Advantages | Disadvantages |
|--------------------|------------|---------------|
| **Predatory**      | Rapid growth | Generally only attack Gram negative bacteria |
| Bacteria           | Effective at removing resistant bacteria | Not very active under anaerobic conditions |
| Bacteriophage      | Non-toxic, many are well characterized | Extremely specific |
|                    | Many produce EPS-degrading enzymes | Laborious culturing process |
| Amoeba and other protozoa | Preferentially thrive on bioms | Ineffective against amoeba resistant bacteria |
| Tardigrades        | Some may be extremely stress tolerant | Limited biofilm associated research available; stress tolerance is not conclusive |
| Probiotics         | Well studied, effective at attacking health-related biofilms | Have potential to enhance new biofilm growth |

The concentration of the wastewater (Table 6) can provide some insight into the amount of biomass that could be produced. The influent water stream contains organic carbon in the form of low molecular weight chemical species such as ethanol, acetic acid, 1,2-propanediol, and lactic acid [9]. These are all fine carbon sources to support micro-

Table 6
Simplified approximate composition of water processor assembly (WPA) influent wastewater.

| Constituent                  | Concentration (mg/L) | Concentration (µM) | Carbon to Element Molar Ratio |
|------------------------------|----------------------|--------------------|------------------------------|
| Total Organic Carbon (TOC)  | 114                  | 9500               | 1.00                         |
| Ammonium (as N)             | 27                   | 1930               | 4.9                          |
| Triethyl phosphate (as P)   | 0.083                | 2.68               | 3500                         |
| Sulfate (as S)              | 0.48                 | 15.0               | 630                          |
sweep flocculation the coagulant of choice is overdosed resulting in large amorphous floccs that encapsulate and capture not only the BDOC but microorganisms, heavy metals, and other contaminants. Enhanced coagulation, on the other hand, uses precise additions of the traditional coagulant to neutralize the charges normally present to keep the particles apart. Once these charges are neutralized the particles are encouraged to flocculate through agitation causing the particles to agglomerate so they can be removed by filtration or clarification [205]. Optimized coagulation is a technique similar to enhanced coagulation in which operational parameters such as pH control or the addition of bi-metallic nanoparticles along with polymer coagulants are added to maximize the efficiency of the coagulation process [206]. While all three methods have been shown effective the latter two are considered the most efficient.

The most common type of water treatment plant design is based around the “conventional” system where coagulation is followed by flocculation, settling, and finally filtration. While this method is effective at removal of turbidity, pathogens, and BDOC it is chemically intensive and is not suited to a microgravity type environment due to the requirement of gravity-assisted settling. Dissolved air flotation (DAF) is similar to the conventional process but the clarification process is accomplished using air to force flotation of the floc instead of sedimentation. Again, this method does not lend itself readily to a microgravity environment [207] since gravity is required for floc flotation and phase separation of the air bubbles. Finally, direct filtration can be used to clarify the water. In this process the treated water is passed through size exclusion media filters to remove the flocs. Unlike the other two methods direct filtration could be configured to be microgravity compatible. This method uses fewer chemical reagents than conventional systems as smaller floc sizes can be removed; however, filter fouling could be problematic, and this method might be questioned as increased backwashing of the filters would be required.

iii. Membrane Filtration. Membrane filters are common in the water treatment industry and they can be used in conjunction with the coagulation flocculation process or as a stand-alone treatment without any additional chemical treatment. There are 4 types of membrane processes: (i) Microfiltration (MF), (ii) Ultrafiltration (UF), (iii) Nanofiltration (NF), and (iv) Reverse Osmosis (RO). Both microfiltration and ultrafiltration are considered to be low pressure processes. MF filters have pore sizes ranging from 0.1 to 0.2 mm while UF filters have pore sizes ranging from 0.01 to 0.05 mm. The pores in the MF membrane are too large to reliably remove BDOC but UF membranes can remove some of the larger molecular weight BDOC compounds (20,000–100,000 MW) from water. These systems generally have a small footprint and low energy consumption; however, the inability to adequately remove BDOC makes them inadequate for effective nutrient removal. NF membranes and RO membranes are considered high-pressure and both are defined by their molecular weight cut off points. Both of these filter membrane systems have adequate pore size that can completely eliminate the passage of BDOC as well as metals and turbidity. However, they are easily fouled by both organic, inorganic, and biological mechanisms (i.e. biofilms) and are energy intensive while in operation [204].

iv. Oxidation Based Systems. Chlorine based oxidants, ozone, permanganate, and air are common oxidants used in the water treatment industry. While these chemicals primarily have been used to control the planktonic bacteria in the water, they will also oxidize larger particulate BDOC to smaller particles and molecules that can then be removed by filtration, slow sand filtration (which typically has associated biofilms [208]), or on activated carbon beds. The use of chlorine as an oxidant is considered problematic because of the potential to form harmful trihalomethane compounds. Ozone is a possible alternative to chlorine. It is a strong oxidant that will attack and break the double bonds in the more hydrophobic and aromatic BDOC species in the water. However, ozone is particularly difficult to handle in a microgravity environment as it requires an effective dispersion of microbubbles within the water to be effective [209]. It is also very reactive with rubber and yellow metals making it incompatible with the water processing and storage systems slated to be used on current and future space expeditions. Advanced oxidation processes (AOP) make use of the very reactive properties of hydroxyl radicals that are formed by the photocatalytic breakdown of ozone or hydrogen peroxide. UV-H2O2 systems have been shown to be more effective at eliminating BDOC than ozone treatment alone especially when used with an activated carbon filter. This method could be used in a microgravity environment; however, because of the short shelf life of hydrogen peroxide it would require an on-demand hydrogen peroxide generator and these systems are not currently commercially available.

v. Adsorption and Ion Exchange. There are numerous adsorbents that have been developed for the water treatment industry. These media work by attaching the species of interest to their surface through intramolecular forces. Granulated activated carbon (GAC) is known to be effective at removing most organics from water; however, pH and ionic strength of the solution can impact the efficacy of the process. For example, it was discovered that at pH 3 more BDOC will be adsorbed onto a GAC filter than will be adsorbed at pH 7 [210]. Adsorbents also tend to have limited capacity for the low molecular weight organics that are the primary nutrients for biofilm growth. Ion exchange is often referred to as “softening” and it works by preferentially replacing one ion from the water phase for another that is bound on the resin surface. Many of the organic species found in water are anionic in nature (they contain a carboxylic acid structure) and they can be removed by an anionic exchange resin. Once exhausted these resins are typically replaced with fresh resin, though they can also be recharged through the addition of salt allowing them to have a longer media lifetime. In recent years, a magnetic ion exchange resin (MIEX) system has been developed that uses beads rather than a traditional resin [211]. The presence of the magnetic beads
allows them to be easily removed and regenerated in a salt solution. While very effective at removing charged organics and inorganics from water, it is ineffective for uncharged compounds. Therefore, ion exchange would be used for removal of nitrogen, phosphorous, and sulfate-containing inorganic species that contribute to biofilm growth.

vi. Removal of Nitrogen and Phosphorous Containing Compounds. Table 8 lists the different techniques and methods that can be employed to remove nutrients such as phosphorous and nitrogen containing compounds. As can be seen in Table 8, many of the methods are similar to those described for the BDOC removal. Although chemical accumulation and precipitation are very effective for high phosphorous removal in terrestrial wastewater treatment, they are not appropriate for microgravity applications as settling would not occur. NF and RO membranes have been shown to produce concentrated nutrient effluent streams with over 80% retention of ammonia and nitrate in the concentrate.

One area of research and development that has gained considerable momentum is that of solid phase adsorbents for nitrate removal. Solid phase denitrification can be broken down into one of two processes, (i) heterotrophic denitrification, where bacteria use an external carbon source such as saw dust, wood chips, straw, and even dead and lysed cells as electron donors while nitrate functions as the electron acceptor source such as saw dust, wood chips, straw, and even dead and lysed cells, and (ii) autotrophic denitrification where the microbes fulfill their energy requirements by reducing nitrates using inorganic compounds as the electron donor species [212–214]. Researchers have demonstrated that using novel inorganic materials such as Mg/Cu bimetallic particles, nano zero valent iron (nZVI) and immobilized Pd/Cu catalysts when used in conjunction with adsorbents, and in some cases ion exchange resins, can improve nitrate reduction when compared to the system without these inorganic additives [215–218].

Biofiltration (reviewed in Ref. [208,219]) represent a mechanism whereby organic material and associated nutrients are removed by microbial communities growing as biofilms on a supporting matrix. Biofiltration may be coupled with other treatments such as ozonation to enhance the efficiency of the process [208]. During operation, microorganisms metabolize organic carbon and assimilate other nutrients including nitrogen and phosphorous, which leads to an increase of biomass (i.e. biofilms) that can clog the filtration system. Normally, the clogging is addressed by backwashing wherein the flow is reversed through the biofilter. Backwashing will remove some of the biomass and regenerate the filtration capability [220]. While biological assisted wastewater treatment including biofiltration is worthwhile investigating in the context of a long-term low gravity environment such as a future Moon or Mars base (addressed below in section G), the generation of biomass from backwashing or simple promotion of growth would create additional engineering problems in the context of space flight and is not a practical approach at present.

Bioelectrochemical systems represent another emerging technology for waste treatment and nutrient removal. The overall concept is that many metabolic reactions involve oxidation and reduction activities, and can be exploited to either generate a current, or else use an applied current to promote desired metabolic activities (reviewed in Refs. [221–223]). One example is the Enhanced Biological Phosphorous Removal (EBPR) process [222]. A key component of this process is the use of phosphate-accumulating organisms (PAO), which have the ability to sequester excess phosphate as intracellular polyphosphate granules in their cytoplasm [224]. The EBPR is an activated sludge process that relies on the ability of the PAO to take up, transform and store phosphate inside the cells. In these bioelectrochemical systems, iron released from the electrode and the electrochemical process frees up insoluble phosphates that are consumed by the PAO’s contained in the activated sludge portion of reactor system, eliminating the phosphates from the water. Other methods to improve on the EBPR process as well as the denitrifying process is to combine microalgae with the bacterial consortium. This process allows for the biological removal of turbidity, nitrogen, phosphorous, and BOD/COD. This technology is suited to large industrial type applications where the algae can be grown in large ponds and fed to the treatment system when needed [225–227].

With magnetic separation adsorption materials tagged with magnetic particles are used as the collection and carrier material for removal of nitrates and phosphates in water. These adsorption materials are the same as the ones (GAC, MIEX) described in the previous section. High gradient magnetic separators are used to sequester the nutrient laden magnetic particles from the solution. These systems have been shown to be 90% effective at phosphorous recovery and do not interfere with other biological processes. However, this technology is still in the developmental phase and there is limited published literature available to fill in the knowledge gap.

In summary, removal of nutrients from water has the potential to reduce the formation and proliferation of biofilms in anthropogenic water handling systems. However, this approach has to be continuous and implemented as part of a long-term strategy. While for terrestrial applications these nutrient removal technologies could have huge environmental impact, for spaceflight applications the generation of waste and the high use of consumables, including water might make these technologies an impractical approach for biofilm control.

3.7. Other strategies

During the space biofilm symposium, several additional biofilm-related ideas were proposed. Many of these proposals involve considerable engineering challenges and payload requirements and as a result would be more appropriate for planned bases on the Moon or Mars, rather than being employed in spacecraft. One suggestion includes incorporating microorganisms into ECLSS. This would facilitate nutrient removal during wastewater recycling. If photosynthetic organisms were involved in this process, it would also contribute to oxygen generation and possibly food production. An example from the biological perspective, touched on in the previous section, is to use organisms (e.g. photosynthetic algae or bacteria) for nutrient removal. At least one genus of algae, *Chlorella*, is being investigated as an easily cultivable food supplement [228–230]. This could have additional positive results, such as CO₂ removal, O₂ production, and generation of edible biomass. Large-scale experimentation with biological nutrient cycling (e.g. Biosphere 2 and bioregenerative life support tests) has been done (reviewed in Refs. [231,232]) on Earth, and at the least feasibility studies would need to be performed in a low gravity condition (i.e. Moon base) prior to consideration for a potential trip to Mars. Another strategy

| Table 8 | Nutrient accumulation technologies for P and N removal from water. |
|---|---|---|---|---|---|
| Nutrient Accumulation | Engineering Feasibility | Technology Maturity | Operability | Operating Cost | Safety Issues |
| Algae Accumulation | Med: complex technology | Med | High | Low | Low |
| EBPR Accumulation | High | High | Med | Low | Low |
| Chemical Accumulation | High | High | High: chemical required | Low | Low |
| Adsorption/Ion-exchange | Med | Low | Med: require adsorbent | Low | Low |
| Membrane Filtration | High | Med | High: membrane clogging and cleaning cost | Low | Low |
| Magnetic Separation | Low | Low | Low | Low | Low |
considered ‘embracing biofilm growth’, i.e. not only allowing biofilm formation but planning for it by inoculating tanks and water systems with a known microbial species. The concept of embracing biofilms is analogous to biofiltration (reviewed in Refs. [208,219]), addressed previously. If inoculation with a specific species or microbial community were to be investigated, notable criteria for the selection would be safety concerns for space crew and the strains that would be non-pathogenic and not have a deleterious effect on equipment. However, biofilms in nature tend to be polymicrobial, so the initial biofilm community could serve as the anchor or stimulus for other strains to attach to the equipment surface. Additionally, the presence of biofilms, regardless of the organisms that form it, would still elicit engineering problems for equipment downstream – e.g. valve clogging, biofouling, potential corrosion, etc. – that would need to be addressed. Given the engineering concerns (fouling, corrosion, etc.), the most reasonable strategy for spacecraft is to contain biofilms rather than promote them.

Alternative engineering and operational approaches may be implemented to mitigate the risks derived from biofilm buildup in spacecraft, namely on water processor assemblies. One strategy to consider is to maintain the components most prone to biofilm formation, e.g. wastewater tank and valves immediately downstream, at temperatures as close to 4 °C as possible. While temperatures below freezing could be achieved, there would be a major risk of equipment damage (notably leaks) due to ice formation and expansion. This approach could result in reduced microbial proliferation, although multiple psychrotolerant bacterial and fungal species can still grow, albeit slower, in these conditions. Nevertheless, this approach may reduce biofilm formation to a level where it is less complicated to handle. Another strategy revolves around the idea of having regenerable or at least readily exchangeable filtration systems installed between the wastewater tank and the first valve downstream. However, a viable approach may be to implement a solution in which the biofilm growth is properly monitored, managed, and contained, thus preventing the release of biomass that can impact the mechanical function of the system. Indeed, ongoing experience with the WRS in the ISS provides valuable data for longer term missions.

Specifically, to the case of the Mars transit vehicle architecture, where ECLSS will stay dormant for a long period of time, in the event that a biofilm-prone component can be identified, one concept would be to replace the item. Using the wastewater tank as one example a novel ‘two-flexible bags’ approach to the wastewater tank is proposed as described in Fig. 3. By the end of the journey to Mars, the original wastewater bag (Bag 1) would remain full, acknowledging that biofilm will grow. After the period of dormancy and prior to return to Earth, the contents of the original wastewater are diverted into a filter that until this point had not been exposed to the contents of the tank, and flushed into a new wastewater bag, having biofilm constituents collected in the new filter (steps B and C). The new wastewater bag (Bag 2) would now contain mostly bacteria-free water. With this approach, the added volume of an empty and a full bag would be similar to that of one hard-shell tank. Challenges that would need to be addressed to implement this approach include pressure rating of the bags to withstand potential biological gas formation and fully disable gas permeating through the bag’s walls.

4. Next steps for research and development

Access to data enables informed decisions and, in the case of biofilm problems in NASA’s water treatment systems. Ongoing experience with the ISS has resulted in the identification of wastewater components (Table 6) and cultivable microorganisms (Table 1). One thing that is not known at present is the changes likely to occur during dormancy. Questions that would need to be addressed are:

i) Would changes in microbial community composition and physiology during dormancy and associated stagnant wastewater [233] affect key ECLSS components either directly or by generating an altered chemical environment?

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**Fig. 3.** Schematic and concept of operations of a proposed ‘two-flexible bags’ approach to the wastewater tank for future ECLSS. Instead of one hard-shell tank, this approach uses two flexible bags, where only one is completely full at any given time. Bag 1 is used for the Earth-to-Mars and Mars-orbit phases of mission (A) similarly to how the current wastewater tank is currently utilized. Before return to Earth, the contents of Bag 1 are transferred through a filter into an unused Bag 2 (B), resulting in the emptying of Bag 1 and the filling of Bag 2 (C), and the collection of biofilm in a filter that will no longer be used (vertical filter in schematic). The Mars-to-Earth mission phase uses the new Bag 2 as wastewater tank (D).
ii) Could the microbial issues be alleviated by enhancing biocide exposure either prior to or following dormancy; or alternatively draining susceptible valves and filter units prior to dormancy?  

iii) Would changes in the microbiome and associated microbial physiology and metabolites occur in individual key components of the WPA prior to and during dormancy? This information would help identifying potential issues of concern.  

iv) Would starvation induce biofilm detachment, and would this require additional filtration for released biomass during the ECLSS startup processes for a return flight to Earth?  

v) Does prolonged growth in microgravity and anticipated increased radiation levels beyond low Earth orbit [234], affect biocide susceptibility of biofilm organisms?  

vi) Would lowering cabin temperature during dormancy represent a potential strategy for decreasing microbial activity? Obviously, care would be needed to avoid damage due to freezing.  

vii) Would the WRS return to an appropriate functioning level following dormancy, and would any specific measures (possibly equipment repair, filtration, chemical treatment) be needed to restore function (also mentioned in point iv, above)?  

New engineering and operational approaches are recommended to be assessed for their effectiveness and impact on biofilm (a) detection, (b) formation inhibition, (c) detachment, and/or (d) filtration. We here describe them by posing key questions that need answers:  

(a) Detection. How can we detect biofilms in tanks and other regions of the WPA as they are forming? How can we differentiate microbial presence in biofilms, from planktonic growth and can this data be used for assessing and adjusting biofouling control strategies and identification of potential problems?  

(b) Formation inhibition. Which of the biocide options here described is most efficient and has the least impact on other WPA processes and in engineering requirements? What is the minimum biofilm inhibitory concentrations (MBIC) of these options in microgravity? Which surface coatings are the most efficient for this specific application, and for how long do they retain their functionality? Would an ionizing radiation approach be worth employing in addition to biocides for controlling microbial populations? Is cabin temperature reduction (e.g. to 4 C) during dormancy a viable option to slow biofouling? Finally, which method or combination of methods would be most effective in controlling biofouling, yet preserve the function and integrity of the WPA?  

(c) Detachment. Would a programmed biofilm detachment strategy be appropriate? If so, would a signal-based approach or alternatively a combination physical (e.g. vibroacoustic, brushing or equivalent physical treatment) and biocide approach be warranted and if so, what parameters should be used?  

(d) Filtration. Is filtration a viable option for controlling biomass including biofilms in the WPA? If so, what would be the mechanisms needed (e.g. filter location, filter pore size, monitoring and replacement schedule during outbound flight, dormancy, startup, and return flight to Earth)?  

(e) Equipment repair or replacement. Is equipment replacement a viable option to address biofouling concerns during dormancy (example of wastewater tank is described in Fig. 3 and adjoining text)? Aside from the tank, are other components identified as “at risk” for biofouling damage, and if so, should replacements be carried?  

5. Conclusions and future directions  

Biofilms are certainly present on human-occupied spacecraft and have been associated with problems associated with life support and other equipment. Biofouling problems are not exclusive to systems operation on Earth, as the Russian Salut 6 and 7, and Mir space stations, as well as the ISS had engineering challenges arise from biofilm buildup [8–10,21,22]. In the case of the ISS, the WPA has presented the most pressing challenges that need to be addressed before human exploration moves forward to Mars and beyond. In these extended duration missions, there are planned periods of ECLSS dormancy, where wastewater will stay stagnant in a tank for periods of months or years. While biofilms could certainly impact other aspects of a spacecraft, the primary focus of the NASA-Montana State University biofilm workshop and this review paper is on biofilm control strategies in the WPA during long-duration space flight.  

The microorganisms associated with the WPA in the International Space Station is frequently monitored (addressed in Section 1 and Table 1) and there have also been some culture-independent studies performed as well (e.g. Ref. [11,12]). Previous biofilm studies performed in space, have shown differences in morphology, sensitivity to disinfectants, viability, biomass, and cell counts compared to matching Earth controls. With the exception of the Biofilm Surfing in Space (BOSS) studies Table 4 (reviewed in Cottin and Retberg [130]), most spaceflight biofilm studies have been conducted over short periods of time with a small number of model microorganisms that have typically been grown in monoculture. While these studies described in previous sections are beneficial, a key research requirement is understanding the changes that may occur in the WPA flora during planned dormancy and startup procedures. A recent report on the ISS by the National Academies proposed the development of a microbial observatory as a high-priority research item [235] and certainly long-term changes in biofilm communities related to space crew health, life support systems and spacecraft integrity in the unique spaceflight environment would be a key beneficial scientific objective. Given payload restrictions during a potential Earth-Mars flight, the use of biocides for controlling WPA biofilms appears to be the most relevant technology to be considered and details are presented in section 4, items i-vii. Related engineering and operation issues are also addressed in section 4.  

While this article focuses on biofilm-related problems and control strategies that impact an Earth-Mars transit vehicle operating under microgravity conditions; biofilm-related issues would also be relevant to life support systems and other facilities during an extended stay on Mars or on the Moon. Microgravity analog devices (described in the introduction) greatly facilitate the number of investigations that can be done. Biofilm growth and biofouling would be anticipated in partial gravity conditions (Moon 0.17g; Mars 0.38 g) [27], but detailed experiments would need to be conducted in order to identify potential risk factors and mitigation strategies. Modeled partial gravity can now be modeled on Earth using RP device technology [27] and RWV (clinostat) technology [236–239], but longer-term tests would await in situ lunar testing. The rapid advancement of technology allows an increasing number of rigorous experimental protocols and equipment fabrication to be done during flights, which enhances the crew flexibility during prolonged mission. Two examples are gene sequencing [240] and three-dimensional printing (3D printing) [241]. We anticipate that ongoing technical and engineering developments along with biofilm research will enhance the success of future extended, crewed, space missions.  

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