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Validation of the International Space Station Smart Sample Concentrator for Microbial Monitoring of Low Biomass Water Samples

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Microbial monitoring on the International Space Station (ISS) is essential to keep astronauts healthy. Current practice involves culture-based methods, but future directives by the National Aeronautics and Space Administration (NASA) will require the use of molecular-based approaches, such as quantitative PCR (qPCR). However, in order to successfully and reliably detect the allowable limit of 5×10^4 colony forming units (CFUs) of bacteria per liter in potable water on the ISS with qPCR, water concentration must first be performed. This report presents the data from a validation study of a NASA-sponsored small business research initiative to develop a microgravity-compatible, automated water concentrator to be used on the ISS, which has been named the ISS Smart Sample Concentrator (iSSC). Efficiency and reproducibility of the iSSC were compared with a ground-based automated water concentrator and the standard Millipore manual filtration. Using 10^4 CFU/L of *Sphingomonas paucimobilis*, *Ralstonia pickettii*, and *Cupriavidus basilensis* and a mixed microbial community, we have shown, through culture and qPCR, that the iSSC is comparable, if not better, at recovering and concentrating bacteria from large volumes of water, with good reproducibility.

KEY WORD: microgravity · molecular biology · qPCR colony counts

INTRODUCTION

In accordance with the International Space Station (ISS) Medical Operations Requirement Documents (MORD NASA-2003), maintaining potable water quality for drinking, food rehydration, personal hygiene, and medical needs is of the utmost priority. This water quality is routinely monitored on the ISS through culture-based approaches; however, because of the operational challenges of culturing samples in space (*i.e.*, length of time before detection, space, labor requirements, and biosafety concerns), the National Aeronautics and Space Administration (NASA) has now made recommendations to implement the PCR and other rapid molecular-based technologies on the ISS.[1]

NASA has recently demonstrated the ability to use PCR technology (SmartCycler and Razor) under microgravity conditions. The SmartCycler (Cepheid, Sunnyvale, CA, USA) has a maximum volume input of 1 μl and needs at least 100 cells per reaction for detection (limit of detection =

1.0×10^2 cells/ml).[1] Razor EX (BioFire Defense, Salt Lake City, UT, USA) has a maximum volume input of 100 μl and needs at least 50 cells per reaction for detection (limit of detection = 5.0×10^2 cells/ml).[1] The ISS Medical Operations Requirement Documents specify that potable water onboard the ISS should not exceed bacterial concentration of 5×10^4 colony forming units (CFUs) per liter;[2] thus, in order to use these molecular biology systems for routine microbial monitoring on the ISS, preconcentration of large volumes of samples (often >1 L) to a few microliters is required before downstream molecular analyses can be performed.

Traditional methods for concentration have involved filtering a sample through a membrane filter and then releasing the trapped microbes by vacuum suction.[3] Although this is currently the gold standard and has been so for many years, it does have some limitations in that it is manual and that the time it takes to filter a sample, plus the resulting concentration, depends on the diameter of the filter. A larger filter size will concentrate a sample quicker than a smaller size; however, the amount of eluant needed to detach the captured microbes is greater than for a smaller filter, resulting in a lower concentration. Thus, for high throughput and low biomass samples, this filtration method is not ideal. The concentrating pipette instrument (CP-150) is a commercially available automated concentrating device

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that was developed by InnovaPrep (Drexel, MO, USA) and can filter large volumes (>1 L) of liquid in minutes (**Fig. 1***A*). It has been used on Earth to concentrate surface samples collected from the ISS[4] and also low biomass surfaces from spacecraft assembly facilities.[5] However, there is currently no such automated system on the ISS to allow for rapid, mechanical concentration of microbes *in situ*, allowing advanced microbial monitoring to occur in space instead of on Earth.

To address this need, under the NASA Small Business Innovative Research Program, NASA sponsored the development of the ISS Smart Sample Concentrator (iSSC), an automated concentrating device that is compatible with microgravity and that is light weight, compact, and self-contained and thus can be flown to and be used on the ISS. The iSSC is based on the underlying technologies of CP-150 but with the necessary modifications to make it spaceflight compatible (Fig. 1*B*). The iSSC can process input volumes as high as 5 L and concentrate them down to as small as 350 µl, providing a concentration factor up to 15,000 times. A more detailed description of the iSSC is presented in the conference proceedings by Page *et al.*[6]

The objective of this paper was to validate the efficacy and reproducibility of the iSSC system in concentrating both cultivable microorganisms and biomolecules from large volumes of water samples. The present study was designed to concentrate microorganisms from 1 L of PBS containing 10^4 CFUs of 3 bacteria that are routinely found in and isolated from the ISS potable water system: *Sphingomonas paucimobilis*, *Ralstonia pickettii*, and *Cupriavidus basilensis*.[7] In addition to these monocultures, 1 L of water containing a mixture of 11 bacteria [mixed microbial community (MMC)] was also tested.[5] The data obtained from the iSSC were compared with those obtained when the above 1-L microcosms were concentrated with CP-150 (commercially available automated system) and Millipore filters (manual gold standard method; MilliporeSigma, Burlington, MA, USA). Efficiency was determined through existing ISS microbial monitoring protocols (colony counts) and future NASA planned molecular microbial burden measurements [quantitative PCR (qPCR)[8]]. Reproducibility was determined by testing biologic and technical replicates.

MATERIALS AND METHODS

Bacterial cultures and growth conditions

S. paucimobilis, *R. pickettii*, and *C. basilensis* were isolated from potable water on the ISS[7, 9, 10] and were obtained from
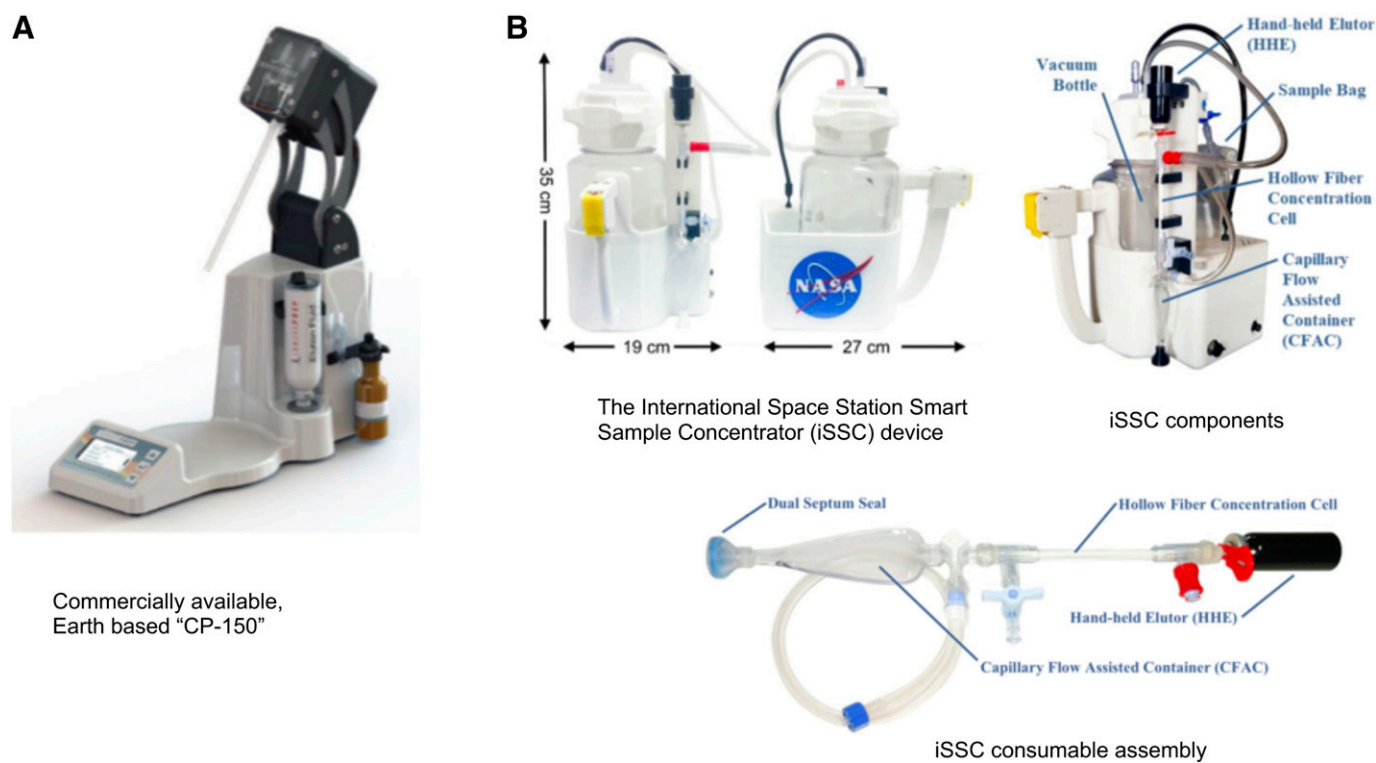
FIGURE 1

Diagram of CP-150 (*A*), the commercially available automated concentrator, and the iSSC (*B*), the NASA-sponsored, spaceflight-compatible automated concentrator.

Kennedy Space Center. Glycerol stocks were streaked out on Reasoner's 2A agar (R2A) plates and incubated at room temperature until adequate growth was observed (~5 d). A single colony was then picked and used to inoculate 5 ml of trypic soy broth and incubated overnight at 120 rpm at either 37°C (for *Sphingomonas* and *Ralstonia*) or 30°C (for *Cupriavidus*). The MMC containing 11 bacteria was established at the Jet Propulsion Laboratory[5] and consisted of a frozen culture of *Aureobasidium pullulans*, *Acinetobacter radioresistens*, *Bacillus megaterium*, *Bacillus pumilus*, *Deinococcus radiodurans*, *Microbacterium imperiale*, *Staphylococcus warneri*, *Micrococcus luteus*, *Cupriavidus metallidurans*, *Clostridium sporosphaeroides*, and *Methanobacterium formicicum* (details listed in Supplemental Table S1).

Concentration of microorganisms

Overnight cultures of *S. paucimobilis*, *R. pickettii*, and *C. basilensis* were measured with DensiCheck (bioMerieux, St. Louis, MO, USA) and then diluted down to 10^4 CFU/ml. The MMC was a frozen culture already determined to be 10^6 16S rRNA gene copies per milliliter.[11] Appropriate aliquots (1 ml) of the 10^4 CFU/ml cultures (referred to as the "inoculum") were then added to 1 L of sterile PBS (pH 7.4), in triplicate (now referred to as the "unconcentrated samples") resulting in 10^4 CFU/L of unconcentrated samples. Within 15 min, the 10^4 CFU/L samples of *S. paucimobilis*, *R. pickettii*, and *C. basilensis* and MMC were concentrated down with 3 different devices: the iSSC, InnovaPrep CP-150, and Millipore filters. The samples were then used for either cultivable microbial examination or qPCR.

iSSC concentration

A 0.2 μM hollow fiber polysulfone filtration system was used to concentrate the samples, and the captured microbes were released using a novel Wet Foam Elution System. A detailed description of the engineering and mechanisms of action of this system has been presented in the conference proceeding by Page *et al.*[6]

CP-150 concentration

A concentrating pipette (InnovaPrep) using 0.22-μM hollow fiber polysulfone tips (CC08022) was used for concentration, and the captured microbes on the hollow fiber tips were released using pressurized canisters containing PBS elution fluid (HC08001).

Millipore filtration

MilliporeSigma microfilm funnels with a 0.45-μm filter (MIHAWG100; MilliporeSigma) were used to concentrate the samples, and a vacuum was used to help pass the samples through the filter. The filter was then removed and added to a 50-ml Falcon tube (Thermo Fisher Scientific, Irwindale, CA, USA). One milliliter of PBS was added, and the filter was vortexed for 30 s at maximum speed to dislodge the bacteria from the filter. The filter was discarded, and the liquid containing the dislodged bacteria was kept.

Assessment of CFUs after concentration

After appropriate serial dilution carried out in sterile PBS, 100 μl of the concentrate from each of the 3 systems was plated on R2A medium in quadruplet and incubated for 24 h at 37°C for *S. paucimobilis* and *R. pickettii* and 30°C for *C. basilensis*. The original inoculum was plated and incubated for 2–3 d in the same way. The percent recovery from each of the 3 concentrators was determined by dividing the CFU counts of the concentrates by the CFU counts of the original inoculum.

qPCR

DNA extraction of the concentrates and the original inoculum was performed with the Maxwell 16 automated system (Promega, Madison, WI, USA), in accordance with manufacturer's instructions using the Maxwell 16 Tissue LEV Total RNA purification kit. The extracted DNA was eluted in 50 μl of molecular-grade water and stored at −20°C. Following DNA extraction, qPCR targeting the partial 16S rRNA gene (universal for all bacteria) was performed with the SmartCycler (Cepheid) to quantify bacterial abundance. Primers targeting the partial 16S rRNA gene were 1369F (5′-CGGTGAATACGTTCYCGG-3′) and modified 1492R (5′-GGWTACCTTGTTACGACTT-3′).[12] Each 25-μl reaction consisted of 12.5 μl of 2X iQ Sybr Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μl each of forward and reverse oligonucleotide primers (10 μM each), and 1 μl of template DNA. Each sample was run in triplicate. DNase/RNase-free molecular-grade distilled water (Promega) was used as the no template control in each run. The reaction conditions were as follows: a 3-min denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, and a combined annealing and extension at 55°C for 35 s. The number of gene copies in the samples were determined by running a standard curve, which was generated using serial dilutions (10^8–10^2) of the synthesized 16S rRNA gene of *B. pumilus* SAFR-032 as described previously.[13] The qPCR efficiency was ~98% for each run. DNA extracted from all 3 test microorganisms and MMC was used as positive control. The negative control (no template nuclease-free water) values were not deducted. The fold efficiency of each of the 3 concentrators was determined by dividing the copy number of the concentrate by the copy number of the original inoculum.

Statistical analyses

Figures and statistical analyses were generated using Prism version 8 (GraphPad Software, La Jolla, CA, USA).

Statistical analysis included a 1-way ANOVA followed by the false discovery rate *post hoc* test. Statistical significance was based on $P < 0.05$ and coefficient of variation to measure variability among the samples and is calculated by dividing the SD by the mean.

RESULTS AND DISCUSSION

The steps involved in microbial sample preparation, concentration of 1-L liquid microcosms, and the downstream assays used during this study are schematically presented in **Fig. 2**.

Recovery volume

The mean recovery volume after concentration was 304 µl for the iSSC, 530 µl for CP-150, and 928 µl for Millipore system (**Fig. 3**), allowing the iSSC to have 3 times more concentrated material when compared with the traditional Millipore filtration system. When these concentrated materials were analyzed, by culture and by qPCR, the elution volume of each respective concentrating system was taken into account and used to calculate the total microbial burden. The elution-based concentration factor was 3.29 for the iSSC, 1.89 for CP-150, and 1.1 for Millipore.

Molecular assays using various PCR systems have reported that a minimum of 100 copies per microliter of target molecules are required in order to reliably and reproducibly detect the targets when universal primer probes are used.[11] Thus, the ability to concentrate a sample in a smaller volume, as can be achieved with the iSSC system, is advantageous because it allows for higher concentrations of the target molecules, especially important when working with low biomass samples. The present ISS operations for microbial monitoring of water samples use syringe-based concentration, where 10 ml of potable water is concentrated using disc filters. However, in order to concentrate volumes in the order of liters, under microgravity, a robust filtration system like the iSSC system is needed.

Percent recovery based on CFU counts

In order to determine the ability of bacteria to survive the concentration process, bacterial plating and subsequent colony counting were performed. The percent recovery of viable organisms was determined by comparing the CFUs of the concentrate with the CFUs of the original inoculum. Percent recovery of CFUs was highest with the iSSC, followed by CP-150 and then Millipore, which was true for all 3 bacterial cultures tested ($P < 0.05$) (**Fig. 4**). *C. basilensis* had the best recovery with the iSSC at 76%, compared with *R. pickettii* at 62% and *S. paucimobilis* at 52% (Fig. 4). These data show that the iSSC system was more efficient at retaining viable organisms. The coefficient of variation was calculated to measure the degree of variability among the different devices and is summarized in **Table 1**. On average, among biologic replicates, the iSSC had the lowest variation (15%) and Millipore the
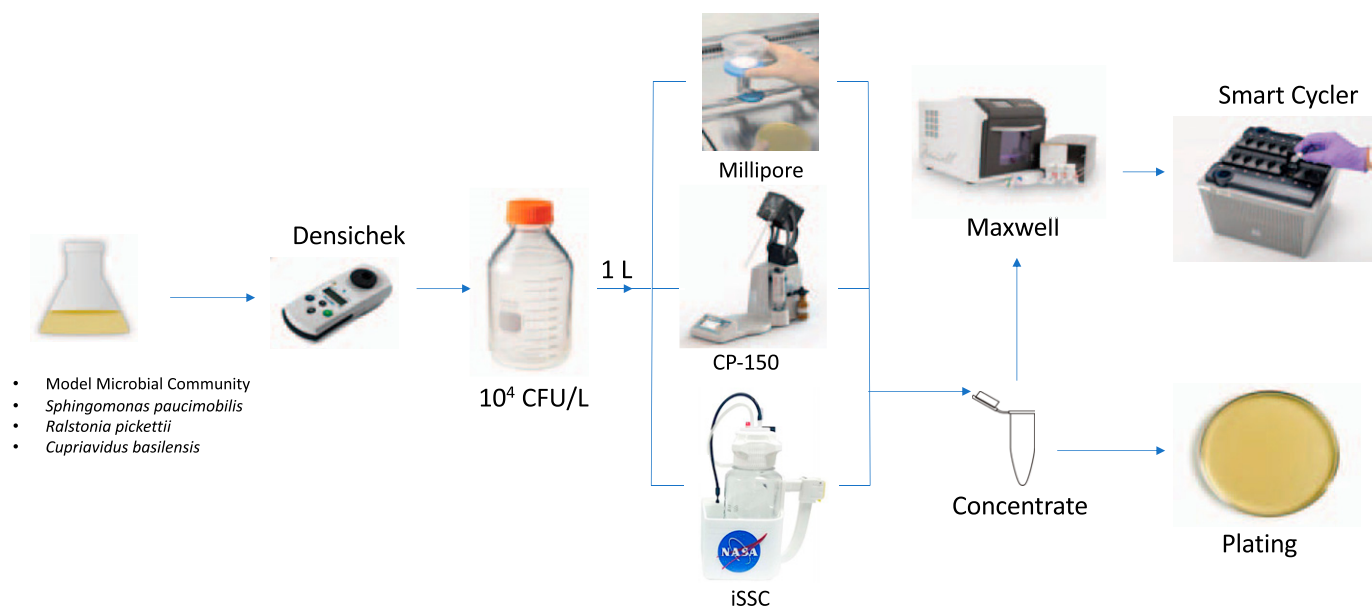
FIGURE 2

Schematic of the workflow to test the iSSC's efficiency. Efficiency was tested by current NASA microbial monitoring culture methods and by NASA planned molecular methods and compared with the CP-150 (Earth-based automated system, InnovaPrep) and the gold standard, manual filtration method from Millipore.
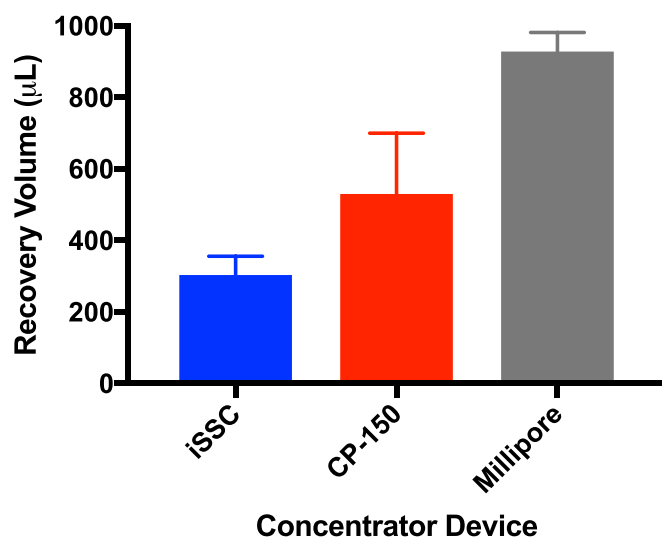
FIGURE 3

Volume of liquid recovered from the various concentrator devices, after concentrating 1 L of PBS containing various bacteria.

highest (56%), indicating that the iSSC system gave the most reproducible results.

The lower efficiency of the Millipore filter method might be due to increased retention of microorganisms on the filter paper compared with the hollow fibers of the CP-150 and iSSC systems either because of the material used or the fact that vortexing of the Millipore filters is used to detach the captured microbes into solution, which may not be as efficient as using pressurized canisters to forcefully eject the captured microbes from the hollow fibers, as is done in the CP-150 and iSSC systems. Additionally, the vacuum

suction pumping used with Millipore filtration could have had negative consequences on viability. Another difference between Millipore and the automated system that could account for the lower CFUs witnessed in this study is the fact that microorganisms are captured in a desiccated state on the filter paper, whereas the hollow fibers used in both CP-150 and iSSC systems retain microbes in a suspended physical status and are designed to retain moisture on the filter cassettes, which could maintain viability. Additionally, because the recovery volume was greater, the samples were less concentrated, and thus fewer CFUs were collected for plating.

Percent recovery based on qPCR data

The concentrator efficiency was also tested using a molecular-based approach, specifically qPCR of the 16S rRNA gene using the Smart Cycler, which is part of the WetLab-2 research platform on the ISS. The concentrates of 10^4 CFU/L were subjected to DNA extraction, and then the 16S rRNA gene copy number of the isolated DNA was measured. The copy numbers after concentration for *C. basilens*, *R. pickettii*, *S. paucimobilis*, and the MMC are summarized in **Fig. 5**. For *R. pickettii* and the MMC, there were no differences in the number of DNA molecules measured after concentration with the iSSC, CP-150, or Millipore. However, for *S. paucimobilis*, the recovery with the iSSC was significantly lower than with CP-150 and Millipore. On the other hand, *C. basilensis* had a significantly better recovery with iSSC compared with CP-150 and Millipore.

The copy number fold efficiency was also calculated by dividing the 16S rRNA gene copy number of the
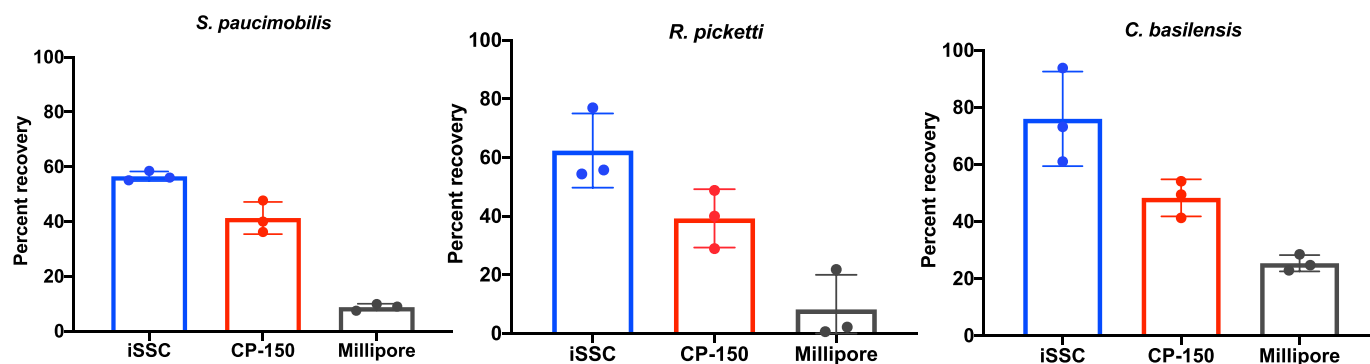

FIGURE 4

One liter of PBS containing 10^4 CFU of either *C. basilensis*, *R. pickettii*, or *S. paucimobilis* was concentrated down with either the iSSC, CP-150, or a Millipore filter. The concentrates were then plated on trypic soy agar plates and the CFUs counted. The initial inoculum of 10^4 CFU was also plated. The percent efficiency was calculated by diving the concentrated CFU counts by the original inoculum counts. For all 3 bacteria tested, the iSSC had a significantly higher efficiency compared with CP-150 and Millipore ($P < 0.05$). Error bars represent the sE of the mean, and the mean is based on 3 biologic replicates. Each point is a biologic replicate that represents the mean of colony counts from 4 replicate plates.

TABLE 1

Coefficient of variation based on the culture data

Bacteria	iSSC	CP-150	Millipore
S. paucimobilis	3.1%	14.2%	13.8%
R. pickettii	20.3%	25.3%	143.8%
C. basilensis	21.8%	13.4%	11.2%
Mean	15%	18%	56%

The values were obtained by dividing the SD by the mean. The values listed for each bacteria and each machine are based on 3 biologic replicates. A lower coefficient of variation value indicates less variability.

concentrated sample by the 16S rRNA gene copy number of the inoculum. For *R. pickettii*, *S. paucimobilis*, and the MMC, the mean fold efficiency for the iSSC was higher than that obtained for CP-150 and Millipore, although statistical significance was not achieved ($P > 0.05$)

(Supplemental Fig. S1). For *C. basilensis*, the means were similar for all 3 concentrator systems. The variability among the 3 biologic replicates for the iSSC was consistent with the variability observed with the other 2 commercial systems (**Table 2**).

The Cepheid software accompanying the real-time PCR instrument was used to evaluate threshold cycle. The sample DNA concentrations are reflected by threshold cycle (C_t values), where larger C_t value indicates lower DNA concentration. The effectiveness of filtration systems was showed by ΔC_t, which was calculated as described previously.[14] Briefly, C_t values of the cell numbers of the sample before concentration (C_{tin}) was subtracted from those of cell numbers of the sample after concentration (C_{tout}). For *S. paucimobilis*, the ΔC_t value ranged from 1.1 to 1.6 for all the 3 systems tested and was consistent for all 3 systems employed to concentrate water samples. The ΔC_t value for *R. picketii* was low for the iSSC (0.69 C_t) when compared
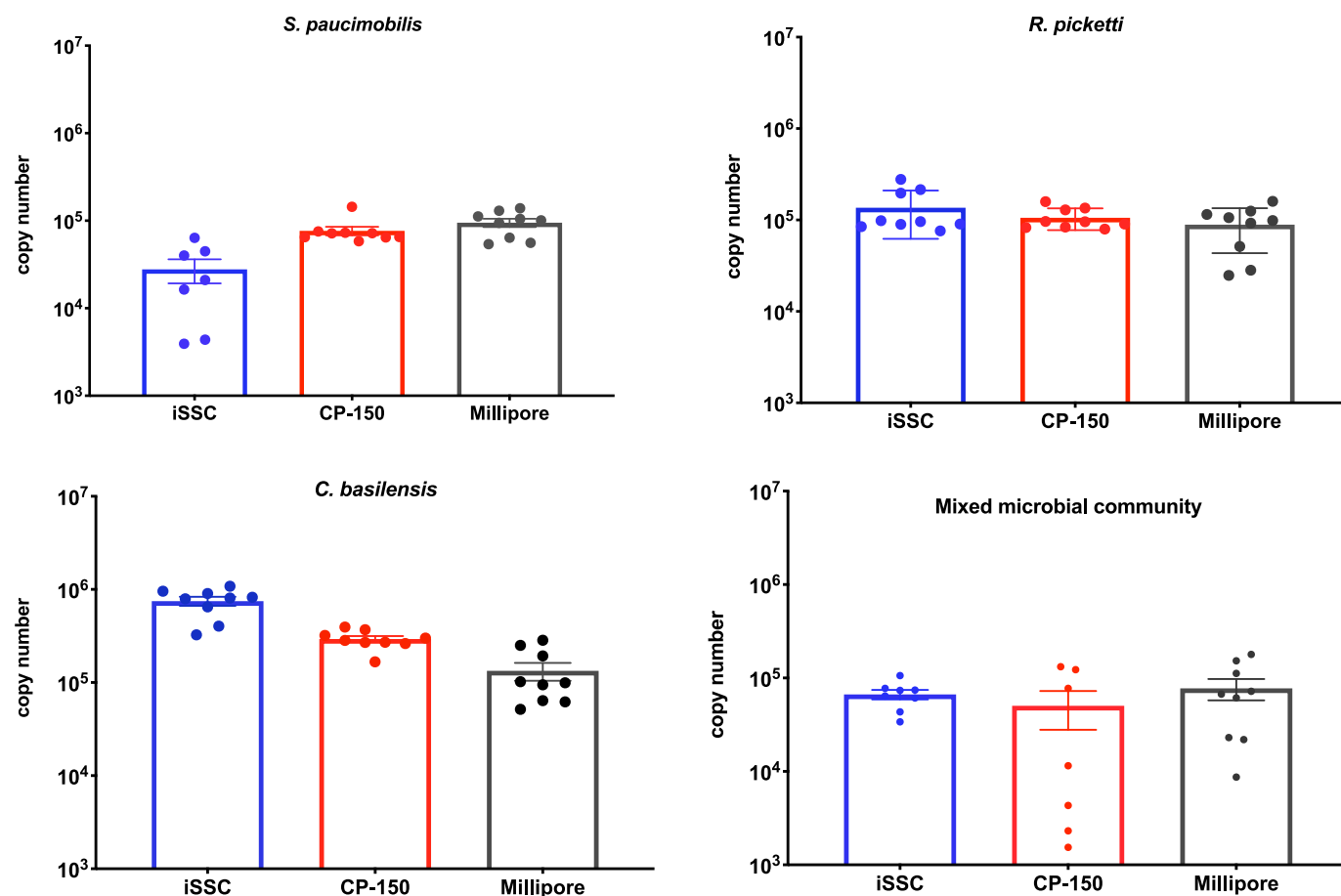

FIGURE 5

One liter of PBS containing 10^4 CFU of either *C. basilensis*, *R. pickettii*, *S. paucimobilis*, or an MMC (consisting of 11 different bacteria) was concentrated down with either the iSSC, CP-150, or a Millipore filter systems. The concentrates were then subjected to DNA extraction and the 16S rRNA copy number assessed by qPCR using the WetLab-2 SmartCycler. Three biologic replicates were run in triplicate on the qPCR, with all data being graphed.

TABLE 2

Coefficient of variation based on the qPCR data

Bacteria	iSSC	CP-150	Millipore
S. paucimobilis	54.1%	77.3%	53.6%
R. pickettii	76.8%	57.8%	160.2%
C. basilensis	82.3%	93.4%	49.1%
MMC	67.9%	24.0%	88.2%
Mean	70%	63%	88%

The values were obtained by dividing the SD by the mean. The values listed for each of the 4 samples and each machine are based on 3 biologic replicates. A lower coefficient of variation value indicates less variability.

with CP-150 (0.73 C_t) and Millipore (1.38 C_t) systems, which means that iSSC system retained considerably more cells than the Millipore system. A similar trend was seen for *R. pickettii* in the colony count assay (Fig. 4). In contrast, for *C. basilensis*, recovery of 16S rRNA gene molecules were higher in all 3 systems employed during this study than their initial inoculum. However, the total number of *C. basilensis* cells recovered from the iSSC system was higher when compared with both CP-150 (0.5-log lower) and Millipore (~1 log lower) systems (Fig. 5). When MMC microcosms were analyzed, the iSSC system performed far better than the other 2 systems in filtering biomolecules of multiple microbial communities. The ΔC_t values of MMC samples for the iSSC system (1.86 ΔC_t) was the least when compared with CP-150 (2.40 ΔC_t), and Millipore system (3.21 ΔC_t), which showed that the iSSC was superior in trapping target biomolecules.

CONCLUSIONS

The validation studies conducted on the NASA-sponsored automated water concentrator (iSSC) to be used on the ISS as part of NASA's *in situ* microbial monitoring initiative has shown great potential. Using 3 bacteria that are routinely found in the ISS potable water system, the iSSC system was shown to be more efficient in maintaining viability (culture data) and in capturing the bacteria that pass through the filtration system (qPCR data) compared with the Earth-based automated concentrator (CP-150) and the gold standard manual system (Millipore). Not only did the iSSC system outrank the other 2 systems in efficiency, the iSSC was also shown to have better reproducibility. An added benefit of the iSSC system is that it could concentrate 1 L of PBS in ~2 min, with a concentrate volume of <1 ml (304 μl), whereas the Millipore filter took 10 min to concentrate and process, and the lowest volume that could be used to unbind the captured microbes was 1 ml. In addition, the iSSC system is automated and self-contained, require less crew time, is capable of filtering large volume of water, is designed to collect filtered water for other use without wasting, and is tested for the microgravity applications.

The system tested in this report was the phase II prototype, but the newly improved current iSSC system is being evaluated for various safety procedures and obtaining approval to spaceflight. This final design will undergo the same validation steps as described above and will also include an "aging" study to determine the reproducibility of the system after pressure loss caused by time in storage (to mimic the spaceflight process). In addition to culture, viability will also be tested by cell counts *via* microscopy. In addition to qPCR, the integrity of the isolated DNA obtained from these concentrated bacteria will also be whole genome sequenced with the MinION (Oxford Nanopore Technologies, Oxford, UK), which has already been used and tested on the ISS.[15] More microbial cultures that are commonly found in drinking water, *Methylobacterium* and *Brevundimonas*, will be tested, and 10 biologic replicates will be carried out. In addition, all the above future tests will be also performed with 10^2 CFU/L and 10^3 CFU/L cultures.

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