Electronic Supplementary Material (ESI) for Environmental Science: Water Research & Technology.
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Mediation of effects of biofiltration on regrowth, *Legionella pneumophila*, and microbial community structure by hot water plumbing conditions

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Supplementary Information

Experimental and Results

SI.1: The 2.5 Years Acclimation Period Prior to Experimental Period

In the first year of acclimation, SWHs were sequentially fed with synthesized water (for 3-4 months, phase A1), a mixture of synthesized water (90% volume) + breakpoint chlorinated and biofiltered Blacksburg tap water (10% volume, microbes in the biofiltered water were not filtered out) first without (for 5-6 months, phase A2) and then with (for 1-2 months, phase A3) supplemented trace nutrients including manganese, iron, zinc and amino acids. Details of the synthesized water, tap water treatment, and supplementation were described in a previous study.[1] Microbes naturally present in the biofiltered Blacksburg tap water during phase A2 and A3, along with the inoculated stationary cultures of *L. pneumophila* (ATCC 33152, 33733, 33734, 33823, total 6.78×10^{6}/mL), *Acanthamoeba polyphaga* (ATCC 30871, 5.42×10^{2}/mL), and *Vermamoeba vermiformis* (ATCC 50237, 1.69×10^{3}/mL) at the beginning of phase A1 and A3 were used to seed SWHs. More details are in the study by Williams *et al.*[1]. All SWHs were incubated at 32°C in the dark.

Beginning at the second year of acclimation, SWHs were modified with each of the eight plumbing conditions described in the plumbing conditions section in Materials and Methods. Water fed to SWHs was biofiltered or unfiltered Blacksburg tap water treated in the same way as described in the Materials and Methods (influent water section), except that sodium thiosulfate (3 mg/L) was used to quench breakpoint chlorine residual instead of heating and cooling. Microbes in the influent water (biofiltered or unfiltered) were removed with a 0.45 μm membrane before feeding SWHs. Water was changed in the same way as described in the Materials and Methods (the water changes section), except with a lower frequency of bi-weekly change rather than three times a week.

At the end of the 2.5-years acclimation period, SWHs were cross-inoculated as described in the Materials and Methods (the acclimation prior to the experimental period section) to initiate the experimental period, which lasted for a total of 448 days. Results presented in this study were acquired from the experimental period if not specified.

SI.2: Town of Blacksburg, VA Municipal Water

Town of Blacksburg VA municipal water was treated conventionally in the facility, without biofiltration and with chloramine as residual disinfectant. This water had an average chlorine residual of 2.2 ppb, a total nitrogen (N) of 0.65 ppm, and copper of 0.06 ppm (90th percentile). More details about this water can be acquired from online reports: 2012 water quality report for the town of Christiansburg. [http://www.christiansburg.org/DocumentCenter/View/3568]

SI.3: Characterization of Inorganic Elements in the Water

Water chemistry of biofiltered and unfiltered influent water was characterized via inductively coupled plasma mass spectrometry (ICP-MS, Thermo Electron, Waltham, MA). Ten milliliters of water was collected in sterile test tubes and acidified by adding 2% nitric acid by mass prior to analysis.

SI.4 Water Chemistry, Organic Carbon, and Heterotrophic Bacteria Monitoring

Water chemistry, including assimilable organic carbon (AOC), biodegradable dissolved organic carbon (BDOC), total organic carbon (TOC), dissolved oxygen, and pH, along with total bacterial growth (heterotrophic plate count (HPC) and 16S rRNA gene copy numbers) and *L. pneumophila* numbers (*mip* gene copy numbers), were occasionally monitored during the later 1.5-years of acclimation. HPC varied between 1×10^5 and 1×10^6 CFU/mL, while 16S rRNA gene copies were 1×10^6 - 10^7/mL in bulk waters of SWHs in the acclimation period.

AOC in influent waters and SWH bulk waters was measured routinely for about one year, but the reproducibility of AOC measurements (with the standard method 9217B[2] and a bioluminescence method[3]) was poor thus results were not publishable. Furthermore, it was questionable whether the two strains implemented in the AOC measurement were fully representative of the thousands of heterotrophic bacteria present in drinking water. Therefore the organic carbon measurement was switched to BDOC and TOC. BDOC in biofiltered water was 0.157±0.146 mg/L, comparable to the amount of consumed organic carbon quantified by TOC measurement (TOC_{in}-TOC_{out}=0.122±0.05 mg/L), which was simpler and more reproducible.

SI.5: Organic Carbon Leaching from PEX Pipes

Leaching of organic carbon from pipe materials was evaluated in SWHs used for this study on days 232 and 448 of the experimental period (i.e., post cross-inoculation), as well as in a similar set of SWHs incubated at higher temperatures. For TOC measurements during the second year of acclimation, the top (5 mm) and bottom (5 mm) layers of bulk water in two sets of SWHs, with PEX pipe and the no-modification Control, were pipetted out slowly while minimizing the disturbance to the water column and biofilms. Each set consisted of 15 SWHs fed with biofiltered or unfiltered water. Leaching of organic carbon was observed only in SWHs fed with biofiltered water. Specifically, SWHs with PEX pipe yielded 0.10-0.19 mg/L more TOC in both top and bottom layers of water column in comparison to the Control (Figure S8), although this difference was not statistically significant due to large variation among SWH replicates (0.07-0.45 mg/L). TOC leached from the PEX pipe (10 cm × ø1.9 cm) at an approximate rate of 0.006 mg/day at 32°C.

On day 232 and 448 of the experimental period of this study, decanted water from triplicate SWHs during water changes were collected, mixed, and pooled for TOC measurement. On day 448, SWHs with PEX pipe contained slightly more TOC (0.05 mg/L) than the Control when both fed with biofiltered water (Figure S8).

Leaching of organic carbon from PEX pipe was further evaluated in a similar set of SWHs, with PEX pipe and with copper pipe as a control, both fed with unfiltered water and incubated at two higher temperatures - 37°C and 53°C. Decanted water from triplicate SWHs during water change were collected, mixed, and pooled for TOC measurement. Leaching of organic carbon from PEX pipe was obviously observed at 53°C, as effluent water in SWHs with PEX pipe had 55% more TOC than SWHs with copper pipe, and 50% more TOC than influent water (Figure S9). TOC leaching from the PEX pipe (10 cm × ø1.9 cm) occurred at a higher rate of 0.018 mg/day at 53°C than at 32°C.

SI.6: Hydrogen Production Associated with the Magnesium Rod

Hydrogen (H_2) concentration in bulk water was measured by dispensing 20 mL water from each SWH into an airtight 40mL glass vial, vigorously shaking the vial to allow equilibration between water and gas, followed by measuring the headspace H_2 with a Trace Analytical™ KAPPA-5/E-002 gas chromatograph with a reduced gas detector (Menlo Park, CA), and a final back calculation into aqueous concentration using Henry's Law.

Bulk water from SWHs with a magnesium rod (MgRod and Combination modifications) contained 530±54 μM H_2, which was 32× higher than that which was measured from SWHs without a magnesium rod (16±7 μM H_2) (Figure S9). The average H_2 generation rate from the magnesium rod was 22.0±2.3 μmol/day.

SI.7: Impact of Plumbing Modifications on Unique OTUs

The impact of individual plumbing modification on the hot water microbiome was examined by identifying OTUs detected in conditions containing a given plumbing modification, but not in the no-modification Control condition. This was achieved using the Venn command in Mothur (1.36.0). The Combination condition had ~500 unique

OTUs, accounting for 16-17% of the microbial community, regardless of whether the influent was biofiltered (Table S3). The addition of an iron rod stimulated unique OTUs to a greater extent with unfiltered water (abundance of these unique OTUs=8.0%) than with filtered water (2.5%). In comparison, unique OTUs stimulated by other modifications ($Fe(OH)_3$, PEX, or Ammonium) represented only a small proportion of the microbial community (0.5-2.5%).

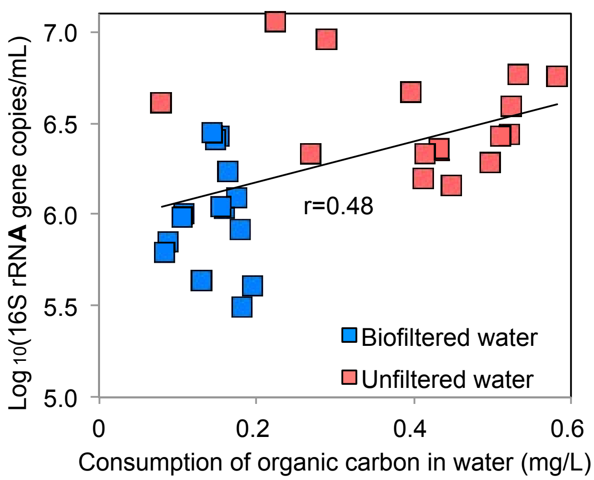

Fig. S1. Positive correlation between total bacterial numbers ($\log_{10}$-transformed) in hot water and consumption of organic carbon in SWHs. Total bacterial numbers were measured as 16S rRNA gene copies/mL. The consumption of organic carbon during each water change cycle was determined as $TOC_{influent}$-$TOC_{effluent}$. Spearman's correlation coefficient r is 0.48 (p<0.001). Blue symbols: biofiltered water; Red symbols: Unfiltered water.

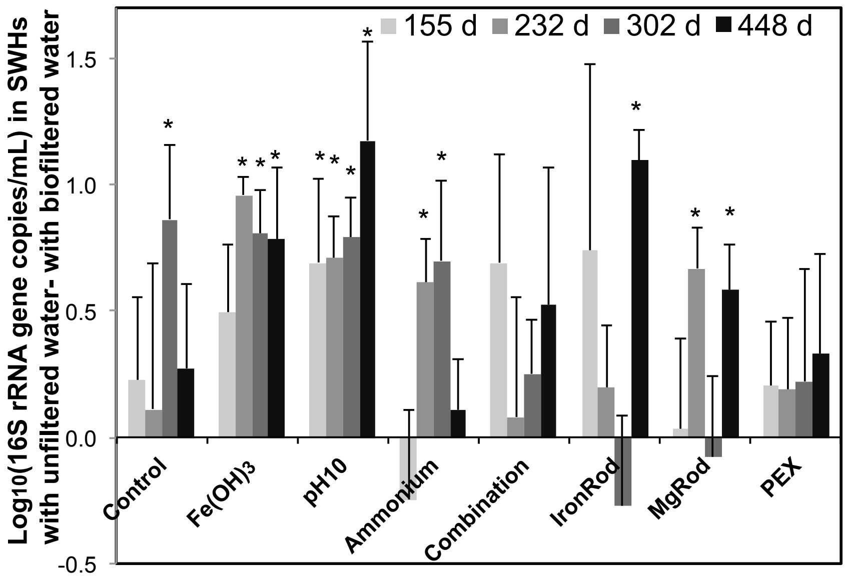

Fig. S2. Reduced bacterial regrowth in hot water attributed to influent biofiltration when modified with plumbing conditions. Reduced bacterial regrowth is shown as total bacterial numbers (16S rRNA gene copies/mL) in SWHs fed with biofiltered water being subtracted from those in SWHs fed with unfiltered water. Error bars are standard deviations calculated from two groups of triplicate SWHs used for the subtraction. * indicates significant difference in total bacterial numbers in SWHs between biofiltered water and unfiltered water ($p<0.05$).

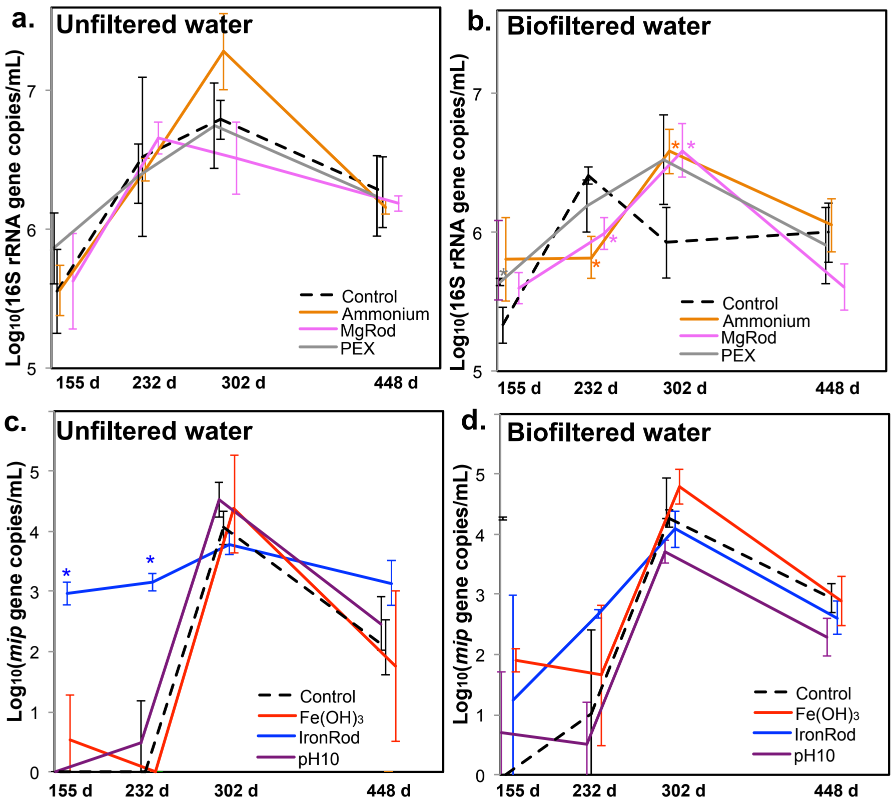

Fig. S3. Effect of other plumbing conditions (not shown in Figure 2) on total bacterial (16S rRNA gene copies/mL) and *L. pneumophila* numbers (*mip* gene copies/mL) in SWHs receiving unfiltered (a, c) or biofiltered (b, d) waters. Other figure legends are the same as in Figure 2.

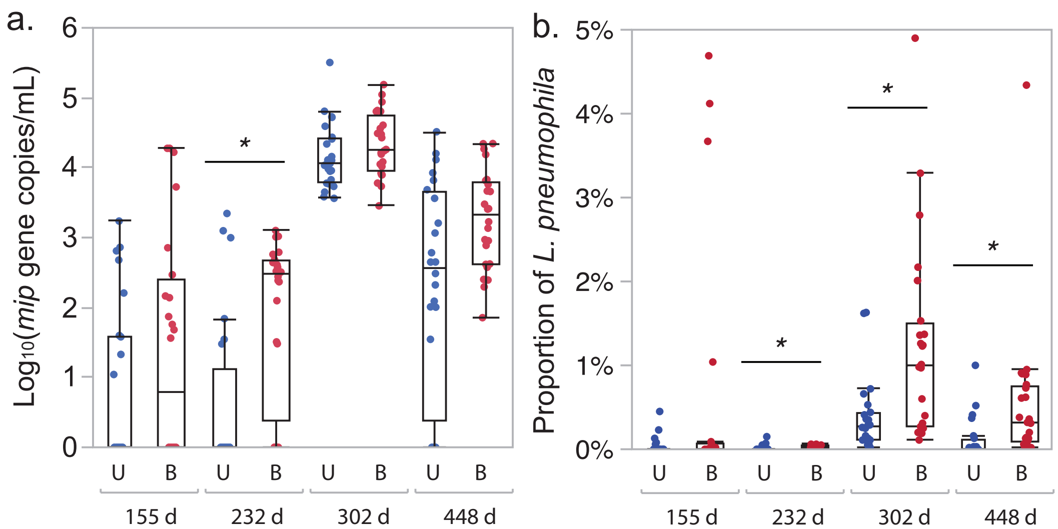

Fig. S4. Effect of biofiltration of influent water on the absolute (a) and relative (b) numbers of *L. pneumophila* in SWHs. *L. pneumophila* numbers are shown as *mip* gene copy number/mL. Proportion of *L. pneumophila* is estimated as the ratio of numbers of *mip* gene to 16S rRNA gene. U: unfiltered water; B: biofiltered water. An outlier box-plot for each group of 24 SWHs is shown on top of single data points. Symbol * indicates significance ($p < 0.05$).

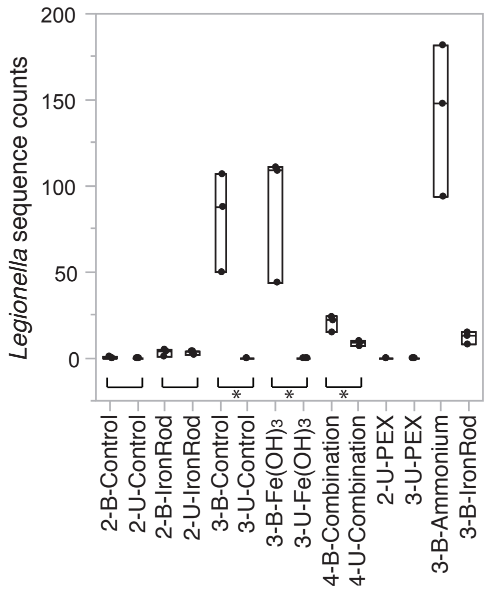

Fig. S5. Number of sequence reads classified as *Legionella* in samples rarefied to 18,490 sequences each. Samples from triplicate SWHs are grouped together. Among each group indicated on the x-axis: the numbers indicate sampling day (2: 232 d, 3: 302, 4: 448 d); U: unfiltered water; B: biofiltered water. * indicates more sequence counts in SWH with biofiltered water than with unfiltered water.

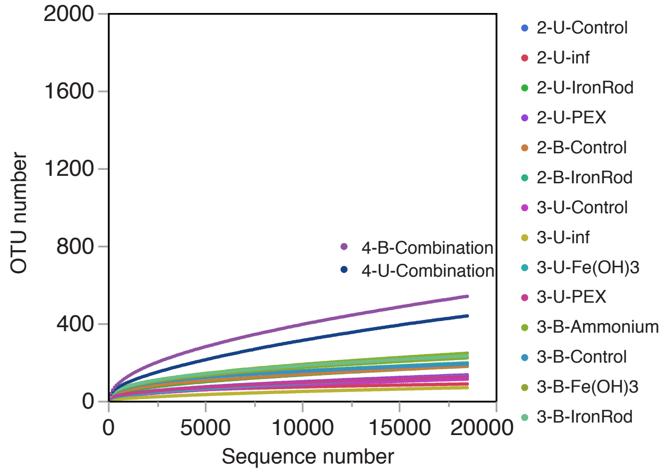

Fig. S6. Mean rarefaction curves of samples from triplicate SWHs. For conditions defined in the legend, the numbers indicate sampling day (2: 232 d, 3: 302, 4: 448 d); U: unfiltered water, B: biofiltered water; inf: influent.

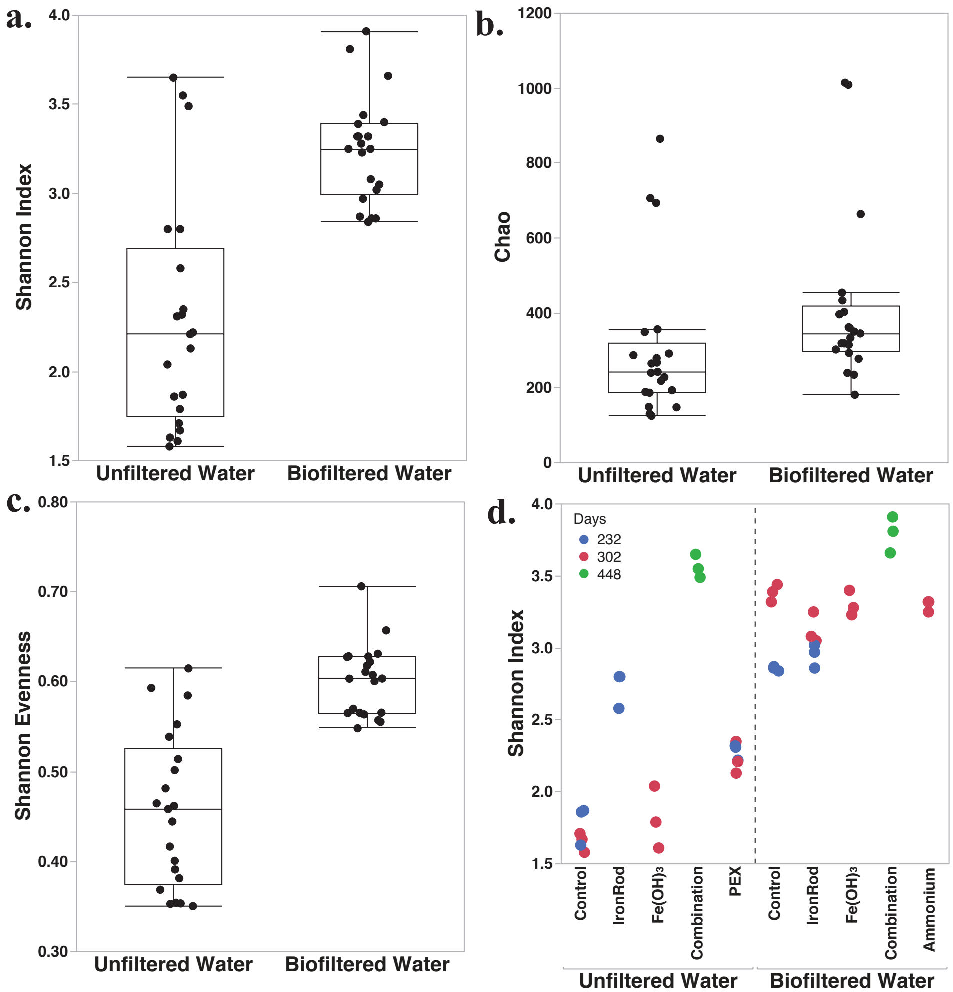

Fig. S7. Effect of the biofiltration and plumbing conditions on alpha diversity of hot water microbiome. The Shannon diversity index (a), estimated richness (Chao) (b) and Shannon evenness (c) of the microbial community in SWHs receiving biofiltered water are shown in comparison to the ones with unfiltered water combining all plumbing conditions. The Shannon diversity index in SWHs is further split into specific plumbing conditions and sampling events (blue: 232 d, red: 302 d, green: 448 d) for both unfiltered and biofiltered water (panel d).

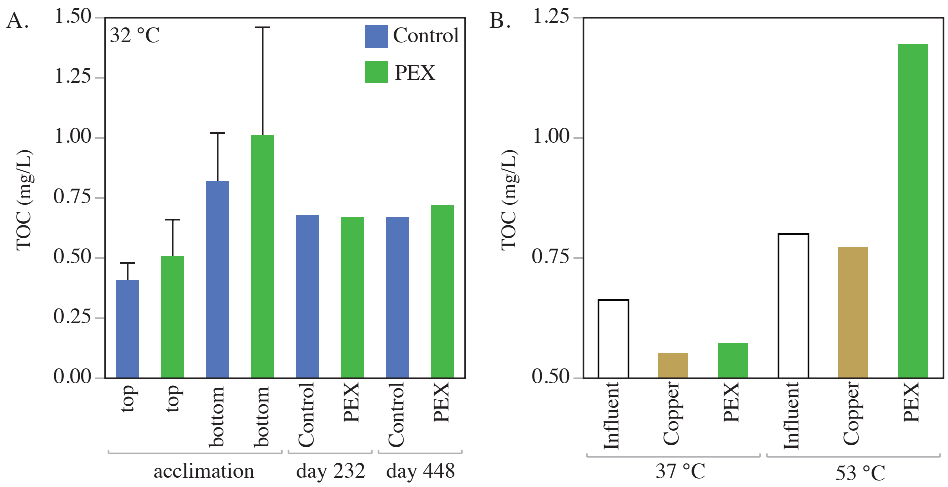

Fig. S8. Evaluation of organic carbon leaching from the PEX pipe in A) SWHs with PEX pipe in comparison to the no-modification Control at 32°C and B) in a similar set of SWHs with PEX pipe in comparison to a copper pipe incubated at 37°C and 53°C. All SWHs were fed with biofiltered waters. Error bars show the standard deviation from 15 SWH replicates. Data without error bars were measurement from pooled water from triplicate SWHs.

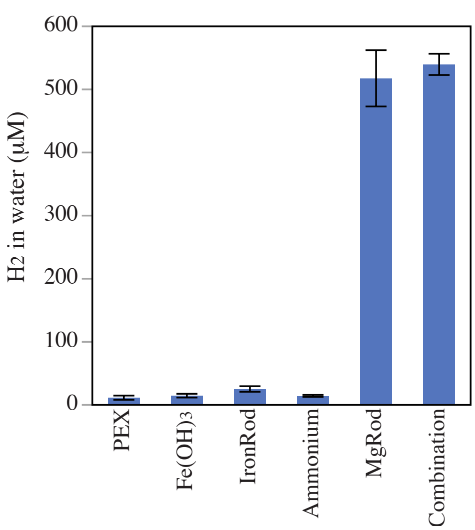

Fig. S9. Aqueous hydrogen concentrations in bulk water from SWHs. Error bars are standard deviations of triplicate SWHs.

Table S1. Water chemistry in unfiltered and biofiltered influent waters. The relative changes (%) show the effect of biofiltration (+ means increase after biofiltration; - means reduction).

Element	Unfiltered influent water (ppb)	Biofiltered influent water (ppb)	Change with biofiltration
Sodium	15120	16740	+10.71%
Magnesium	3508	3566	+1.65%
Aluminum	20.00	11.23	-43.85%
Silica	2998	3117	+3.97%
Phosphorous	203.0	132.8	-34.58%
Potassium	1520	1694	+11.45%
Calcium	9017	9417	+4.44%
Vanadium	0.765	0.826	+7.97%
Chromium	0.132	0.081	-38.64%
Iron	9.296	0.517	-94.44%
Manganese	0.557	2.723	+388.87%
Nickel	0.345	0.477	+38.26%
Copper	64.01	12.61	-80.30%
Zinc	67.80	85.68	+26.37%
Lead	0.350	0.143	-59.14%

No error bars were available as only one measurement was conducted.

Table S2. Cumulative abundance of OTUs unique to a plumbing modification in comparison to the no-modification Control

Abundance of OTUs unique to a plumbing modification	**Unfiltered water**	**Biofiltered water**
$Fe(OH)_3$	0.6%	0.6%
PEX	0.5%	na
Ammonium	na	2.5%
IronRod	8%	2.5%
Combination	16%	17%

na: data not available because the samples were not sequenced.

Reference:

1. Williams, K.; Pruden, A.; Falkinham, J. O.; Edwards, M., Relationship between organic carbon and opportunistic pathogens in simulated glass water heaters. *Pathogens* **2015,** *4*, (2), 355-72.

2. 9217 Assimilable organic carbon. http://www.standardmethods.org/store/ProductView.cfm?ProductID=104

3. Weinrich, L. A.; Giraldo, E.; Lechevallier, M. W., Development and application of a bioluminescence-based test for assimilable organic carbon in reclaimed waters. *Appl. Environ. Microbiol.* **2009,** *75*, (23), 7385-90.