

Impact of water heater temperature setting and water use frequency on the building plumbing microbiome

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Supplementary Information

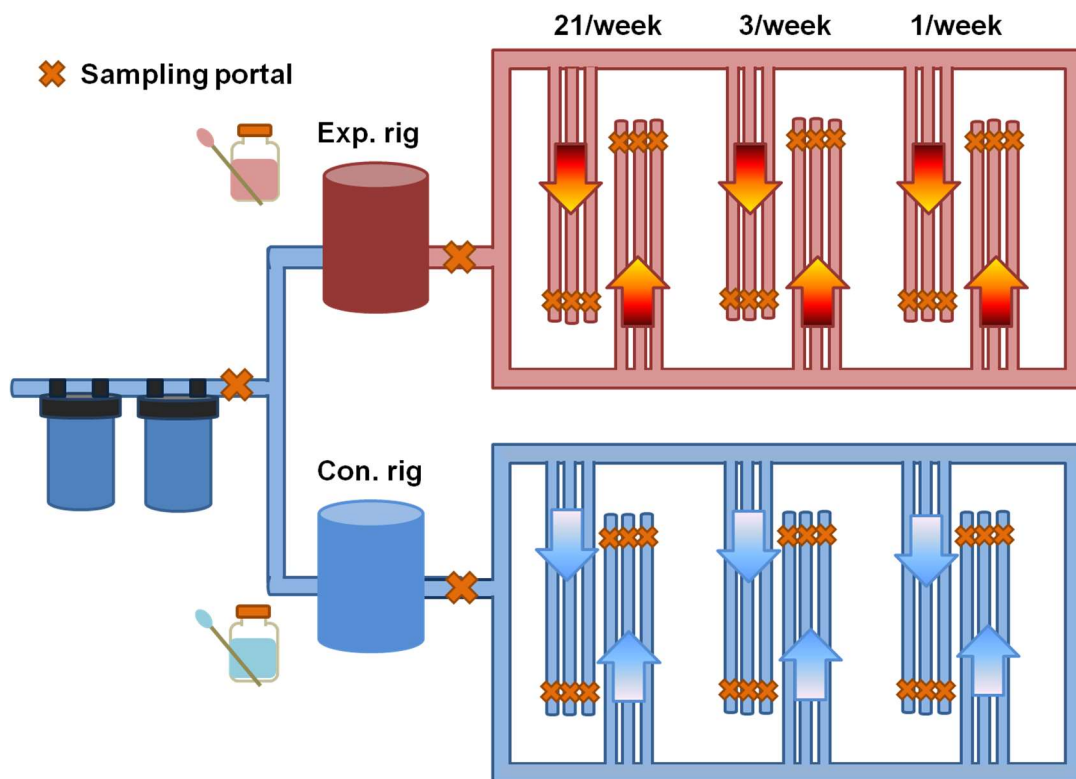
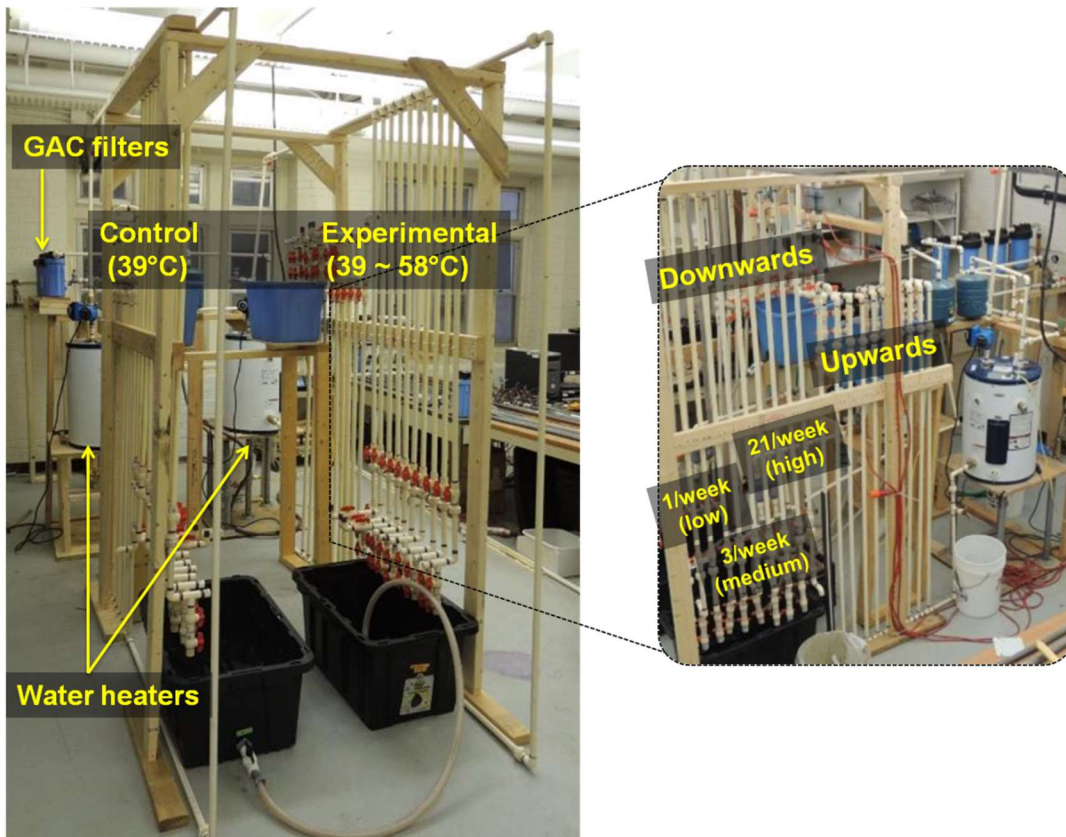


Figure S1. Design of the lab-scale hot water heater rigs.

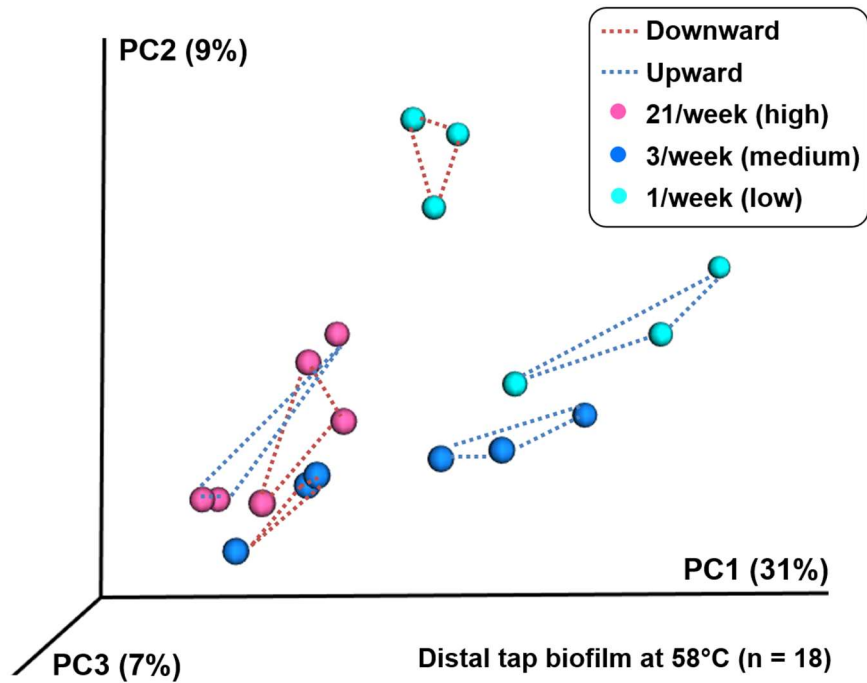


Figure S2. Phylogenetic dissimilarity pattern among biofilm samples categorized by water use frequency (colored points) and pipe orientation (dotted lines) (n=18, distal tap biofilm samples at water heater temperature setting of 58°C, 15 months). Weighted UniFrac distance matrix was used (rarefied 100 times).

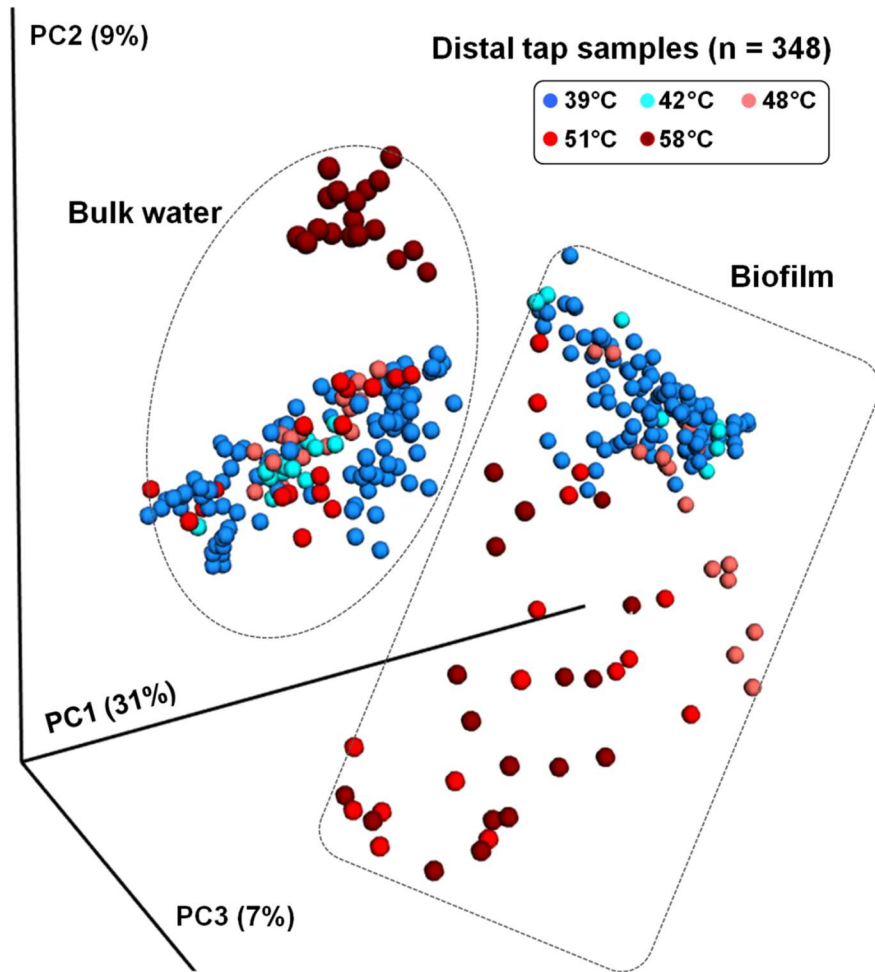


Figure S3. Distal tap phylogenetic dissimilarity pattern across elevated water heater temperature setting (n = 348). Weighted UniFrac distance matrix was used (rarefied 100 times).

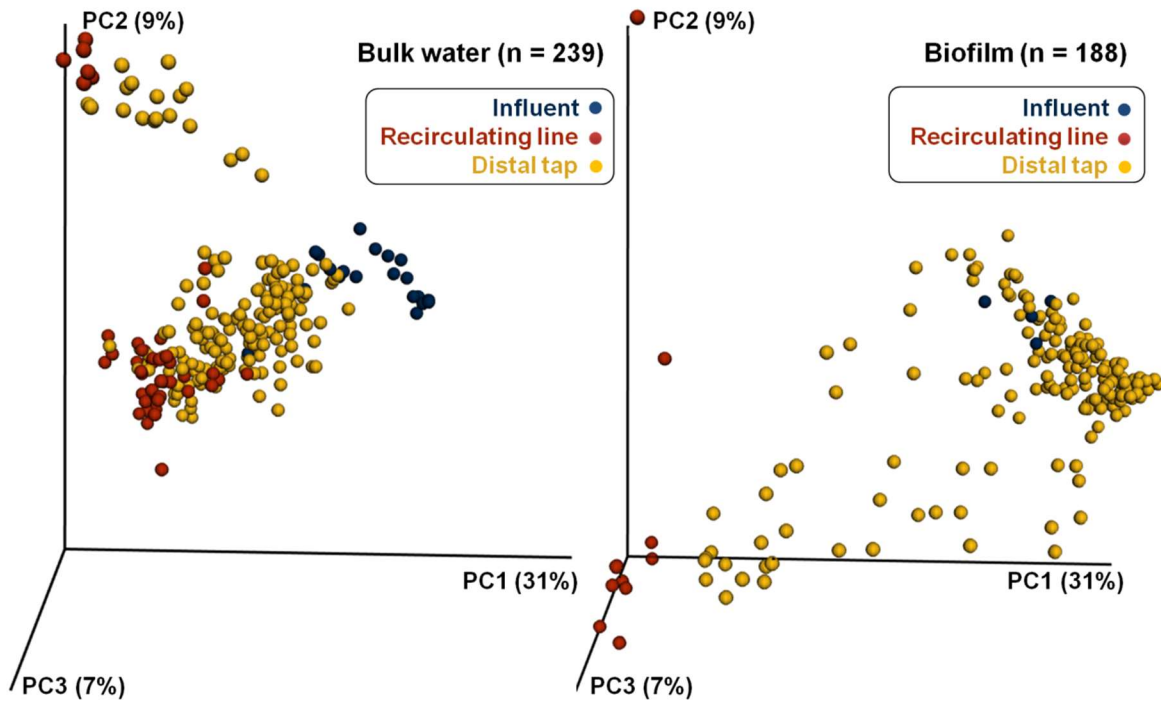


Figure S4. 3D beta diversity plot of phylogenetic dissimilarity patterns comparing sampling location across all samples of each phase (bulk water n=239, biofilm n=188).

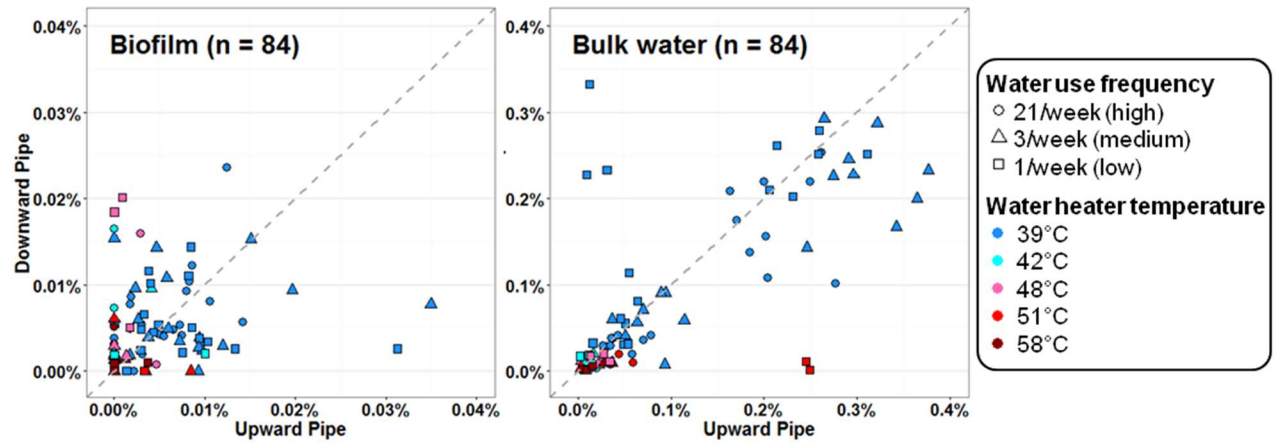


Figure S5. Paired relative abundance of *Legionella* spp. in downward and upward pipes (n = 84 pairs of samples for each). The grey dashed line demarcates a 1:1 ratio (downward = upward), indicating no effect of pipe orientation.

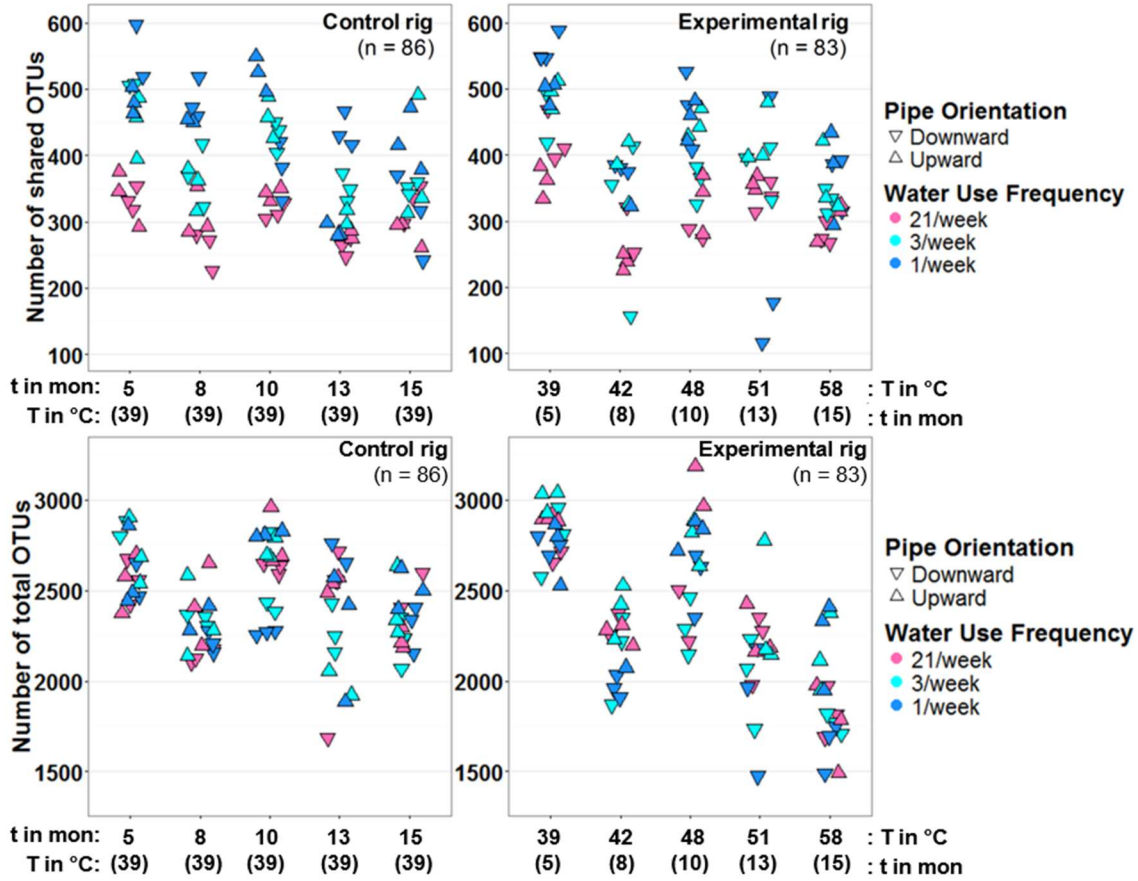


Figure S6. Number of shared and total OTUs in paired bulk water and biofilm samples at distal taps. Time is abbreviated as t in month (mon) with temperature as T in °C.

Table S1. Quality control for PICRUSt analysis. Weighted nearest sequenced taxon index (NSTI), ratios of PICRUSt-compatible OTUs and sequences are reported.

	Min	1st Quantile	Median	Mean	3rd Quantile	Max	Standard Deviation
NSTI	0.050	0.084	0.096	0.099	0.112	0.246	0.022
% of PICRUSt-compatible OTUs	0.078	0.126	0.148	0.162	0.175	0.679	0.066
% of PICRUSt-compatible sequences	0.492	0.774	0.820	0.813	0.854	0.960	0.063

Table S2. Ammonia concentration across time and water heater temperature setting. Measurements were taken at influent and recirculating line sampling portals. Net decrease in ammonia concentration was calculated as the difference between influent and corresponding recirculating line ones.

Influent	Recirculating Line					
	Control rig			Experimental rig		
Ammonia (mg/L)	t (months)	Ammonia (mg/L)	Net decrease (mg/L)	T (°C)	Ammonia (mg/L)	Net decrease (mg/L)
0.56	5	0.57	-0.01	39	0.49	0.07
0.48	8	0.16	0.32	42	0.15	0.33
0.41	10	0.08	0.33	48	0.10	0.31
0.42	13	0.06	<u>0.36</u>	51	0.34	<u>0.08</u>
0.46	15	0.08	<u>0.38</u>	58	0.36	<u>0.10</u>

Table S3. Relative abundance of *Nitrosomonas* spp. at recirculating line across time and water heater temperature setting. Relative abundance was calculated using aggregated sequences of samples from the same group.

Control rig			Experimental rig		
t (months)	Bulk water	Biofilm	T (°C)	Bulk water	Biofilm
5	3.88×10^{-5}	0	39	2.30×10^{-5}	0
8	7.79×10^{-5}	0	42	8.45×10^{-6}	0
10	NA	0	48	NA	0
13	2.16×10^{-5}	0	51	5.50×10^{-6}	0
15	3.51×10^{-5}	0	58	2.45×10^{-6}	0

Table S4. Impact of water heater temperature on the proportion of operational taxonomic units and sequences shared between biofilm and bulk water sample pairs. Tukey Honestly Significant Differences test at 95% confidence intervals were applied to One-way analysis of variance results (n = 68 sample pairs at t = 13 months or 15 months in both rigs). Statistical significance was set at 0.05 and italic grey font suggested non-statistically significant.

Groups	Proportion of shared OTUs		Proportion of shared sequences	
	ΔMean	P_{adjusted}	ΔMean	P_{adjusted}
51°C _{13 month} vs 39°C _{13 month}	0.026	0.046	<i>0.004</i>	<i>0.992</i>
58°C _{15 month} vs 39°C _{15 month}	0.031	0.005	<i>0.026</i>	<i>0.258</i>
39°C _{15 month} vs 39°C _{13 month}	<i>0.010</i>	<i>0.717</i>	<i>0.018</i>	<i>0.559</i>
58°C _{15 month} vs 39°C _{13 month}	0.041	<0.0002	0.044	0.013
51°C _{13 month} vs 39°C _{15 month}	<i>0.016</i>	<i>0.341</i>	<i>-0.014</i>	<i>0.759</i>
58°C _{15 month} vs 51°C _{13 month}	<i>0.016</i>	<i>0.357</i>	<i>0.040</i>	<i>0.038</i>
Levene's test Pr (>F)	0.392		0.062	

Table S5. Impact of flushing frequency on the proportion of operational taxonomic units and sequences shared between biofilm and bulk water sample pairs (n = 169). Kruskal and Wallis test with Tukey and Kramer (Nemenyi) test was applied with statistical significance set at 0.05 and non-statistically significant ones in italic grey font.

Groups	Proportion of shared OTUs		Proportion of shared sequences	
	Δ Median	P _{adjusted}	Δ Median	P _{adjusted}
3/week (high) - 21/week (medium)	0.03378	<0.0001	0.058321	<0.0001
1/week (high) - 21/week (low)	0.05115	<0.0001	0.062514	<0.0001
1/week (medium) - 3/week (low)	<i>0.01738</i>	<i>0.14</i>	<i>0.004193</i>	<i>0.97</i>
Levene's test Pr (>F)	< 0.001		< 0.0001	

Table S6. Impact of flushing frequency on the proportion of operational taxonomic units and sequences shared between biofilm and bulk water sample pair from each rig and time/temperature. One-way ANOVA with Tukey HSD test or Kruskal and Wallis test with Tukey and Kramer (Nemenyi) test was applied depending on Levene's test results. Statistical significance set at 0.05, with non-statistically significant values indicated in italic grey font.

Proportion of shared OTUs (control rig)										
Water use frequency	5 months		8 months		10 months		13 months		15 months	
	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}
3/week(medium) - 21/week (high)	0.039	0.001	0.030	0.003	0.047	<0.001	0.040	0.007	<i>0.025</i>	<i>0.059</i>
1/week (low) - 21/week (high)	0.067	<0.001	0.083	<0.001	0.055	<0.001	0.031	0.024	<i>0.019</i>	<i>0.173</i>
1/week (low) - 3/week (medium)	0.029	0.016	0.054	<0.001	<i>0.008</i>	<i>0.570</i>	<i>-0.008</i>	<i>0.735</i>	<i>-0.006</i>	<i>0.818</i>
Levene's test Pr (>F)	<i>0.444</i>		<i>0.613</i>		<i>0.178</i>		<i>0.150</i>		<i>0.202</i>	
Sample size (pairs)	16		17		18		17		18	
Proportion of shared OTUs (experimental rig)										
Water use frequency	39°C		42°C		48°C		51°C		58°C	
	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Median	P _{adjusted}
3/week(medium) - 21/week (high)	0.031	0.009	<i>0.036</i>	<i>0.083</i>	0.045	<0.001	<i>0.029</i>	<i>0.341</i>	<i>0.015</i>	<i>0.284</i>
1/week (low) - 21/week (high)	0.054	<0.001	0.071	0.003	0.059	<0.001	<i>-0.025</i>	<i>0.562</i>	<i>0.031</i>	<i>0.099</i>
1/week (low) - 3/week (medium)	0.024	0.040	<i>0.036</i>	<i>0.111</i>	<i>0.014</i>	<i>0.120</i>	<i>-0.054</i>	<i>0.103</i>	<i>0.016</i>	<i>0.851</i>
Levene's test Pr (>F)	<i>0.587</i>		<i>0.702</i>		<i>0.241</i>		<i>0.161</i>		0.009	
Sample size (pairs)	18		15		17		15		18	
Proportion of shared sequences (control rig)										
Water use frequency	5 months		8 months		10 months		13 months		15 months	
	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Median	P _{adjusted}	Δ Mean	P _{adjusted}	Δ Mean	P _{adjusted}
3/week(medium) - 21/week (high)	0.085	<0.001	0.084	<0.001	0.095	0.034	0.067	0.035	0.034	0.003
1/week (low) - 21/week (high)	0.107	<0.001	0.1012	<0.001	0.098	0.002	<i>0.029</i>	<i>0.430</i>	0.028	0.010
1/week (low) - 3/week (medium)	<i>0.022</i>	<i>0.43</i>	<i>0.018</i>	<i>0.441</i>	<i>0.004</i>	<i>0.662</i>	<i>-0.038</i>	<i>0.284</i>	<i>-0.005</i>	<i>0.790</i>

Levene's test Pr (>F)	<i>0.288</i>		<i>0.063</i>		0.001		<i>0.173</i>		<i>0.573</i>	
Sample size (pairs)	16		17		18		17		18	
Proportion of shared sequences (experimental rig)										
Water use frequency	39°C		42°C		48°C		51°C		58°C	
	ΔMean	Padjusted	ΔMean	Padjusted	ΔMedian	Padjusted	ΔMean	Padjusted	ΔMean	Padjusted
3/week (medium) - 21/week (high)	<i>0.048</i>	0.002	<i>0.051</i>	<i>0.110</i>	0.093	0.014	<i>0.038</i>	<i>0.463</i>	<i>0.007</i>	<i>0.822</i>
1/week (low) - 21/week (high)	0.067	<0.001	0.078	0.023	<i>0.093</i>	<i>0.071</i>	<i>-0.080</i>	<i>0.136</i>	<i>0.006</i>	<i>0.862</i>
1/week (low) - 3/week (medium)	<i>0.018</i>	<i>0.266</i>	<i>0.027</i>	<i>0.528</i>	<i>0.0003</i>	<i>0.804</i>	<i>-0.118</i>	<i>0.024</i>	<i>-0.0009</i>	<i>0.997</i>
Levene's test Pr (>F)	<i>0.143</i>		<i>0.728</i>		0.049		<i>0.051</i>		<i>0.069</i>	
Sample size (pairs)	18		15		17		15		18	