Distribution of Bacteria within Operating Laboratory Water Purification Systems

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Experiments were conducted to measure communities of bacteria within operating ultrapure water treatment systems intended for laboratory use. Samples from various locations within Milli-Q Plus and Milli-Q UV Plus systems were analyzed for populations of planktonic bacteria at weekly intervals over 3 months of operation. Relatively high initial densities of planktonic bacteria (10^8 to 10^9 bacteria per ml) were seen within both units when they were challenged with source water of poor quality, although the product water continued to be acceptable with regard to bacterial numbers, resistivity, and endotoxin concentration. Under more normal operating conditions, significant differences were seen in planktonic populations throughout the systems with excellent product water quality. A great deal of variability was observed in biofilm populations analyzed from various system surfaces after 3 months of operation. The concentrations of planktonic bacteria and biofilm densities were much lower in the unit containing a UV lamp. These findings suggest that a range of microenvironmental conditions exist within purified water systems, leading to variable populations of bacteria. However, product water of excellent quality was obtained despite the bacterial communities.

The efficient operation of water purification systems is important since the microbiological quality of ultrapure water is of fundamental concern in laboratory science, medicine, and a wide range of industries. Bacterial growth and biofouling within these systems present potentially serious problems for these applications because of biodeterioration and the presence of bacteria or their products in the purified water (19, 20). Despite this significance, surprisingly little research has focused on the microbiology of these systems, and no comprehensive studies have addressed their microbial ecology.

Oligotrophic bacteria are capable of reproduction under conditions that are usually considered nutrient restricted (12). Such organisms are found in low-nutrient environments like drinking water (14, 15, 23) and attain population densities of 10^6 to 10^7 cells per ml in distilled water (3). Oligotrophic aquatic environments are also favorable for the colonization of surfaces (16), where bacteria may be physiologically more active than their planktonic counterparts (5, 8). The extremely high surface/volume ratio in ultrapure water systems, including tubing and tank surfaces, activated carbon and ion-exchange particles, and filters, is conducive to the formation of biofilms that are very difficult to control (20). Circumstances are, therefore, favorable for the establishment and maintenance of prolific bacterial communities within purified water systems.

Planktonic bacteria and biofilms have been observed previously in operating purified water systems (17–20). Although none of these reports provided detailed or comprehensive information on the distribution of these bacteria throughout ultrapure water units, they documented the occurrence of relatively dense communities. Quantitative information is available describing heterotrophic (HPC) bacteria within potable water distribution systems, which resemble ultrapure water devices in some respects. For example, biofilm communities have been implicated as the primary source of planktonic bacteria in drinking water distribution systems, where they are thought to cause unexplained occurrences of indicator bacteria of sanitary significance (14, 28). The concentration of dissolved organic material (i.e., total organic carbon [TOC]) and the concentration of assimilable organic carbon (AOC) have also been quantitatively correlated with the tendency of water to support the regrowth of bacterial indicators and HPC bacteria within drinking water distribution systems (14, 24–27).

This study was initiated to describe the density of HPC bacteria that colonized operating ultrapure water systems. Planktonic and attached populations of these bacteria were enumerated at various points within two operating versions of the recently introduced Milli-Q Plus laboratory water purification unit.

MATERIALS AND METHODS

Laboratory water systems studied. Two commercially available, compact Milli-Q Plus water systems (Millipore Corp., Bedford, Mass.) were examined in this study. These systems were designed to produce high-purity water for laboratory use. The Milli-Q Plus water systems that were tested each included a four-cartridge purification pack (Qpak) that is intended for use where either reverse-osmosis (RO), deionized, or distilled water is used as the feed water for the system. This module included activated-carbon and ion-exchange resins housed within four polypropylene cylinders. Water flowed sequentially through the four cylinders. The tubing, fittings, and pumps were made of polytetrafluoroethylene, polyamide, Viton, and stainless steel. The other unit examined was a Milli-Q UV Plus system, which was identical to the Milli-Q Plus system but also included a high-intensity 185-nm UV lamp plus a polishing cartridge. The UV light and polisher were located immediately downstream from the Qpak module in this unit. Both systems were designed to eliminate stagnant side streams and included an intermittent recirculation function which operated automatically during periods when there was no demand for

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product water. A final filter (pore size, 0.22 μm), within a polycarbonate housing, was also a part of the dispensing point of both units. Figure 1 shows the design of the systems. Both systems were operated to simulate normal laboratory use by running 34 to 42 liters of water through them each week. Between the two experiments performed, new components were exchanged for all replaceable items, and the remainder of each system was cleaned and disinfected according to the manufacturer’s suggestions.

Sample collection. All glassware was cleaned in RBS-35 cleaning solution (Pierce, Rockford, Ill.), rinsed three times in tap water and then three times in Milli-Q-treated water, and then heated to 180°C overnight for sterility and elimination of endotoxin.

Samples were collected from various points within each system at weekly intervals for determination of planktonic bacterial levels and endotoxin concentration by disconnecting tubing at a connection, swabbing the tubing with 70% ethanol, and flushing water at that point for at least 2 minutes. A 500-ml sample was then collected in a sterile flask, and the tubes were reconnected. A modified sanitary sampling port (catalog no. YY20040S; Millipore) was incorporated in each unit immediately upstream from the three-way valve (Fig. 1). The run-to-waste interval was randomized between 2 and 10 min to minimize sampling artifacts within the systems. The temperature and resistivity of the product water were recorded for each sample interval, when possible.

Bacterial testing. Planktonic bacteria were enumerated by filtering appropriate aliquots (generally 10 to 100 ml) in triplicate through sterile 0.45-μm-pore-size filters (catalog no. AWG 047S1; Millipore). The filters were then placed on plates containing Difco R2A agar (23) supplemented with 0.002% tetrazolium chloride, and the plates were incubated at 25°C for 1 week prior to enumeration. The bacterial densities reported below, expressed in CFU per milliliter, are averages of triplicate enumerations.

The bacterial populations attached to the particulate matrix within each of the Opak cylinders were enumerated in a comparable manner. Sampling ports were cut near the tops and bottoms in each housing with a drill press following alcohol disinfection of the drill and the housing surface. Aliquots of the contents were removed with a sterile spatula and gently rinsed, and 1.0-g portions were treated with a procedure to desorb the bacteria from the surface of the solid matrix particles, as described by Camper et al. (1). This technique utilizes a cocktail of surfactants and protein in 0.1 M Tris buffer (pH 7.0) with blending (Ultra Turax; Tekmar, Cincinnati, Ohio) in an ice bath for 2 min at ca. 13,000 rpm. The resulting suspensions were spread onto R2A agar plates containing tetrazolium chloride that were incubated and counted as described above. The bacterial densities of these populations were expressed in CFU per gram of Opak matrix.

Densities of attached bacteria were also determined on the inner surfaces of system components such as tubes and housings following the experiments. Sections of the components were aseptically removed with sterile knives or an alcohol-disinfected (flamed) hacksaw. Bacteria were removed from measured areas of flat surfaces by scraping with a sterile spatula. The scraped material was then added to 10 ml of the blending cocktail described previously (1). Measured segments of tubing were also removed aseptically from Milli-Q units. Biofilm material was removed by brushing the interior of each segment with a small, sterile brush (Wilton catalog no. 418-1123). The interior of each segment of tubing and the brush were both rinsed with a standard volume (10 ml) of the cocktail. The resulting suspensions were then blended, plated, and counted as described above. The bacterial densities were expressed in CFU per square centimeter.

Endotoxin analysis. Bacterial endotoxin lipopolysaccharide (LPS) concentrations were measured in water samples from the test systems. The gel-clot method of the Limulus amebocyte lysate test was used to measure bacterial LPS concentrations according to the procedure described by the supplier of the Limulus amebocyte lysate reagent (Pyrotel; Associates of Cape Cod, Woods Hole, Mass.) (6). Pyrotel (0.1 ml) was mixed with 0.1 ml of the undiluted sample, and the preparation was incubated for 60 min at 37°C. A positive reaction was indicated by a firm clot which did not break when the tube was inverted. Endotoxin standards (Associates of Cape Cod) were used as positive controls for each test.

Organic carbon analysis. The concentrations of TOC in water samples from the test systems were determined with an Anatel model A-100P TOC analyzer.

Statistical analysis. Data for each sampling location were subjected to a one-way analysis of variance after log₁₀ transformation (0.5 was added to values of 0) to calculate the standard deviation. The error was then applied to the individual data points (which were the means of three replicate counts) that made up the curve for that sampling location.
RESULTS

The first phase of this study was conducted to examine the relative levels of planktonic bacteria and endotoxins within the test systems when source water of poor bacterial quality was used. The Milli-Q Plus systems were provided with RO-treated source water containing relatively high concentrations of bacteria for the first 13 weeks of the 16-week experiment. The results observed with both test systems were similar in this phase of the study. The plate counts of bacteria were between 10^6 and 10^8 CFU/ml (Fig. 2), and the mean endotoxin concentration was 0.6 ng/ml in the source water. Water samples collected before the final filter during this period of bacterial challenge contained higher levels of planktonic bacteria for the first month of operation (Fig. 2). This was followed by a reduction in bacterial levels of more than 1 order of magnitude. Following a transition to RO source water of better bacterial quality at week 13, the concentrations of planktonic bacteria remained relatively constant at the preshift levels. Throughout the experiment the densities of bacteria were markedly lower in the product water than in water samples taken from within the systems (Fig. 2). In addition, endotoxin concentrations were below the level of detection (3 pg/ml) in all samples throughout the 16-week experiment, and the resistivity of the product water remained acceptable at 18.2 MΩ-cm.

An independent experiment was performed with Milli-Q Plus and Milli-Q UV Plus systems under comparable conditions to follow the levels of TOC in the product water. With feed TOC levels of 300 μg/liter, the product water from the Milli-Q Plus system averaged 10 μg of TOC per liter, while the Milli-Q UV Plus unit reduced the TOC concentration to 5 μg/liter or less. The bacterial levels in the feed water increased over time from 10^8 to 1.8 × 10^6 CFU/ml. When the systems were challenged with 6 × 10^6 CFU/ml, corresponding to 1.8 × 10^5 μg of TOC per liter, the Milli-Q Plus unit yielded an average TOC level of 35 μg/liter, while the Milli-Q UV Plus system produced water with an average TOC concentration of 15 μg/liter.

During the second phase of this study, an experiment was performed to measure planktonic bacterial concentrations in the bulk water, as well as biofilm community densities, at various locations within each of the systems for 13 weeks. The source water was again produced by an RO unit, although the bacterial density in the water was much lower than the density in the water used for the early part of the previous experiments. The mean bacterial concentration in the source water samples analyzed at weekly intervals during the experiment was 0.38 CFU/ml (Fig. 3). As before, the resistivity of the product water remained at 18.2 MΩ-cm throughout the experiment. The levels of planktonic bacteria in the product water from both systems varied somewhat, but the average levels were consistently less than 10^2 CFU/ml (Fig. 3). In general, the bacterial densities in the bulk water from within the units were lower for the UV-containing water system. For example, samples taken before the final filter consistently revealed somewhat higher bacterial concentrations in the Milli-Q Plus system, with more fluctuation, than in the Milli-Q UV Plus unit (Fig. 3). Also, the levels of planktonic bacteria appeared to increase with
TABLE 1. Population densities of biofilm communities within components of Milli-Q Plus laboratory water systems

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sample location</th>
<th>Milli-Q UV Plus unit</th>
<th>Milli-Q Plus unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Feed to pump</td>
<td>$3 \times 10^2$ CFU/cm²</td>
<td>$3 \times 10^2$ CFU/cm²</td>
</tr>
<tr>
<td>2</td>
<td>Pump to Qpak</td>
<td>$7 \times 10^1$ CFU/cm²</td>
<td>$4 \times 10^1$ CFU/cm²</td>
</tr>
<tr>
<td>3</td>
<td>Qpak to UV light</td>
<td>$1 \times 10^1$ CFU/cm²</td>
<td>NA²</td>
</tr>
<tr>
<td>4</td>
<td>UV light to polisher</td>
<td>6 CFU/cm²</td>
<td>NA²</td>
</tr>
<tr>
<td>5</td>
<td>Qpak to sample port</td>
<td>NA</td>
<td>$1 \times 10^4$ CFU/cm²</td>
</tr>
<tr>
<td>6</td>
<td>Sample port to three-way valve</td>
<td>$2 \times 10^2$ CFU/cm²</td>
<td>$2 \times 10^2$ CFU/cm²</td>
</tr>
<tr>
<td>7</td>
<td>Three-way valve to final filter</td>
<td>$3 \times 10^1$ CFU/cm²</td>
<td>$4 \times 10^1$ CFU/cm²</td>
</tr>
<tr>
<td>8</td>
<td>Recirculation loop</td>
<td>$1.6 \times 10^4$ CFU/cm²</td>
<td>$3 \times 10^4$ CFU/cm²</td>
</tr>
<tr>
<td>9</td>
<td>T-connector to pump</td>
<td>$3 \times 10^2$ CFU/cm²</td>
<td>$9 \times 10^2$ CFU/cm²</td>
</tr>
<tr>
<td>10</td>
<td>T-connector</td>
<td>$2 \times 10^2$ CFU/cm²</td>
<td>$2 \times 10^2$ CFU/cm²</td>
</tr>
<tr>
<td>11</td>
<td>UV housing</td>
<td>NA</td>
<td>$2 \times 10^1$ CFU/cm²</td>
</tr>
<tr>
<td>12</td>
<td>Contents of Qpak cylinder 1-inlet</td>
<td>$6 \times 10^5$ CFU/g</td>
<td>$4 \times 10^4$ CFU/g</td>
</tr>
<tr>
<td>13</td>
<td>Contents of Qpak cylinder 1-outlet</td>
<td>$6 \times 10^5$ CFU/g</td>
<td>$1 \times 10^4$ CFU/g</td>
</tr>
<tr>
<td>14</td>
<td>Contents of Qpak cylinder 2-inlet</td>
<td>$2 \times 10^1$ CFU/g</td>
<td>$5 \times 10^3$ CFU/g</td>
</tr>
<tr>
<td>15</td>
<td>Contents of Qpak cylinder 2-outlet</td>
<td>$1 \times 10^1$ CFU/g</td>
<td>$1 \times 10^4$ CFU/g</td>
</tr>
<tr>
<td>16</td>
<td>Contents of Qpak cylinder 3-inlet</td>
<td>$5 \times 10^1$ CFU/g</td>
<td>$8$ CFU/g</td>
</tr>
<tr>
<td>17</td>
<td>Contents of Qpak cylinder 3-outlet</td>
<td>$9$ CFU/g</td>
<td>$7$ CFU/g</td>
</tr>
<tr>
<td>18</td>
<td>Contents of Qpak cylinder 4-inlet</td>
<td>$6$ CFU/g</td>
<td>$8$ CFU/g</td>
</tr>
<tr>
<td>19</td>
<td>Contents of Qpak cylinder 4-outlet</td>
<td>$3$ CFU/g</td>
<td>$6$ CFU/g</td>
</tr>
</tbody>
</table>

¹ Sample numbers correspond to numbers in Fig. 1.
² NA, not available.

the flow between the UV module and the final filter in the Milli-Q UV Plus unit. This trend was even greater between the Qpak and the final filter in the Milli-Q Plus unit.

Bacterial biofilms were also assessed within each of the test systems at the conclusion of the second phase of the study. The populations of attached bacteria were markedly less dense on the surfaces of common components in the Milli-Q UV Plus system (Table 1). However, the levels of biofilm bacteria observed within the recirculation loop tubing and T-connectors of both systems were similarly higher than the levels in most of the other components. Interestingly, some evidence of a biofilm was found on the UV lamp housing. Relatively high densities of bacteria were also found on the surfaces of the granular matrixes from some of the Qpak cylinders (Table 1). However, differences were observed between the numbers of biofilm bacteria in the Qpak modules of the two systems. With the exception of the initial stage of the first cylinder, the densities of attached cells in the UV-containing system were as much as 2 orders of magnitude lower for the first two cylinders of the Qpak module than for the comparable components in the Milli-Q Plus unit. Control samples from unused Qpak modules revealed a mean concentration of 3.1 CFU/g (range, 0 to 13 CFU/g), which was only slightly lower than the values found in the third and fourth cylinders in Qpak modules from operating systems (Table 1).

**DISCUSSION**

Only limited previously published information describing the occurrence and distribution of bacteria in ultrapure water systems is available. Therefore, some of the previously published papers describing work on drinking water systems are used in the discussion below because the systems described may be the oligotrophic environments most similar to the environments described in this report with respect to data on biofilms, HPC bacteria, and dissolved organic carbon (i.e., TOC/AOC ratio).

Mittleman (17, 19) has reviewed problems resulting from bacteria in ultrapure water systems and has provided evidence that between $10^2$ and $10^6$ bacteria per ml have been found in the final product water of an operating 18-MΩ·cm unit. Our findings demonstrated that both Milli-Q Plus systems produced water containing much lower numbers of heterotrophic bacteria, although the levels of these bacteria were higher prior to the final filter when the systems were challenged with elevated levels of bacteria in the source water (Fig. 2). These results are similar to the results of reports which documented the different bacterial populations with flow (14, 24), water from domestic RO units (19), and other home water treatment devices (9). Such results are not surprising when viewed within the context of oligotrophs such as pseudomonads, which can grow to levels of $10^6$ to $10^7$ bacteria per ml in distilled water (3). On the other hand, lower levels of HPC bacteria were found within both Milli-Q Plus systems and in the product water when source water of better microbiological quality was used, although the UV-containing system consistently contained fewer bacteria (Fig. 3). In addition, the low concentrations of bacterial endotoxin (i.e., concentrations below the level of detection) in the product water of both units confirmed the excellent microbiological quality of the product water (21).

The growth of bacterial populations in oligotrophic aquatic environments, such as treatment and distribution systems for potable and ultrapure water, is controlled by factors such as the concentration and nature of nutrients, temperature, disinfectants, turbidity, and pH (11, 14, 15, 17, 20, 24). Of these factors, nutrients have probably received the most attention from researchers studying aquatic systems without disinfectants because oligotrophic bacteria are capable of attaining relatively high numbers in low-nutrient water (3, 12, 14, 24). Bacterial growth, for which ambient nutrients were used, was probably responsible for increases in HPC bacterial numbers with flow (time) within both of the Milli-Q Plus systems in the second phase of this study (Fig. 3). Similar increases in planktonic heterotrophic bacterial levels have been described as water moved within operating municipal drinking water distribution systems (14, 24), as well
as within ultrapure water systems (20). The suggestion that the increases in bacterial numbers resulted from growth for which ambient nutrients were used is supported by the concomitant decreases in both TOC and AOC levels with flow (time) in those studies. AOC is defined as the fraction (ca. 1 to 10%) of the TOC within water that can be utilized by oligotrophic bacteria (25–27). As a result, AOC has been demonstrated to be an accurate predictor of the potential for bacterial growth within potable water systems (14, 15, 24). In addition, statistical correlations have been made between the concentrations of both TOC and AOC and the levels of HPC bacteria in potable water (15, 24, 27) and ultrapure water systems (17, 20). Similar correlations have been reported between endotoxin and TOC levels, suggesting that the two parameters may be interrelated in a closed-loop purified water system (18).

An examination of the Milli-Q Plus test systems for densities of attached bacteria also provided insights into the microbial ecology of laboratory ultrapure water units. It has been proposed that biofilm growth and detachment account for most of the planktonic bacteria in drinking water systems (20, 28). Conditions within the recirculation loop of both Milli-Q Plus units promoted the occurrence of the highest levels of biofilms observed (Table 1), except for the Qpak module. This observation might be explained by more flow in the loop, imposed by the recirculation cycles that operated automatically in the Milli-Q Plus units, since positive relationships have been demonstrated between shear force and biofilm processes, including attachment and substrate loading rates (4). The presence of biofilms within different parts of the Milli-Q Plus systems (Table 1) examined in this study is consistent with previously published reports documenting biofilms within purified water units (18, 19, 22), as well as within operating municipal potable water systems (14, 28). However, the previous studies did not examine biofilm populations in the various parts of laboratory water systems. The range of biofilm densities at the sampling locations suggests that factors such as substrate material, the chemical or physical properties of the substrate, and the entrapment of planktonic bacteria from the bulk liquid during recirculation cycles may have affected the magnitude of the attached microbial community at the various sampling sites. For instance, materials within the Qpak modules, such as ion-exchange resins (7) and activated carbon (2), are known as surfaces that favor biofilm development and the matrix could have acted as a physical filter. These mechanisms might explain the observation of higher density biofilms at the inlet end of both Qpak modules (Table 1). In addition, the high surface area/volume ratios of ultrapure water systems, as well as oligotrophic conditions, also promote biofilm formation (16, 20).

Differences in bacterial populations and TOC concentrations between the Milli-Q Plus and the Milli-Q UV Plus systems were noteworthy. In our second-phase experiment, in which source water of better microbiological quality was used, markedly lower levels of bacteria were observed in most parts of the UV-containing system (Fig. 3 and Table 1). These observations suggest that concentrations of bacterial nutrients were lower in that unit than in the Milli-Q Plus system and that the reduced biofouling within the Milli-Q UV Plus unit should extend the functional life of components such as the Qpak module. On the other hand, the observation of similar product water quality in the two units might bring into question the utility of the UV-polisher module. However, the markedly reduced biofilms seen in the UV-containing unit could lead to significant operational differences with long-term use beyond the time frame of the experiments reported here. The observation that biofilms of relatively high density penetrated further into the Qpak module of the unit without the UV lamp (Table 1) supports this idea. Furthermore, this observation may be explained by the polisher module that is unique to the UV-containing system, although it has been demonstrated that UV irradiation increases AOC concentrations in aquatic systems (10). In addition, the observation of attached bacteria on the surface of the tube surrounding the UV lamp was somewhat surprising, although a similar finding has been reported previously in an aquatic setting (13).

In summary, our results showed that Milli-Q Plus laboratory water systems contained a range of environmental regimes that resulted in different levels of planktonic and attached microbial communities. Several factors, including flow rates and properties of component surfaces and nutrients, were probably responsible for these differences. The incorporation of the UV lamp plus the polisher module in the Milli-Q UV Plus system reduced levels of planktonic bacteria and biofilm formation. Although bacterial densities were relatively high in various parts of the units examined, the quality of the final product water, as judged by conductivity and concentrations of both bacteria and endotoxin, was excellent. This information will be useful in the design of new ultrapure water treatment devices and in guiding the maintenance of various types of clean-water systems.

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