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Persistence of MS-2 and PRD-1 bacteriophages in an ultrapure water system

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The persistence of bacteriophages MS-2 and PRD-1 was evaluated in tap water, in reverse osmosis (RO) permeate, and in three locations within an ultrapure water system; ultrapure samples included pre- and post-UV sterilization and post-mixed bed ion exchange tank. The inactivation rates for MS-2 were calculated as $\log_{10}$ reduction per hour and per day: $k = -(\log_{10} C_t/C_o)/t$. PRD-1 was found to persist with no significant loss of infectivity in all water purity environments evaluated. Inactivation of MS-2 was dependent on water quality and pH. Short-term inactivation rates for chlorinated tap water, post-RO, pre-UV, post-UV and post-ion exchange sample locations were 0.028, 0.455, 0.231, 0.191 and 0.168 $\log_{10}$ h^{-1}, respectively. Long-term inactivation rates for chlorinated tap water, post-RO, pre-UV, post-UV and post-ion exchange sample locations were 0.485, 0.911, 0.605, 0.632 and 0.684 $\log_{10}$ day^{-1}, respectively. Since phages were found to remain intact as well as to lyse in the ultrapure water environment, the phages have the potential to contaminate the ultrapure water environments of the microelectronics, pharmaceutical and power generation industries in both colloidal and dissolved form. Further work is proceeding to generate standardized and cost-effective methods to detect viruses in water environments.

Keywords: viruses; coliphage MS-2; coliphage PRD-1; inactivation; reverse osmosis; ion exchange; ultraviolet light (UV)

Introduction

The microelectronics industry has made extraordinary advances in device manufacturing technologies in recent years. Order of magnitude escalations in device complexities have pushed the technologies to the megabyte (Mb) levels found today, and can push the same technologies to the gigabyte (Gb) regimes and beyond in a few years. The devices are currently manufactured in nearly one hundred specific processes that, when assembled, create the three-dimensional microelectronic processors that are integral components of today's personal computers. A significant fraction of these process steps involves a rinse step where the microelectronics devices are contacted with ultrapure water; the quality of the ultrapure water is therefore critical to the overall microelectronics device manufacturing process.

Ultrapure water is a dynamic entity whose definition has changed as the device contamination limits have become more stringent (Table 1). In the case of ultrapure water, a contaminant is typically defined as any element or chemical compound that isn't H^+, OH^-, or H_2O. Major measurables of the quality of ultrapure water can include total oxidizable carbon (TOC), resistivity (a measure of the total ionic contamination in water), viable bacteria (colony forming units per liter, CFU L^{-1}), silica in parts per billion (ppb, $\mu g\ L^{-1}$), colloidal particles (count per liter @ 0.05-μm diameter and larger), and elemental chemical contaminants from aluminum to zinc. It is important to understand that equivalent standards do not at this time exist for the detection or enumeration of viruses in ultrapure water systems, or at the points of water use.

Although bacteria have been given some attention in the ultrapure water environment [8], viruses have not received similar study. One explanation for this phenomenon involves the relative size of viruses compared to the critical particle size or 'killer defect' size [9]. As shown in Table 2 [9], the summary of future semiconductor product size shows that today's circuits are constructed with line widths smaller than a typical 1-μm sized bacterium, but larger than a 50-nm sized virus. A killer defect can occur on today's circuits when a particle of 80 nm deposits on a device with a 350-nm line width [9]; however, in 1998, it is projected that a 50-nm sized particle can cause a killer defect when deposited on a device with a 250-nm line width. Since viruses are in the size ranges of 50 to 500 nm [2] and since the microelectronic devices will continue to decrease in size and increase in complexity, the importance of viruses in ultrapure water used in the microelectronics industry should increase. The problems associated with viruses and their breakdown products towards the microelectronics industry may be encountered within the next few years.

Unlike potable water treatment systems [12], chemical additives such as chlorine [11], chloramines, halogens and transition metals such as copper [4,13] and silver [11] cannot be used for disinfection in the ultrapure water environment as they are considered contaminants towards the ultrapure points of use [7]. Only the use of trace amounts of dissolved ozone has been recognized in the ultrapure water environment as a potentially effective anti-microbial agent. Although proven as a non-specific oxidizing agent, ozone decays upon reaction to dissolved oxygen. Dissolved oxygen, as shown in Table 1, is also viewed by many as a contaminant in the ultrapure water environment and therefore may have to be minimized.

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Table 1 Evolution of ultrapure water quality guidelines

Measure	Ultrapure water (1985)	Ultrapure water (1988)	Ultrapure water (1992)	Ultrapure water (1996)
TOC (ppb)	<20	<10	<5	<0.5
Resistivity (MΩ-cm @ 25°C)	17.9	18.0	18.2	>18.2
Viable bacteria (CFU per liter)	<60	<50	<10	<0.1
Silica (ppb)	5	3	1	<0.5
Particles (cpm @ 0.05 μm)	N/A	N/A	1000	<500
Dissolved oxygen (ppb)	N/A	N/A	100	<15
Aluminum	2	0.05	0.05	<0.05
Barium	N/A	0.05	0.05	<0.05
Calcium	N/A	2	<2	<0.1
Chloride	0.05	<0.05	<0.05	<0.05
Copper	0.02	<0.02	<0.02	<0.02
Iron	0.1	0.1	<0.02	<0.005
Lithium	N/A	0.05	<0.05	<0.05
Magnesium	N/A	0.05	<0.05	<0.05
Manganese	0.5	0.05	<0.05	<0.01
Potassium	0.3	0.1	<0.1	<0.1
Sodium	0.2	0.1	0.05	<0.05
Sulfate	0.1	0.05	<0.05	<0.05
Zinc	0.1	0.05	0.02	<0.02

Table 2 Summary of future semiconductor product characteristics

Type	1995	1998	2001	2004	2007
Line width[a] (μm)	0.35	0.25	0.18	0.13	0.10
DRAM (bits)	64M	256M	1G	4G	16G

[a]Line width: the limiting distance between component connections on an electronic circuit. If a line is damaged, communication between the device components will not occur correctly and result in a defect. It is important to note that a defect can result from the deposition of a particle that is a fraction of the actual line width.

This study was designed to evaluate the persistence of the bacteriophages MS-2 and PRD-1 in various treatment stages of an ultrapure water production facility.

Materials and methods

Water

Source water from the Martin Street well, located at the University of Arizona, Tucson, Arizona was transported to a pilot Ultrapure Water Pilot System [7]. Chemical and physical properties of water samples (Table 3) including pH and free chlorine were determined according to Standard Methods for the Examination of Water and Wastewater Analysis [1]. Total organic carbon (TOC) and resistivity were measured using an Anatel A-100 on-line TOC monitor (Anatel, Boulder, CO, USA). One-liter high density polyethylene containers were used to collect water samples. The containers were rinsed with ultrapure water and sterilized prior to use by autoclaving. Water was stored at 21°C for the duration of the experiment (10 days).

Preparation and assay of purified coliphages MS-2 and PRD-1

Cultures of *Escherichia coli* (ATCC 15597) and *Salmonella typhimurium* (ATCC 19585), grown for 18 h in tryptic soy broth (TSB; Difco, Detroit, MI, USA) at 37°C without shaking, were used to inoculate fresh TSB. These inocula were incubated for 3–6 h at 37°C with continuous shaking to obtain a fresh culture. Stock MS-2 (ATCC 15597B) and PRD-1 were serially diluted in Tris buffer pH 7.3 (Trizma base; Sigma, St Louis, MO, USA) to approximate concentrations of 10^5 pfu ml^{-1}. To tubes of molten overlay agar (TSB with 1% agar), 0.1-ml MS-2 phage dilution and 1-ml *E. coli* culture as well as 0.1-ml PRD-1 phage dilution and 1-ml *S. typhimurium* culture were added and mixed.

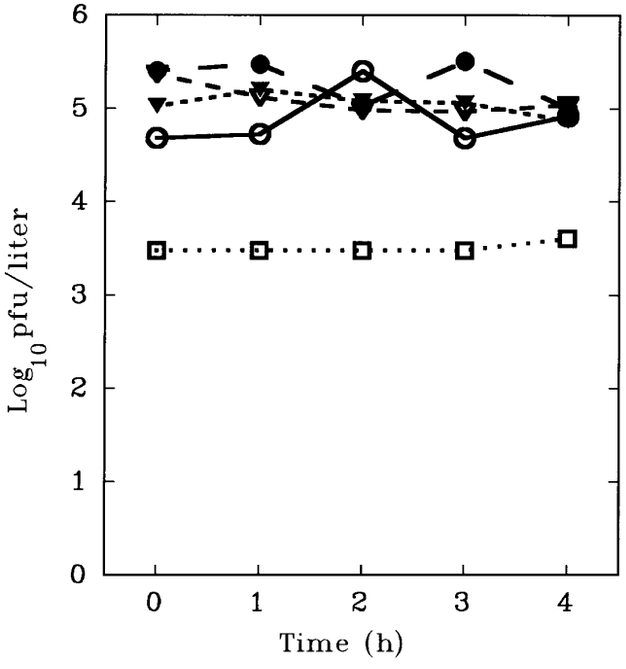

Figure 1 Short-term survival of bacteriophage PRD-1 in city water (○—○), post-RO water (● - - ●), pre-UV water (▽ --- ▽), post-UV water (▼ ---- ▼) and post-IE water (□ ..... □).

Table 3 Physical and chemical properties of water used in this study

Selected water environment	pH	TOC (μg L^{-1})	Resistivity (MΩ-cm)	Free Cl_2 (mg L^{-1})
Tap water	7.6	1000	0.0025	0.02
Post-RO[a]	8.9	100	1.0	0.0
Pre-UV[b]	6.7	3.5	18.1	0.0
Post-UV[b]	6.4	2.5	17.0	0.0
Post-IE[c]	7.0	3.0	l8.2	0.0

[a]RO: reverse osmosis.
[b]UV: ultraviolet sterilizer.
[c]IE: mixed bed ion exchange tank.

The mixtures were poured into petri dishes containing tryptic soy agar (TSA; Difco). After 18–24 h at 37°C, 6–7 ml Tris buffer was added to plates with confluent plaques and allowed to incubate further for a maximum of 1 h to induce the phage particles to the agar surface for separation and concentration. The liquid fraction was recovered from the plates and centrifuged (15 300 × *g* for 10 min at 10°C), and the resulting supernatant was centrifuged again (100 000 × *g* for 3 h at 10°C). The pellet was resuspended in sterile Tris buffer and stored at 4°C. The phage stocks were titered prior to use.

Serial dilutions were made in Tris buffer, added to test tubes containing 3 ml of molten overlay agar and 1 ml of 3- to 6-h cultures of *E. coli* and *S. typhimurium*, and poured onto TSA plates. The plates were incubated for 18–24 h at 37°C, after which the plaques were enumerated and the log reduction and inactivation rates of MS-2 and PRD-1 were calculated.

Experimental design

Water for this study was collected: (i) at the tap; (ii) downstream of the reverse osmosis unit (Post-RO) (Millipore, Bedford, MA, USA); (iii) upstream of the ultraviolet sterilizer (Aquafine, Valencia, CA, USA) in the polishing loop [7] of the ultrapure water system (Pre-UV); (iv) downstream of the ultraviolet sterilizer in the polishing loop of the ultrapure water system (Post-UV); and (v) downstream of the mixed bed ion exchange [7] tank in the polishing loop of the ultrapure water system (Post-IE). Experiments were performed in duplicate at room temperature (23 ± 2°C). Purified stock viruses were added to the various samples at time zero. At time intervals of 0, 1, 2, 3, 4 h and 1, 2, 4, 7, 10 days, 1-ml samples were assayed in triplicate for bacteriophage concentrations. The experiments were run in triplicate.

Data analyses

Linear regression analyses were used to calculate inactivation rates for each experimental system. The inactivation rate (k) can be expressed by the equation:

$$k = -(\log_{10} C_t / C_o)/t$$

where C_t and C_o are the final and initial viral concentrations, respectively, and t represents time in hours for the short-term persistence and days for the long-term persistence. The data were also calculated as the mean $\log_{10} N_t/N_o$ pfu ml^{-1}, which expresses the reduction in viral numbers at each time interval; N_t and N_o are the final and the initial numbers of viruses, respectively. The Student's t-test [10] was used for analysis of variance to determine significant differences in the inactivation rates among the various water environments.

Results

The ultrapure water system was designed to produce water with contaminant levels at or below those recommended for ultrapure water in 1992 (Table 1). Chemical analyses of selected water samples were performed and the results are shown in Table 3. The TOC, conductivity (reciprocal of resistivity), and the free chlorine level decreased as the water proceeded through the treatment components. The pH, however, increased from 7.6 to 8.9 as the water passed through the reverse osmosis unit. The pH of the ultrapure water returned to 7.0 as the water recirculated in the polishing loop of the ultrapure water pilot system.

No apparent ($P \leq 0.05$) decrease in the concentration of PRD-1 phage was noted in the time interval used for analysis, nor was any apparent decrease in concentration noted as a function of water purity (Figure 1).

Significant decreases in the concentrations of MS-2 were observed in the different types of water. The MS-2 phage was inactivated by less than 0.1 log in the tap water environment over a 4-h time period (Figure 2); however in ultrapure water, inactivation proceeded rapidly. The high pH environment provided by the reverse osmosis permeate was found to be the most aggressive environment of those studies; over the 4-h time period, the concentration of MS-

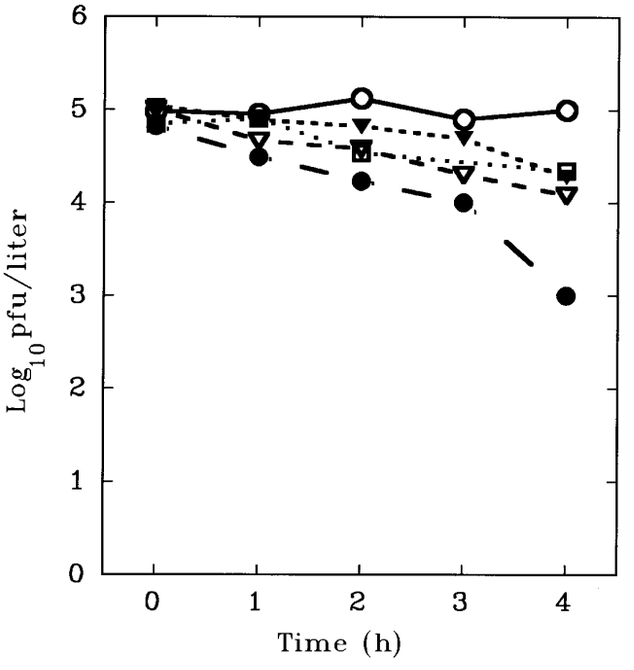

Figure 2 Short-term survival of bacteriophage MS-2 in city water (○—○), post-RO water (● -- ●), pre-UV water (▽ --- ▽), post-UV water (▼ ---- ▼) and post-IE water (□ ..... □).

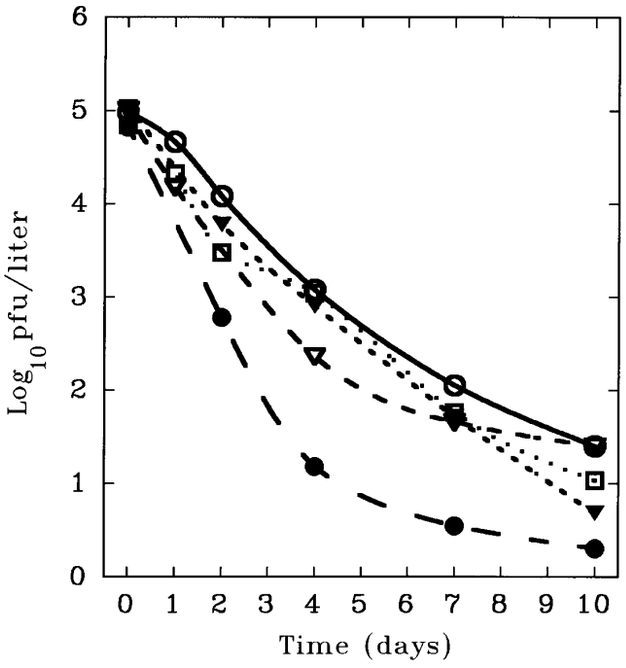

Figure 3 Long-term survival of bacteriophage MS-2 in city water (○ — ○), post-RO water (● -- ●), pre-UV water (▽ --- ▽), post-UV water (▼ ---- ▼) and post-IE water (□ ..... □).

2 was reduced by 99%. A three log reduction in MS-2 was observed within 3 days in the high pH environment of the Post-RO water, 6 days in the environment of the ultrapure water and 7 days in the remaining samples, respectively (Figure 3).

The inactivation rates (*k* values) for MS-2 in the various waters observed are shown in Table 4. Initially, the inactivation rate of MS-2 was greater by a factor of seven in ultrapure water (Pre-UV, Post-UV, Post-IE) when compared with the inactivation rates observed in the chlorinated tap water. The inactivation rate of MS-2 in the Post-RO water was approximately 16 times greater than the inactivation rate observed in the chlorinated tap water.

Discussion

No information is currently available on the mechanisms of viral inactivation in ultrapure water systems. It is believed that ultrapure water, being a powerful solvent, can become more thermodynamically unstable as it is brought towards theoretical purity limits. Previous work has shown [7] that highly reactive radicals in the ultrapure water environment including hydroxyl and superoxyl ions have relatively long lifetimes; these reactive radicals can effectively oxidize matter on contact in the water environments. It has also been previously demonstrated that MS-2 is sensitive to inactivation above pH 9.0 [3].

Table 4 MS-2 inactivation rate constants

Selected water environment	*k* ($\log_{10}$ h^{-1}) short-term	*k* ($\log_{10}$ day^{-1}) long-term
Chlorinated tap water	0.028	0.485
Post-RO	0.455	0.911
Pre-UV	0.231	0.605
Post-UV	0.191	0.632
Post-IE	0.168	0.684

In the case of the aggressive solvent-like nature of ultrapure water, the virus surfaces (capsid, tail fibers, etc) may be attacked through the mechanism of direct oxidation when exposed to these environments. The virus head can degrade and lyse from the action of the reactive radicals in the ultrapure water environment [6]; dispersions consisting of capsid fragments, tail fiber fragments as well as partially oxidized viral genetic material can be released into the water environment, potentially into the points of use and can have the potential to contaminate the product. Based on previous work [7], it is speculated that these viral reaction products will be composed of low molecular organic compounds that can be highly recalcitrant in ultrapure water systems.

There exists a significant difference between the inactivation rates of MS-2 and PRD-1 in different waters. PRD-1, a DNA virus, is more thermally stable in water environments [13] than MS-2, an RNA virus. This may be due to the greater stability of DNA in water environments than RNA. Since the water becomes a more powerful solvent as contaminants are removed, the potential to degrade the genetic material may also increase. The PRD-1 virus also has an internal lipid which may make the internal structure of the capsid more resistant to degradation in the ultrapure water environment.

PRD-1, a DNA-containing *S. typhimurium* phage, and MS-2, an RNA-containing *E. coli* phage were selected as the test viruses for this initial study. They represent two different types of DNA and RNA viruses which have been used in many studies in the environment as well as showing resistance to commonly used water disinfectants. It should be noted that typical enteric viruses were not chosen for this study, as one would not expect to find enteric or fecal host bacteria in an oligotrophic environment such as that produced by an ultrapure water system. This study was designed to show the effects of water chemistry on viruses representative of those viruses that could exist in this environment. The experiment times were designed to represent the maximum amount of time a virus would be expected to navigate an entire water treatment system. The break at 4 h was chosen to represent an estimate for the amount of time a virus would potentially require to navigate an industrial-sized ultrapure water polishing loop system.

The effect of pH compared to the solvent effect of water can be shown using the MS-2 concentration profiles as seen in Figures 2 and 3. Note that the water at the post-RO sample location is in a transition stage between tap water and ultrapure water with respect to TOC, resistivity, and total dissolved solids levels. The water's pH at this location, however, is elevated. The high concentration of hydroxyl ions in water was far greater than the concentration of free reactive radicals and, therefore, dominated the viral inactivation mechanisms.

The importance of the viruses and their decay products in the ultrapure water-requiring industries (microelectronics, pharmaceutical, and power generation) can be related to the

viruses' potential to exist as both colloidal and dissolved contaminants in the ultrapure water environment. Viruses, based on the results generated, may therefore have the potential to contaminate an expensive end-product such as a semiconductor device, cause an electrical defect, and destroy that device. A 50-nm virus can additionally have the effect of depositing on the electrical device and altering the device's electrical current-carrying characteristics, using mechanisms currently employed by microbes during adhesion to fixed surfaces in water treatment systems [5]. The viral fragments (dissolved and/or colloidal contaminants) could additionally deposit on the wafer and alter the electrical characteristics of the wafer in a fine dispersion as well as contributing to the background TOC level in the ultrapure water system. Previous work has shown [8] that detection of TOC and particle concentrations was beyond the scope of the on-line analyzers currently used in the microelectronics industry. Decay products can be tested through the collection of contamination onto a filter and viewing under a scanning electron microscope for increases in visual and elemental contaminant profiles; this is the approach of future work.

In this study, we demonstrated that bacteriophages MS-2 and PRD-1 could persist in ultrapure water systems in between the various stages of treatment. Until cost-efficient and standardized methods are available to characterize and enumerate viruses in the ultrapure water environment, the relationship between viruses and the loss in yields in a microelectronics fabrication facility may continue to be poorly defined. Further work is proceeding towards characterization and detection of viruses in water.

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References

1 American Public Health Association. 1992. Standard Methods for the Examination of Water and Wastewater, Washington, DC.
2 Atlas R. 1984. Classification and identification of microorganisms: systematic of fungi, algae, protozoa and viruses. In: Microbiology Fundamentals and Applications (Aker K and G Payne, eds), p 452, MacMillan Publishing Company, New York, NY.
3 Battigelli DA and MD Sobsey. 1993. The inactivation of hepatitis A virus, poliovirus and coliphage MS-2 by lime softening and chlorine/monochloramine disinfection. In: Proceedings of the Water Quality Technology Conference. pp 479–492, American Water Works Association, Miami, FL.
4 Bitton G and V Friehofer. 1978. Influence of extracellular polysaccharide on the toxicity of copper and cadmium toward *Klebsiella aerogenes*. Microb Ecol **4**: 119–125.
5 Caldwell D and J Lawrence. 1986. Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb Ecol 12: 299–312.
6 Chang SL. 1970. Modern concept of disinfection. In: Proceedings of the National Specialty Conference on Disinfection. pp 635–682, American Society of Civil Engineering, New York.
7 Governal R, A Bonner and F Shadman. 1991. Oxidation and removal of organic particles in DI water polishing loops using combined ozone and UV treatment. In: Proceedings of Watertech '91 Conference and Exposition, pp 74–88, San Jose, CA.
8 Governal R. 1992. Overview of the concept of ultrapure water. In: Characterization and Removal of Organic Contaminants in Ultrapure Water Systems. pp 39–45, University Press, New York.
9 Henley M. 1995. Market review: semiconductor industry expansion should continue. Ultrapure Water 13(5): 15–20.
10 Hoerl A, M Nashed, J McKetta and I Silverberg. 1984. Mathematics. In: Perry's Chemical Engineering Handbook (6th edition) (Perry RH, DW Green and JO Maloney, eds), pp 85–87, McGraw-Hill Book Company, New York.
11 Thurman RB and CP Gerba. 1989. The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. CRC Crit Rev Environ Control 18: 295–315.
12 United States Environmental Protection Agency. 1975. National interim primary drinking water regulation. Fed Regist 40: 59 566–59 574.
13 Yahya MT, TM Straub, CP Gerba and AB Margolin. 1991. Inactivation of bacteriophage MS-2 and poliovirus in copper, galvanized and plastic domestic water pipes. Int J Environ Health Res 1: 76–96.