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Microbiological Quality Assurance of Purified Water by Ozonization of Storage and Distribution System

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ABSTRACT Purified water storage and distribution systems at ambient temperature are highly susceptible to microbial contamination and formation of biofilm. The impact of two disinfection regimens with ozone as a function of time, the heterotrophic plate counts (HPC), and the concentration of total organic compounds (TOC) in purified water were investigated over a period of 4 years. We have established that concentrations of ozone of 70 ± 20 ppb in the production regimen and 250 ± 50 ppb in the disinfection regimen are sufficient to maintain a low bioburden and low TOC in a recirculating distribution system. The purified water that entered into the distribution system has low HPC (0.01 CFU/mL), indicating a reduction by ozone in the storage tank by up to approximately 120-fold. Over 4 years, 94–98% of the microbial counts were in the category 0–5 CFU/mL, and none in category ≥50 CFU/mL. In spite of increased TOC in the inlet water, up to 40 ppb, the microbial counts in purified water in the distribution loop were unaffected. The study emphasizes that the critical points regarding microbial contamination of the purified water system are user point valves and the tubes used for transferring water to equipment. The specified ozone level prevents microbial growth and formation of biofilm in the distribution system that might otherwise endanger the water quality by sporadic release of microbes.

KEYWORDS Ozone, Purified water, Heterotrophic plate count, Total organic carbon, Disinfection

INTRODUCTION

Storage and distribution of purified water at ambient temperature greatly increases the risk of microbial contamination. Pretreatment and treatment steps for preparing purified water in pharmaceutical industry are well known and accepted. However, much less is known about avoiding degradation of quality during storage and distribution. Given that isolated mobile bacterial cells can attach to almost any surface in an aqueous environment, a biofilm can develop, especially in a closed-loop water system (Mittelman et al., 1987; Riedewald, 1997). Biofilms can be considered as microbial ecosystems, comprising different microbial strains and species in aggregates, which cooperate

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efficiently in protecting the component cells against environmental stress and in facilitating nutrient uptake for survival (Gillis & Gillis, 1996; Schaule & Flemming, 1997; Chandy & Angles, 2001; Sihorkar & Vyas, 2001; Stoodely et al., 2001). Biofilms in water systems act as reservoirs of microorganisms that are sporadically released into the water, causing great increase of heterotrophic plate counts (HPC). Biofilms are difficult to detect, inactivate, and remove. In industry, such serious contamination of purified water systems can lead to contamination of products (LeChevallier et al., 2003).

Only limited studies have been published regarding control and prevention of microbial contamination of purified water by biofilm (Gillis & Gillis, 1996, Riedewald, 1997, Schaule & Flemming, 1997). Even fewer studies have been published on the use of ozone as a promising disinfectant of pharmaceutical water at ambient temperature. Current knowledge of the behavior of biofilms results from extensive studies of drinking water over the past two decades (Viera et al., 1999; Liu et al., 2002; Ndiongue et al., 2005).

The main heterotrophic microorganisms that contribute to high bacterial counts in distribution systems use biodegradable organic compounds as a source of carbon. However, there is no universal understanding as to what level of organic carbon is needed to support the microbial growth and how to measure it. It is reported that total organic carbon (TOC) greater than 2.4 mg/L could be associated with regrowth of coliform bacteria in chlorinated drinking water systems (LeChevallier et al., 1991). The level of biodegradable organic carbon, measured as the concentration of assimilable organic carbon (AOC), is the main factor associated with bacterial growth. AOC levels below 10 μg/L limit the growth of heterotrophic bacteria (Van der Kooij, 1992).

Proper design of the purified water system, in combination with effective disinfection, should prevent microbial contamination and formation of biofilm (Meltzer, 1997; Bruno & Lorenz, 1999; Stucki et al., 2005). The system can be disinfected, either thermally or chemically (hydrogen peroxide, ozone). However, several studies have shown that bacteria in biofilms are less susceptible to killing than planktonic cells when treated with antimicrobial agents (Elvers et al., 2002). This can be attributed to several factors, including reaction and diffusion processes that limit the agent's accessibility to bacteria, and phenotypic changes caused by stress and adaptation of the bacteria.

Of all the physical and biocidal treatments of pharmaceutical water systems, ozone appears to hold the greatest promise as an effective, product-compatible biocide. Although its use has been known since the beginning of the 20th century for disinfecting drinking water, its utilization for pharmaceutical water treatment has been mentioned mainly since the beginning of the 1990s only (Governal & Shadman, 1992; Meltzer, 1997; Collentro, 1999). Recently, it was reported that hydroxyl radicals, resulting from ozone decomposition, play a significant role in microbial inactivation and oxidation of organic material (Lee et al., 1991; Siddiqui et al., 1997; Summerfelt, 2003). Ozone is relatively unstable, its half-life in distilled water being about 25 min at 20°C due to autodecomposition and oxidation, yielding hydroxyl radicals that initiate a radical chain reaction. These radicals can damage the active ingredients in products (Zhou & Smith, 1995).

Based on this fact, it has been recommended that ozone should be added constantly during disinfection (Collentro, 1999). Since a biocide is itself a contaminant in purified water it must be removed prior to use (Meltzer, 1998). Ultraviolet light at 254 nm rapidly converts ozone into oxygen and an excited oxygen intermediate.

The degree of disinfection by ozone correlates with its concentration (C) and contact time (t), from which values required to reduce different types of heterotrophic bacteria to given levels under specified conditions can be derived. Total inactivation of suspensions containing 10^6/mL and 10^7/mL of *Staphylococcus aureus* has been reported using ozone concentrations of 0.3 mg/L and 2.61 mg/L, respectively. A standard planktonic strain of *C. albicans* was inactivated after 5 min of exposure to 3.3 mg/L of ozone; however, fresh isolates of *C. albicans* from saliva showed higher resistance (Faria et al., 2005). A total of 50% of a planktonic isolate of *B. cepacia* was inactivated by 0.2 mg/L ozone in 10 min at 25°C; however, 50% removal of a biofilm of *B. cepacia* required an ozone concentration of 2 mg/L and 2-h contact time (Koenig et al., 1995).

The majority of studies of bacterial resistance to ozone have been conducted on standard strains, and a very limited number on naturally occurring heterotrophic bacteria. No studies of growth of the naturally occurring heterotrophic bacteria following ozonization in an industrial purified water distribution system have been published.

In the present study, we present the design of a new purified water system. The effectiveness of ozone on the inactivation of heterotrophic bacteria, and the growth that occurs after ozonization in the purified water storage and distribution system, have been evaluated. The impact of two disinfection regimens as a function of time, the concentration of total organic compounds (TOC), and the heterotrophic microbial counts (HPC) in purified water were determined. The day of the week for sampling, the production environment, together with the mode of sampling and the influence of production procedures on microbial growth were also considered. The results of 4 years of experience with an ambient temperature purified water system are presented.

MATERIALS AND METHODS

Design of Purified Water System

The inlet water to the purified water system is drinking water that complies with EU regulations. It is produced from ground water and has an average TOC concentration of 0.78 ± 0.46 mg/L. The purified water system comprises a pretreatment plant (filtration, ion exchange), a two-stage reverse osmosis unit (RO), a storage tank for purified water, an ozone generator and UV lamp, and a distribution loop with 26 user points, 2 of them connected to washing machines (Fig. 1).

The system was designed to minimize dead legs by using pressure gauges with diaphragm seals. The pumps use water as the seal lubricant, all fittings are of sanitary type, air gaps are provided at all drain lines, backflow is prevented, the system is drainable, no filter is used on the distribution loop, and constant flow and recirculation are ensured. The smoothness and resistance against corrosion of the inner surfaces was ensured by appropriate selection of the materials (stainless steel type 316L, roughness of surfaces less than 1 μM, RA (*define*) < 0.8 μM) and proper welding techniques. The gaskets are made of poly tetra fluoro ethylene (PTFE) and Viton. A storage tank with a capacity of 3 m^3 is used as an ozone contact chamber and is continuously ozonized. From Monday to Friday, the average residence time of water in the tank is from 0.5 to 2 h, depending on the use. No water is consumed on weekends and it is recirculated back to the tank. The flow velocity through pipes with 2.5–5-cm diameter is 0.8 to 1.2 m/sec. The length of pipes constituting the distribution loop is 100 m.

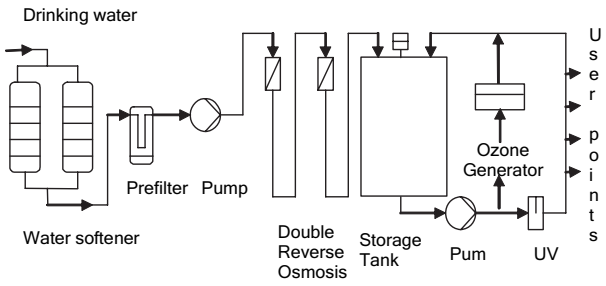

FIGURE 1 **Scheme of the Recirculating Purified Water System and Its Functioning Using Ozone Disinfection at Ambient Temperature.**

The recirculating purified water system is computer controlled (Fig. 1). The chemical quality of the purified water is controlled by an in-line Endress + Hauser conductivity sensor (Endress + Hauser, Reinach, Switzerland), by an off-line Anatel monitor of TOC (Anatel Corporation, Loveland, CO, USA) and by an in-line ozone concentration monitor, Orbisphere model 3600 (Orbisphere, Geneva, Switzerland).

The purified water system complies with U.S. pharmacopoeia. The target requirements for monitoring parameters are: conductivity, not more than 1.3 μS/cm at 25°C, TOC, not more than 500 ppb, microbial quality, alert level ≥5 CFU/mL, and action level ≥50 CFU/mL.

Ozone Generation

Ozone is generated by electrolysis of purified water (MEMBREL MkII; Ozonia, Duebendorf, Switzerland). The maximum capacity is 3 g of ozone per hour. The flow of water through the generator is between 60l/h (minimum) and 160l/h (maximum). During electrolysis, the hydrogen formed at the cathode is released to the atmosphere while the ozone generated at the anode is released into water. Water electrolysis takes place at a potential of 3 to 5 V with an applied current density of 0.5 to 2 amp/cm^2. The target ozone concentration was controlled by an ozone detector calibrated periodically on air according to the manufacturer's instructions.

Ozone Concentration

Different concentrations of ozone are used for the production and disinfection regimens. During production, the average residence time of the water in the ozonized storage tank was 0.5 to 2 h at 70 ± 20 ppb of

ozone (the average C^*t was 2.1 mg*min/L to C^*t 8.4 mg*min/L). The time period between two disinfections was 5 to 7 days. The complete storage and distribution system was disinfected weekly with 250 ± 50 ppb of ozone, for a specified time. The conductivity of the resulting purified water was below 1.3 µS/cm at 25°C and its average temperature was 21.2°C (range 18.7–23.5°C).

Ozone Destruction

Before water enters the distribution piping, residual ozone is destroyed by UV light (model RBE-8R; Aquafine Corporation, Valencia, CA) at 254 nm, the germicidal frequency, in a fraction of a second. Following UV treatment the water is monitored by an in-line ozone detector (Orbissphere) for the absence of ozone. The water transferred into the distribution loop had no detectable residual ozone. The availability of in-line sensors with improved accuracy (±3 ppb) and reproducibility (±2% of reading) has made it possible to detect lower ozone residuals.

Design of the Study

The efficiency of ozone disinfection in the storage and distribution system at ambient temperature and its ability to prevent bacterial growth after the weekly disinfection were studied over 1 year to give the performance qualification, PQ. PQ was divided into PQ 1 (study of the system itself, 4 weeks) and PQ 2 (study of specific impact on the purified water quality, 48 weeks). To study the effectiveness of ozone and microbial growth following ozonization, more than 5000 samples were analyzed in the 4-year investigation period and associated with the concentration of ozone, duration of disinfection, fluctuation of the content of the organic compounds expressed as TOC, and HPC in the inlet purified water. The absence of coli forms, *Pseudomonas aeruginosa* and *Pseudomonas cepacia*, was determined. Growth after ozonization and impact of the external environment on microbial counts were evaluated by statistical treatment of the microbial data collected during the period between two disinfections and by comparison of microbial samples collected by different ways of sampling. The conductivity was below 1.3 µS/cm at 25°C during the whole study period.

Microbial Sampling and Methods

Samples of purified water (at least 400 mL), taken after the reverse osmosis, at the outlet of the storage tank, and from all user points on the production line, were collected in sterile flasks after flushing for 2 min. Total viable counts, and viable counts of the coli forms *Pseudomonas aeruginosa* and *Pseudomonas cepacia*, were determined by filtering 100 mL of purified water through a 0.45-µm membrane filter. The membrane with total cells was incubated on R2A-agar (Oxoid; Basingstoke, Hampshire, England) for 5 days at 30–35°C. The membrane with coli forms was incubated on Endo agar (Becton Dickinson, Sparks, MD) for 48 h at 30–35°C. The membranes with *Pseudomonas aeruginosa* and *Pseudomonas cepacia* were incubated on Cetrimid agar (Merck KgaA, Darmstadt, Germany); the first incubated at 43 ± 2°C for 48 h and the second at 30–35°C for 48 h. A media control plate was always prepared to eliminate error due to media contamination.

Biofilm samples were scraped from surfaces using sterile swabs. The swab was then transferred to R2A-agar plates and incubated for 5 days at 30–35°C. Colonies were counted and expressed as CFU/swab.

Chemical Sampling and Methods

TOC and conductivity measurements were performed in compliance with the current U.S. pharmacopoeia. TOC was measured on-line with an Anatel monitor (Anatel Corporation, Loveland, CO, USA), which was calibrated once a year by the manufacturer. Conductivity and temperature were measured on-line with an Endress + Hauser conductivity sensor (Endres & Hauser, Reinach, Switzerland). The system was calibrated according to the requirements of the current USP pharmacopoeia.

Statistical Analysis

HPC levels are reported as geometric mean and range, and TOC as mean ± standard deviation. Statistically significant differences of the influence on the bacterial count of sampling days, personnel, and other variables were assessed with the software package SAS. Significance was tested at the 0.05 level of probability.

RESULTS

Impact of the Sampling Day on the Microbial Quality of Water in PQ 1

The PQ 1 study assessed the performance of the storage and distribution system without interference from the external environment (personnel, dust from process operations). The objective was to compile baseline chemical and microbial features to establish the effectiveness of ozone in the storage tank in the production regimen, and the effectiveness of the weekly disinfection of the storage tank and distribution loop. Water samples were taken daily direct from the valve at each user point and their microbial quality determined (Fig. 2). On comparing the microbial content of samples taken from the loop each day from Monday (first day after disinfection) to Friday (last day before disinfection), no significant variation in HPC was observed. HPC values in 725 samples taken from the distribution loop were all in the category 0–5 CFU/mL. HPC in the inlet purified water following RO and entering the tank was 0.10 CFU/mL (range, 0–0.21 CFU/mL) and TOC was 5.8. ± 6.1 ppb. The HPC in the distribution system was 0.30 CFU/mL (range, 0–3.56 CFU/mL). The water in the ozonized tank was a combination of inlet purified water and recirculated water from the loop. Ozonization in the storage tank with 70 ± 20 ppb of ozone for 0.5 to 2 h reduced the HPC to a level in the tank below 0.01 CFU/mL. The results confirmed that microbial growth in the distribution system was still controlled at least 5 days after the weekly disinfection of the whole system.

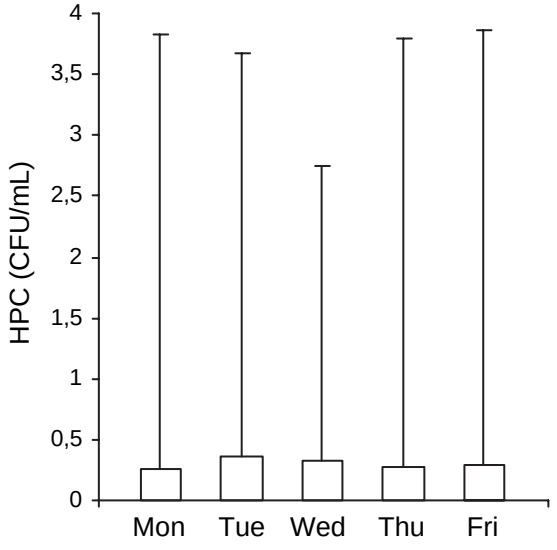

FIGURE 2 **Number of Heterotrophic Plate Counts (CFU/mL) in PQ 1 From Monday to Friday per Weekday Over 4 Weeks (Mean, Range 0–*x*; *N*=725).**

Impact of External Environment on the Water Quality in PQ 2

The objective of the PQ 2 study was to further assess the effectiveness of the earlier established ozone disinfection regimes, taking into consideration the impact of external contamination caused by dust generating operations and human procedures. Samples for microbial control were taken each day, with or without tubes, from different locations in the system, in the same manner as for normal water take-off. Conductivity and TOC were monitored continuously and the values were found to be in the same range as during PQ 1. TOC in the distribution loop was 6.1 ± 5.3 ppb. The HPC of inlet water was 0.19 CFU/mL (range, 0–1.67 CFU/mL) and TOC was 6.1 ± 5.2 ppb. The HPC in the distribution system was 1.22 CFU/mL (range, 0–104.00 CFU/mL). Ozonization of water in the storage tank reduced the HPC by 120-fold. HPC at the outlet of the tank was below 0.01 CFU/mL (Table 1).

Comparing results from PQ 1 and PQ 2, some adverse microbial trends were noticed (Table 1, Fig. 3). The values for HPC differed significantly. Further, the proportions of HPC in category 0–5 CFU/mL decreased to 93.77%, in category 5–50 CFU/mL was 6.16%, and category ≥50 CFU/mL was 0.08%. Furthermore, 92.45% of samples (278) taken on Monday were in category 0–5 CFU/mL Mondays and 93.88% of samples (245) taken on Fridays. No significant impact of the day of sampling on the HPC was observed.

The external environment and the mode of sampling were found to have a significant impact on the HPC measured on purified water. To control the deterioration of microbial water quality we considered the general contamination of the system, the mode of sampling, and external, localized contamination of the user points. Samples were taken at user points (UP) located after the reverse osmosis unit, UP RO, in the production areas, UP 6, and in the laboratory, UP 11. All samples taken without tubes from UP RO and UP 11 were in category 0–5 CFU/mL, but only 83.83% of those taken using tubes in dust generating areas (user point UP 6 in granulation area) (Fig. 4).

These results were confirmed by a detailed inspection of the cleanliness of user points in production and

TABLE 1 **Heterotrophic Plate Counts (HPC) and Total Organic Carbon (TOC) Analyses During the PQ 1, PQ 2 Phases and the Further Three Years of Monitoring**

Parameter	PQ 1	PQ 2	Year 1	Year 2	Year 3
HPC inlet water	0.10	0.19	0.14	0.13	0.17
(CFU/mL)MeanRange	0–0.21	0–1.67	0–0.49	0–2.78	0–2.36
HPC outlet water	below 0.01	below 0.01	below 0.01	below 0.01	below 0.01
(CFU/mL)Mean					
HPC all user points	0.30	1.22	0.75	0.40	0.49
(CFU/mL)MeanRange	0–3.56	0–104.00	0–53.20	0–51.20	0–52.00
HPC on Mondays	0.26	1.53	0.77	0.58	0.62
(CFU/mL)MeanRange	0–3.56	0–52.00	0–51.00	0–34.20	0–18.00
HPC on Fridays	0.30	1.03	0.44	0.31	0.27
(CFU/mL)MeanRange	0–3.56	0–18.70	0–22.50	0–51.20	0–52.00
HPC in category 0–5 CFU/mL (%)	100	93.77	96.73	98.39	98.59
Number of samples for HPC level	725	1316	1077	968	970
TOC in inlet water (ppb, mean ± SD)	5.8 ± 6.1	6.1 ± 5.2	16.0 ± 8.0	26.0 ± 0.18	41.0 ± 27.0
TOC at all user points (ppb, mean ± SD)	6.0 ± 5.4	6.1 ± 5.3	16.6 ± 7.9	22.9 ± 8.3	34.7 ± 17.8
Number of samples for TOC	29	240	256	266	244

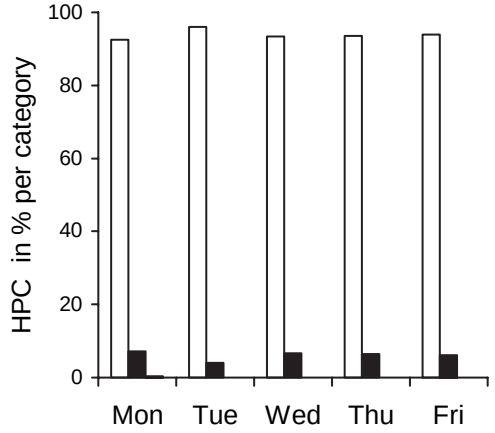

FIGURE 3 **Microbial Counts at the User Points per Weekday in PQ 2 in % for Different Categories:(□) 0–5 CFU/mL, (■) 5–50 CFU/mL, *N*=1316.**

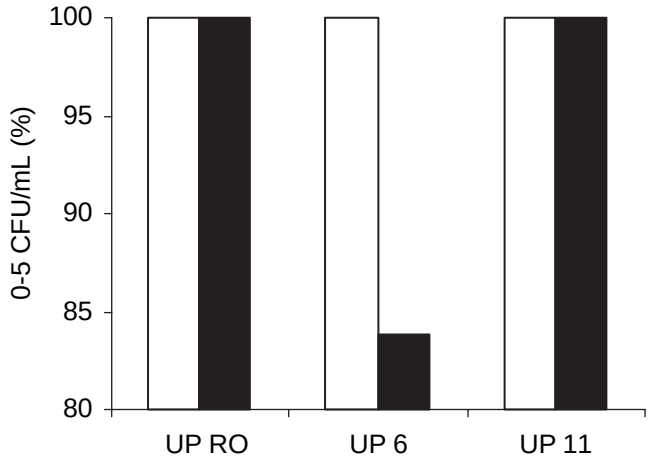

FIGURE 4 **Comparison of Microbial Counts in Category 0–5 (%) of all Samples Over the PQ 1 (□) and PQ 2 (■) Periods at Specific User Points (UP): UP RO, Point After Reverse Osmosis, Before Ozonization; UP 6, Dust Generating Area (Direct Sampling From the System in PQ 1 (*N*=29) and Sampling With Tube in PQ 2 (*N*=68); UP 11, Laboratory Point as a Non-Dust-Generating Area (Direct Sampling From the System in PQ 1 (*N*=29); and Sampling With Tube in PQ 2 (*N*=220).**

the procedures for tube disinfection and handling. The user points in the rooms where preparation of granulate, final blend, and coating took place were partly covered with powder and swabbing revealed microbial contamination. Biofilm was observed as a thin, gelatinous film at the edge of the pipe valves. The specified routine disinfection of the storage tank and distribution loop with ozone did not prevent external contamination of the user points. Several corrective actions were implemented: replacement of user point valves to increase contact with ozonized water, additional external cleaning and disinfection of user points, improved disinfection procedure for tubes used for accessing water, and training of the production personnel in appropriate procedures. The effectiveness of these corrective actions was monitored over the following 3 years. HPC were always smaller than in PQ 2 and always within category 0–5 CFU/mL (Table 1).

These excellent microbial results, coupled with the low bioburden and low TOC of the inlet purified water, justified the gradual shortening of the duration of disinfection of the storage and distribution system from 48 h (PQ) to 2 h. The time between two disinfections was increased from 5 days to 6 days 22 h.

Microbial counts remained in the same range, although TOC increased.

Impact of Increased TOC on HPC Level

TOC in the inlet purified water increased from the initial 5.8 ± 6.1 ppb in PQ 1 to 41.0 ± 27.0 ppb over the 3 years of monitoring (Fig. 5). In spite of this increase in organic nutrients, however, only slight variations in HPC were observed from PQ 1 over the same period (Fig. 5, Table 1). Bacterial retention capacity and susceptibility to microbial growth of reverse osmosis subsystems remained unchanged and yielded inlet purified water of adequate quality. The mean HPC of the recirculated purified water from the distribution system ranged from 1.22 CFU/mL (range, 0–104.00 CFU/mL) in PQ 2 to 0.49 CFU/mL (range, 0–52.00 CFU/mL) in the third year.

The same trend of increasing concentration of TOC was observed in the purified water in the distribution system, but TOC was always slightly lower than in the inlet water. The 6-fold increase in concentration of organic nutrients did not lead to increased bacterial growth in the distribution system.

Control of HPC Increase after Weekly Ozonization

Despite the disinfection time being shortened to 2 h at 250 ppb of ozone, comparison of the results in the PQ 2 phase with those of the following 3 years of monitoring revealed that the percentage of HPC in

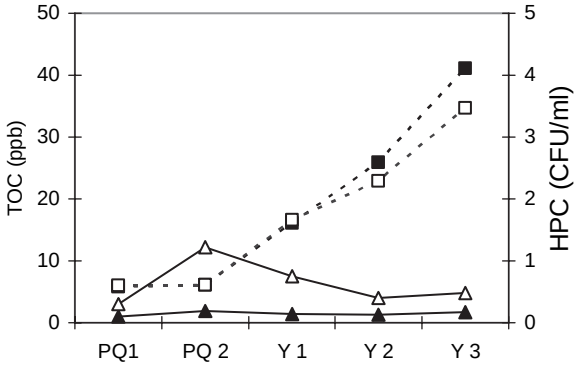

FIGURE 5 Mean of Total Organic Carbon (TOC, ppb) of Inlet Purified Water (■) and of Water in Distribution Lines (□); HPC (CFU/mL) in Inlet Water (▲) and at all User Points (Δ) From PQ 1 to PQ 2 Period, and First Year (Y 1), Second Year (Y 2), and Third Year (Y 3) of Monitoring.

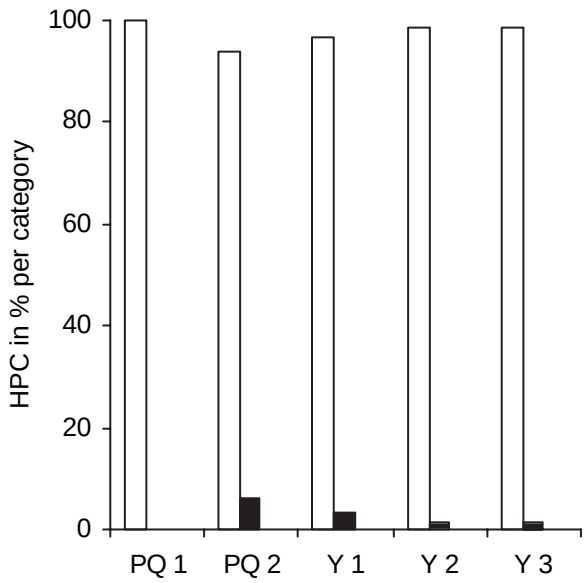

FIGURE 6 Microbial Results of the User Points Expressed as HPC in % for Different Categories: (□) 0–5 CFU/mL, (■) 5–50 CFU/mL From PQ 1 to PQ 2, First Year (Y 1), Second Year (Y 2), and Third Year (Y 3) of Monitoring.

category 0–5 CFU/mL increased from 93.77% to 98.59% in the third year and HPC at all user points decreased (Table 1, Fig. 6).

Levels of bacterial contamination were independent of the day of sampling. Mean HPC on Mondays were 0.26 CFU/mL in PQ 1, reached a maximum of 1.53 CFU/ml in PQ 2 and decreased to 0.62 CFU/mL in the third year of monitoring. The corresponding results for samples taken on Fridays were 0.30 CFU/mL, 1.03 CFU/mL, and 0.27 CFU/mL. In the approximate 7-day period between two disinfections, no increase of planktonic heterotrophic bacteria occurred within the distribution loop over the further 3 years of monitoring (Table 1). Over the total 4-year period slightly higher numbers of bacteria were always detected in the distribution system than at the outlet of the ozonized storage tank (Fig. 5). Thus, the HPC in the distribution loop increased along the distribution system from 0.01 CFU/mL at the outlet to a mean HPC that was 30 times (in PQ 1) to 120 times higher (PQ 2) than at the outlet.

DISCUSSION

In the 4-year assessment period it was demonstrated that 0.5- to 2-h treatment with 70 ± 20 ppb of ozone in the storage tank resulted in up to approximately 120-fold reduction of the number of planktonic

heterotrophic bacteria. Consequently, HPC in the purified water that entered the distribution loop was below 0.01 CFU/mL. Similar findings have been reported for treatment of drinking water (Lee et al., 1991).

This treatment schedule suppressed microbial growth in the distribution loop for approximately 7 days after its weekly disinfection by 250 ppb of ozone for 2 h. Reported ozone concentrations for control of microbes in water vary from 0.10 ppm to several ppm; however, other parameters that affect its effectiveness were not defined (Riedewald, 1997; Viera et al., 1999; Stucki, 2005). We have established that a concentration 70 ppb of ozone is sufficient to maintain low bioburden and TOC in a recirculating purified water distribution system. This, however, requires low bioburden and TOC content of the inlet water, coupled with good engineering practice. The fact that no variations in microbial counts were observed from Monday to Friday also confirmed the effectiveness of the ozone treatment regimens (Figs. 2 and 3). The low concentrations of ozone used also increase the life of the system components.

However, different findings were reported regarding growth after ozonization of the drinking water distribution systems. Studies of the effectiveness of much higher ozone concentrations (2.64 to 0.52 mg/L of ozone) revealed increasing bacterial growth 2 h after ozonization of drinking water. Significantly higher concentrations of ozone did not prevent the rapid bacterial growth after ozonization. This contradiction with our results could be explained by different chemical, physical, and microbial environments in the purified water system. The very low content of organic compounds in purified water increased the duplication time of heterotrophic bacteria to several days (Mueller, 1996). The long replication times of heterotrophic bacteria restricted growth for 7 days after ozonization. Additionally, purified water was constantly recirculated back to the ozonized tank, again reducing HPC of planktonic bacteria.

Microbial growth depends on the availability of TOC (Mittelman et al., 1987; Chandy & Angles, 2001; Lehtola et al., 2001; Liu et al., 2002). However, in our case, a 6-fold higher mean TOC did not affect HPC in the distribution system.

A slightly higher value of TOC in inlet water was observed than in distribution water. Lower TOC values in the distribution system could be caused by consumption of biodegradable organic compounds by bacteria, as reported several times for drinking water systems (LeChevallier et al., 1991; Liu et al., 2002).

In spite of the effective disinfection described above, approximately 30 to 120 times higher HPC were found repeatedly in samples from the distribution system than in samples taken from the outlet of the ozonized tank (Table 1). The sampling procedure was the same and the higher HPC counts were not the result of sampling error. Due to the short residence time of the water in the distribution loop (less than 3 min), the increased HPC were attributed to detachment of planktonic bacteria from biofilm as reported elsewhere. It was suggested that the critical parts of the system were the user point valves.

Investigation of the increased HPC at user points in dust generating production rooms showed that biofilm developed inside the user point valves. Similar results are known from the literature (Flemming & Kemkes, 1999; Klauer, 2001). Organic dust from the environment contaminated the exterior of the valve, enhancing the proliferation of the bacteria in the valve. The weekly disinfection of the system was not effective, due to only partial contact of the inner surfaces of the valves with ozonized water. After replacement with valves that gave increased contact with ozonized water, and after training the personnel in procedures, the HPC decreased. On the other hand, swabbing the interior of pipelines of the distribution system during maintenance works now showed biofilm in the system.

Samples of flowing water only indicate the concentration of planktonic (free floating) microorganisms present in the system and do not include the concentration of microorganisms attached to the surfaces as biofilm. However, the consistent appearance of elevated planktonic levels is usually an indication of advanced biofilm development, which requires remedial control.

CONCLUSIONS

More than 4 years of experience with a recirculating purified water system at ambient temperature has demonstrated that ozone is an effective and reliable disinfectant for controlling the microbial level in purified water. Furthermore, it prevents the formation of biofilm to an extent that could result in sporadic release of microbes to the water. Low TOC and low bioburden of the inlet purified water, proper selection of materials, and design of the system further contribute to the effectiveness of ozone. The study emphasizes that the critical points regarding microbial

contamination are the construction of user point valves and the mode of use of the tubes used for transferring water to equipment.

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