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Use of a laser diffraction particle counter to monitor integrity of reverse osmosis membranes and its inefficiency to detect bacteria in product water

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Summary

Submicron particle counting was evaluated as a method to monitor the integrity of reverse osmosis (RO) membranes continuously during operation. For this purpose, the method proved to be superior to conductivity measurements which are widely used. At retention rates of $\leq 10^4$, particle rejection corresponded well to microbial retention. Unfortunately, this method is susceptible to distortion by particles that are released from the inner surface of the cartridges. Therefore, it is suitable only for long term surveillance after sample washout time. Although the counter had a detection limit of 0.5 μm, it failed to detect bacteria that grew in the cartridge. Likewise, water-borne bacteria cultivated in bottles filled with pure water produced particle counts far lower than expected from their number which was determined by the spread plate technique.

Introduction

In a recent study (März et al., 1989), we found large differences in microbial retention rates of commercially available reverse osmosis (RO) cartridges. Apart from those differences already present in new membranes, damage to membranes during operation or sanitization has also been reported (Jacobs, 1981; Ridgway et al., 1984). As determination of microbial counts takes several days, a quick test for membrane integrity would be most desirable. As shown in our previous paper (März et al., 1989), conductivity measurements are not a suitable indicator for microbial retention. Other attempts to check the integrity of RO and UF membranes by determination of removal of macromolecular substances (Jacobs, 1981; Cooper and Derveer, 1982; Sarbolouki, 1982) proved not to be very sensitive either. Moreover, because this method implies the addition of such foreign substances it is not practicable for in-process controls in pharmaceutical production.

Faced with these problems, particle counting seemed to be a viable solution, since tapwater usually contains large numbers of particles (McCoy and Olson, 1986). Such particles should be completely removed by flawless RO membranes. Modern laser scattering particle counters are able to recognize particles ≥ 0.5 μm in diameter. Thus, it seemed reasonable to perform an investigation

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of whether microorganisms themselves are detected by such counters. This would be valuable for detection of the beginning of microbial growth on the product side.

Materials and Methods

RO system

The RO apparatus used is based on a Hemo-RO system (Millipore Corp., Eschborn, F.R.G.) stripped of all parts except the pump, pressure gauge, pressure vessel and the valve for adjustment of pressure. To the product and reject outlets of the pressure vessel, an inline sampling device was mounted, diverting part of the outflow to the counter (see Fig. 1). This device allowed product sampling directly at the outlet of the cartridge. It was verified that the polytetrafluoroethylene (PTFE) tubes did not release particles into the samples.

For the experiments, the following spiral wound RO cartridges were used:

	CA1	CA2	PA	PS
Membrane Material	cellulose triacetate	cellulose triacetate	polyamide	polysulfone
Manufacturer	Millipore	Millipore	Millipore	Millipore
Type	CDROOO25SH	CDROO25SH	CDRCO25SH	CDRSO25SH
Lot no.	C5P0649832	C3A7758403	119373	C7B1480550

At the time of this investigation, each of the RO elements had been in use for about 6 months.

Before being fed to the RO system, local tapwater was passed through a 3 μm carbon filter and adjusted to pH 7.1 $\pm$ 0.1 by injection of hydrochloric acid. When the polysulfone cartridge was used, the feedwater was softened because this membrane does not tolerate calcium.

After thorough sanitization with 3% formaldehyde solution as described in März et al. (1989), the system was run continuously.

Cultures in bottled RO water

Thoroughly cleaned and rinsed glass bottles were filled with 10 l of sterile filtered RO water. The bottles were inoculated with small numbers of bacteria that have been cultured in particle-free FIP (1976) phosphate buffer whose NaCl content

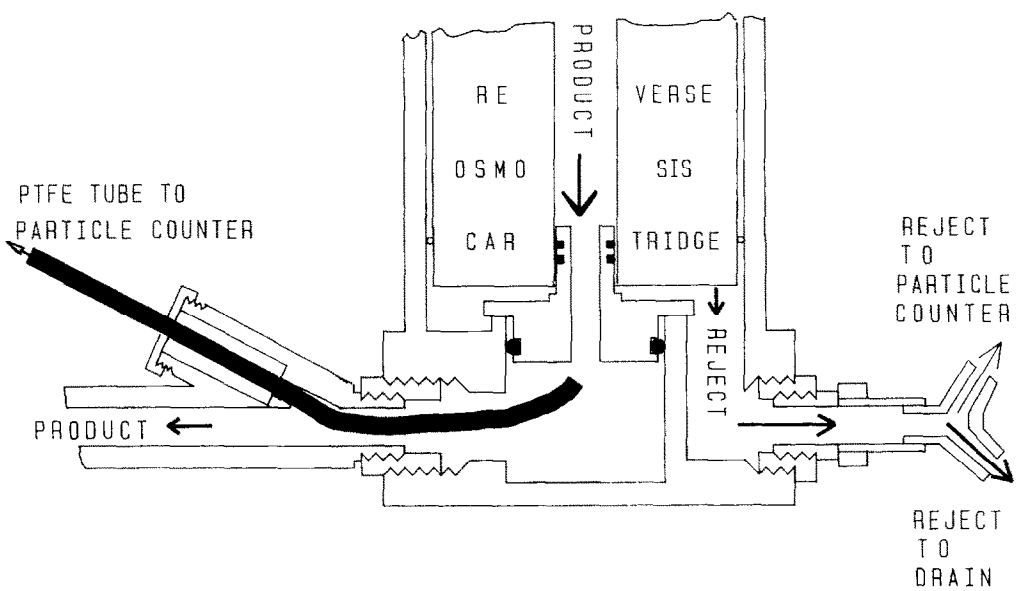

Fig. 1. Inline sampling system for RO apparatus. All tubes and the particle counter are flushed continuously even when not in use.

was reduced to 0.05%. *Flavobacterium capsulatum* DSM 30196 and *Chromobacterium violaceum* DSM 30191 were obtained from the DSM (German Collection of Microorganisms, Braunschweig). *Pseudomonas fluorescens* was isolated from product water of the CA2 cartridge, whereas the *Ps. paucimobilis* strain had been isolated from the polyamide cartridge during earlier experiments. *Xanthobacter* sp. was isolated from distilled water left standing for several weeks. For the experiments, the bottles were kept at $21 \pm 1°$ C. Samples were pumped to the counter through tubes made of PTFE by means of a silicone-tubed peristaltic pump (Heidolph PK2, F.R.G.). Pulsation of the pump was minimized by proper adjustment of the flow controller (see below). Tubing and pump were sanitized and flushed free of particles prior to sampling.

To determine the size of the bacteria, they were stained with carbol fuchsin and photographed under oil through a microscope. On enlarged prints, the bacteria were measured and compared to a micrometer scale that was photographed and enlarged under identical conditions.

Particle counting and microbiological analyses

Particles were detected using a Hiac Royco model 346b laser scattering sensor with counter model 4100 obtained from Pacific Scientific (Leonberg, F.R.G.). Flow through the counter was adjusted to 100 ml/min using a model 800 flow controller (Hiac Royco). The counter had been calibrated by the manufacturer with latex microspheres. Each count was repeated ten times and mean counts were calculated. On the bottled cultures, mean counts were determined from five repeats only.

Reduction factors for particles as well as for bacteria were calculated dividing the reject water counts by the permeate counts. We decided to perform the calculations on the reject rather than the feed water as the former is a better indicator of particle concentration on the 'dirty' side of the membrane. When feedwater enters the cartridge its particle concentration is altered by particles that have been deposited on the membrane during earlier experiments. Furthermore, new particles such as $CaCO_3$ crystals form during the process.

For the microbiological analyses, the PTFE tube was removed from the counter and disinfected with isopropyl alcohol. Samples were taken aseptically after flushing for 5 min. Depending on the expected number of bacteria, microbial counts were determined either by the membrane filter technique or by spreading serial dilutions on low-nutrient agar plates (Nutrient Agar, Merck, Darmstadt, F.R.G.). Incubation time was 7 days at 25° C.

Results and Discussion

Comparison of particle retention with bacterial retention

On comparing the bacterial reduction level of a low-performance cartridge (reduction factor about 10^2) with a reduction level for particles 0.5–1 μm, we observed a good correlation. In an experiment with a high-performance cartridge whose logarithmic reduction factor was impaired to 3.8 because of a worn O-ring, we observed almost identical reduction factors for particles and bacteria (see Table 1).

When testing cartridges with good bacterial rejection properties, we often detected high initial particle counts which declined in a doubly exponential fashion over several days and eventually stayed almost constant. Shutdown of the apparatus for a short time and subsequent restart sometimes also led to temporarily elevated counts as seen in Fig. 2. These effects were observed mainly when the cartridges had been recently mounted after storage in 3% formaldehyde solution. Thus, we attribute this problem to contamination of the product side of the membranes with particles washed away from the reject side by the storage solution. After several sanitization cycles without removal of the cartridge from the apparatus, initial counts were lower and constant counts were reached after only 1 day of operation.

The origin of these particles was verified as being the product side by lowering feedwater particle load by passage through cellulose asbestos depth filters (Seitz EKS, Bad Kreuznach, F.R.G.). Though particle counts in reject waters were then about 100-fold lower than before, the counts in

TABLE 1

Median logarithmic reduction factors for bacteria and particles after washout period during several runs with different RO cartridges

Cartridge	Log microbial reduction		Log particle reduction		Median reject particle load (ml^{-1})	Median product particle count (ml^{-1})
	Median	95% confidence interval	Median	95% confidence interval		
CA1	1.80	1.17–1.94	1.70	1.61–1.90	3.1×10^3	60
CA1	2.13	1.69–2.63	1.67	1.53–1.87	5.6×10^3	155
CA2 [a]	3.72	2.88–4.30	3.79	3.70–3.97	3.2×10^3	0.48
PS	5.18	4.85–5.39	4.54	4.47–4.76	5.0×10^3	0.13
PA	5.64	n.a.	3.85	3.67–3.93	3.2×10^3	0.44
CA2	6.3	n.a.	4.77	4.61–4.83	5.2×10^3	0.09

[a] Low rejection because of worn O-ring.

n.a. not applicable; the reduction factor was calculated from the cumulative samples because most samples contained no viable bacteria.

product waters of the high-performance cartridges did not change significantly. Contrastingly, changing particle concentrations in the reject water of the low-performance CA1 cartridge made product water counts fluctuate accordingly. Therefore, we conclude that the product water of RO cartridges may be contaminated by submicron particles released very slowly by the inner surface of the cartridges. With new cartridges, this effect may be less distinct as the membrane is not yet loaded with particles. This aspect was not investigated further.

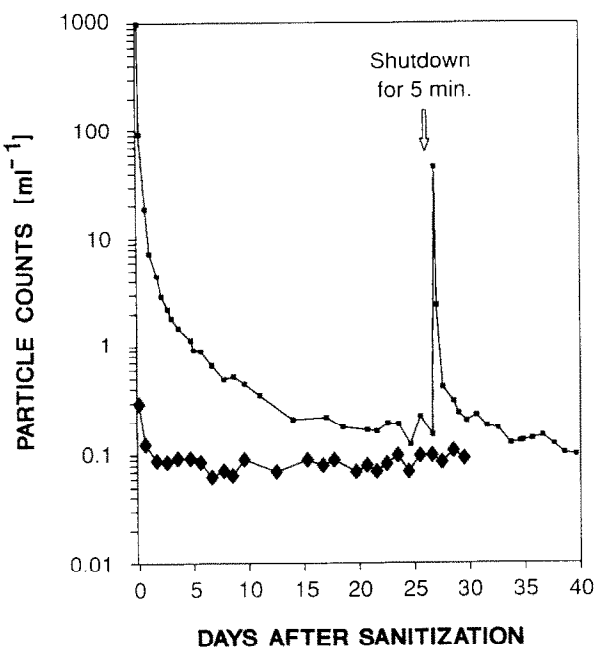

Fig. 2. Flush out of particles from the product side of the RO cartridges. (■) Polysulfone cartridge newly mounted after storage in formaldehyde solution. (◆) Cellulose triacetate cartridge after six sanitization cycles without removal from the pressure vessel.

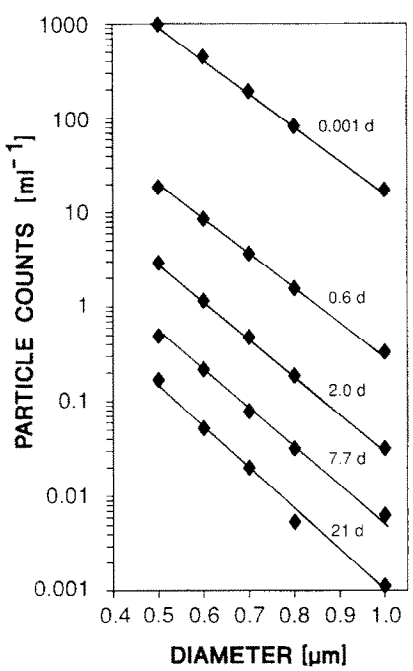

Fig. 3. Size distribution of particles in the product water at different points of time during the flush-out period. Regression coefficients: $R_{0.001d} = 0.9998$; $R_{0.6d} = 0.9998$; $R_{2.0d} = 0.9999$; $R_{7.7d} = 0.9991$; $R_{21d} = 0.9981$.

In our experiments, constant counts were reached at levels ranging from 0.1 to 0.5 particles > 0.5 μm per ml. As our reject water counts were $2-5 \times 10^3$/ml, it is obvious that logarithmic particle reduction factors were 4.5 at best even when microbial retention was far better (cf. Table 1).

Nevertheless, it should be emphasized that such water is of excellent quality with respect to particulate matter. As can be seen in Fig. 3, after the washout period there are only between one and three particles ≥ 1 μm present in 1 l product water of a high-performance cartridge. Thus, these water samples almost complied with particle limits established in the proposed VDI guidelines (1989) for the production of 4 MB semiconductor chips demanding less than 0.1 particle ≥ 0.5 μm per ml (cf. Table 1). Therefore, the water surpasses any limits for particulate matter in large-volume parenterals by several orders of magnitude. Actually, the British Pharmacopoeia (1988) allows up to 500 000 particles ≥ 2 μm to be present in 1 l (determined with the light blockage method). The USP XXI (1984) admits 50 000 particles ≥ 10 μm (filtration/microscope method).

For particles in the range of 1–50 μm, several authors reported a log-log distribution for the cumulative number of particles-particle size relation when they examined injections using both Coulter counter and light blockage methods (Groves and Wana, 1977; Montanari et al., 1982). Surprisingly, our data fitted better to a log cumulative number-linear size system during all phases of the flush-out process (Fig. 3). It should be borne in mind, however, that the size range measured is quite small and that any method of particle counting usually yields a different size distribution curve (Leschonski, 1987).

Inefficiency of particle counter to detect bacterial growth

During monitoring of both microbial growth and particle counts in the CA2 cartridge, we found

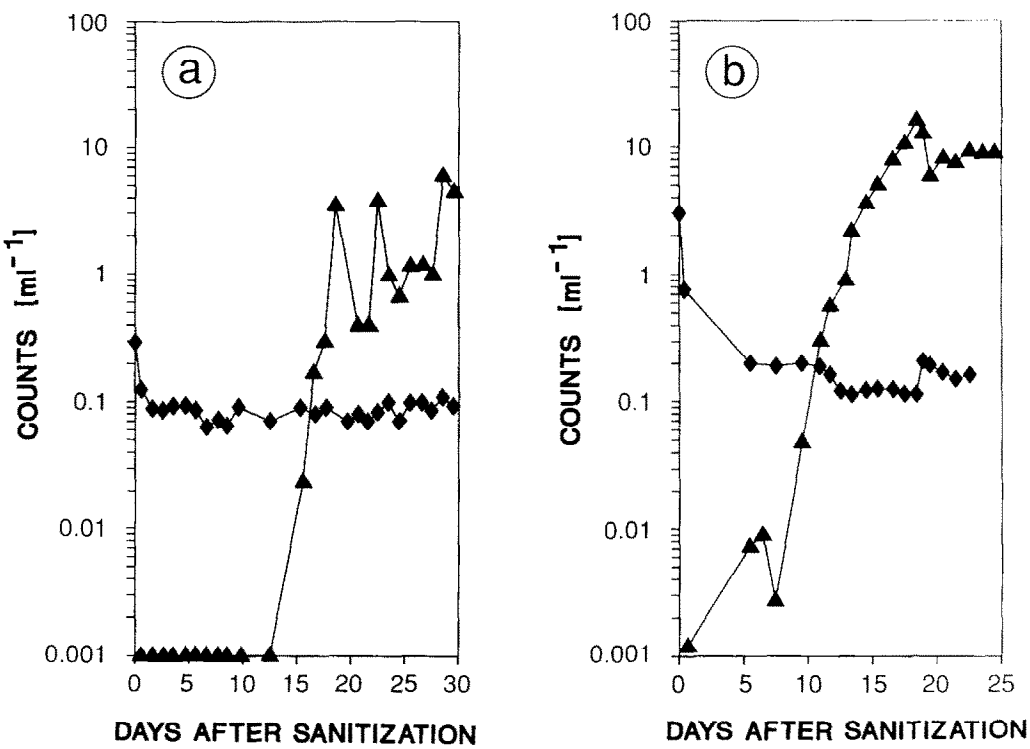

Fig. 4. Microbial counts (▲) and particle counts (◆) during two runs of CA2 cartridge. The small break in the curves of panel b at day 18 is caused by switching the feed from cellulose asbestos-filtered water to 3 μm carbon-filtered water.

that rising bacterial counts did not affect the particle count level. As demonstrated in Fig. 4, microbial counts exceeded particle counts 10–100-fold in those experiments. Microbial growth was found to be caused by *Ps. fluorescens* and *Ps. alcaligenes* (Fig. 4a) and by *Ps. fluorescens* alone, respectively (Fig. 4b).

In order to obtain data on a wider variety of species, we measured particle counts in bottled RO water which was deliberately contaminated with bacteria that are often involved in microbial growth in pure water. Although all strains were selected for their ability to multiply rapidly in deionized water, only a few were able to grow in our RO water (cf. März et al., loc. cit.). The growth curves and corresponding particle counts of those strains are shown in Fig. 5. The yellow-pigmented *Ps. paucimobilis* strain grew to levels of almost 10^4 CFU/ml whereas particle counts throughout remained below 10 per ml. Contrastingly, during its logarithmic phase of growth, a reddish pigmented *Xanthobacter* sp. strain produced particle counts increasing at the same rate as the colony counts although 8-times lower. When the growth curve reached its stationary region, the difference between microbial and particle counts increased to more than 1 log unit.

Ps. fluorescens bacteria cultivated in phosphate buffer died rapidly after inoculation into the RO water. Yet inoculation with 0.5 l of unfiltered RO water containing the same strain resulted in bacterial growth beginning 3 days after inoculation. Up to a level of 10^2 CFU/ml, this *Ps. fluorescens* strain was the prevailing organism but was then outgrown by *Ps. alcaligenes*. Fig. 5c shows that particle counts in this mixed culture increased with microbial counts but at a 15–30-fold lower level. During the stationary phase the particle counts declined by up to 1 log unit.

When small amounts of nutrients were supplied by addition of 5 ml of particle-free 0.067 M phosphate buffer containing 0.1% peptone (FIP, 1976), a pure culture of *Ps. fluorescens* rapidly grew to levels of 10^6 CFU/ml as did other bacteria that

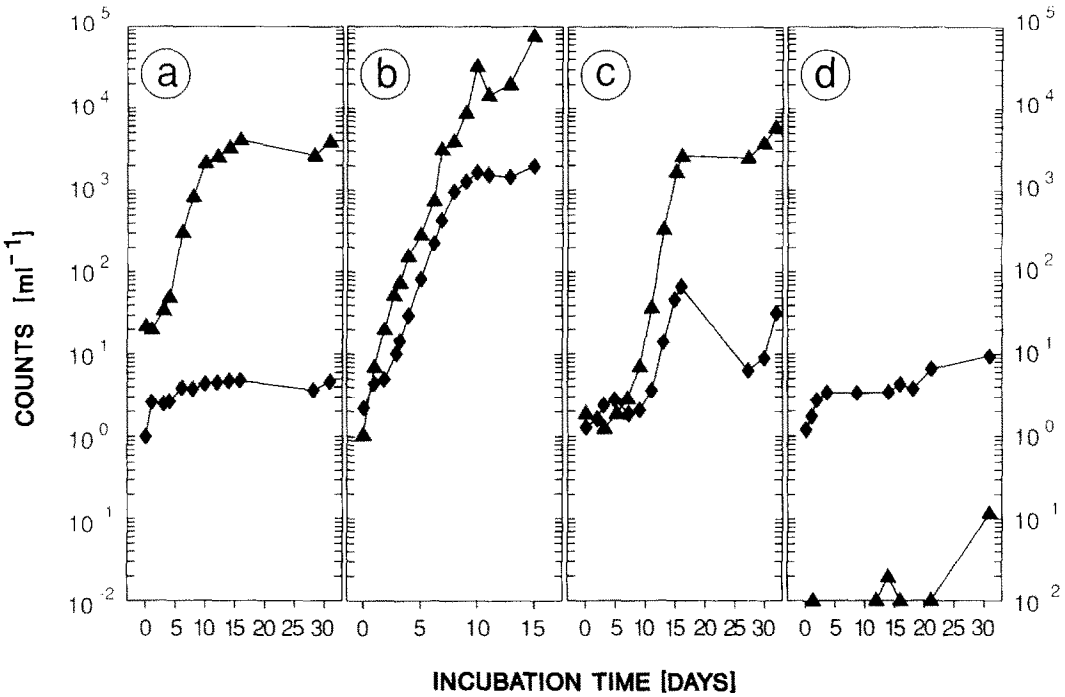

Fig. 5. Microbial counts (▲) and particle counts (◆) during incubation of pure bottled RO water inoculated with different bacteria. (a) *Ps. paucimobilis*; (b) *Xanthobacter* sp.; (c) mixed culture of *Ps. fluorescens* and *Ps. alcaligenes*; (d) control, no microorganisms added.

failed to grow in the aforementioned experiments. Thereby, *Ps. fluorescens* produced particle count patterns very similar to those observed with the mixed culture in the pure water. Microbial counts were 15–30-fold higher than particle counts during the log phase of growth and declining particle counts could be seen in the stationary phase. With *F. capsulatum* and *C. violaceum* similar results were obtained (Fig. 6). Contrary to its behavior in pure RO water, our *Ps. paucimobilis* strain produced increasing particle counts when grown in water to which phosphate buffer had been added, but the difference between microbial and particle counts still amounted to about 2 log units.

Poor detection of the bacteria is consistent with observations of microbial counts in water of up to 10^7 that produced no visible turbidity (Carson et al., 1973), and is probably due to several reasons.

Firstly, the size of the microorganisms seems to be significant. In the literature, there are reports of pure water bacteria being only half to one-third the size of those cultured in broth (Carson et al., 1973). Schmidt-Lorenz (1975) reports that cell diameters of autochthonous water bacteria which mostly are of coccoid shape are often below 0.5 μm. Table 2 lists the sizes of our reference bacteria during growth in RO water. With the exception of *Xanthobacter* sp., all of the reference organisms were smaller than 0.5 μm in width. Their length, however, was found to be markedly greater than 0.5 μm in most cases. Thus, spheres of equivalent volume and spheres of equivalent surface were both calculated to be mostly larger than 0.5 μm in diameter. As the *Xanthobacter* strain indeed proved to be the largest organism, it is not surprising that this strain produced the highest counts. However, though most *Xanthobacter* cells measured more than 0.5 μm in width, the counter failed to detect about 90% of them. Moreover, the complete failure of the counter to detect *Ps. paucimobilis* in pure RO water cannot be explained by the size of the bacteria alone. Obviously, there must be other causes for the weak signals.

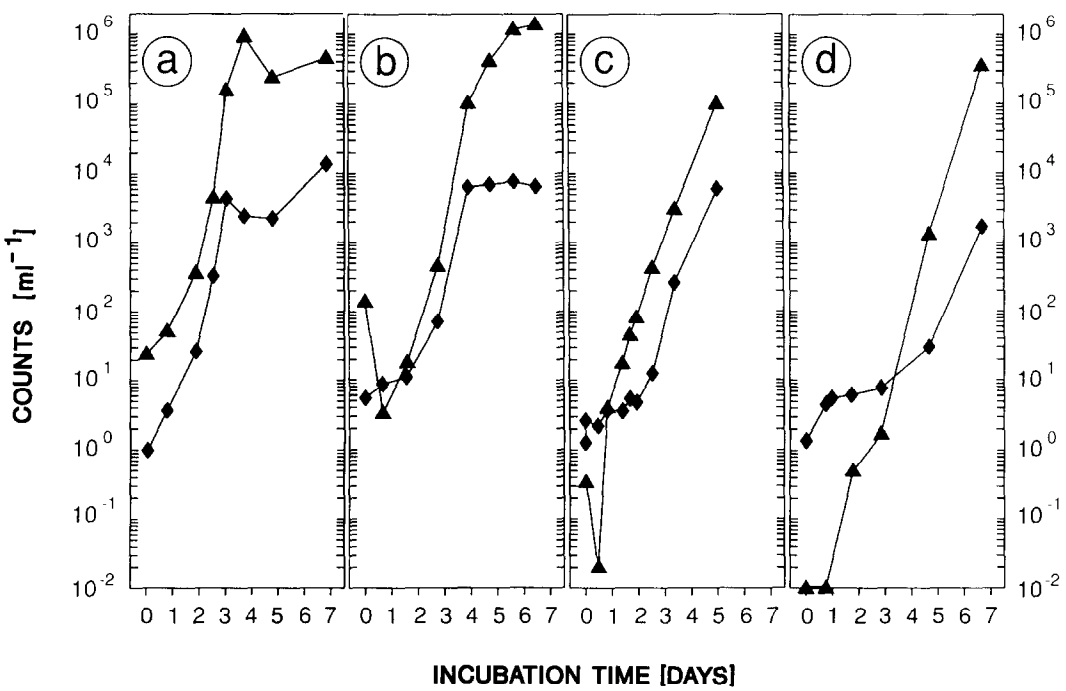

Fig. 6. Microbial counts (▲) and particle counts (◆) during incubation of bottled RO water to which small amounts of phosphate buffer were added. The bottles were inoculated with the following bacteria: (a) *Ps. fluorescens*; (b) *Chromobacterium violaceum* DSM 30191; (c) *Flavobacterium capsulatum* DSM 30196; (d) *Ps. paucimobilis*.

TABLE 2

Sizes of bacteria growing in bottled RO water

	0.05% buffer added	Incubation time	Diameter of sphere with same volume		Diameter of sphere with same surface		Length		Width	
			Mean	Range	Mean	Range	Mean	Range	Mean	Range
C. violaceum	+	3	0.55	0.42–0.67	0.62	0.46–0.77	0.95	0.50–1.27	0.34	0.25–0.40
	+	7	0.45	0.36–0.55	0.50	0.38–0.60	0.65	0.33–0.80	0.31	0.25–0.38
F. capsulatum	+	3	0.64	0.55–0.80	0.72	0.62–0.91	1.11	0.83–1.55	0.40	0.33–0.47
	+	5	0.61	0.46–0.75	0.68	0.51–0.86	1.01	0.60–1.50	0.39	0.32–0.50
Ps. fluorescens	+	3	0.57	0.48–0.69	0.64	0.53–0.79	0.96	0.66–1.27	0.36	0.30–0.42
	+	7	0.51	0.41–0.62	0.56	0.47–0.70	0.77	0.65–1.10	0.34	0.25–0.38
Ps. paucimobilis	+	3 [a]	0.53	0.47–0.62	0.59	0.51–0.69	0.81	0.67–1.00	0.35	0.32–0.40
	+	7	0.46	0.36–0.51	0.51	0.39–0.58	0.65	0.45–1.05	0.32	0.25–0.38
Ps. paucimobilis	–	4 [b]	0.52	0.49–0.56	0.60	0.57–0.63	0.98	0.88–1.07	0.32	0.27–0.37
	–	7	0.69	0.52–0.87	0.74	0.57–0.94	0.78	0.60–1.00	0.53	0.40–0.67
Xanthobacter sp.	–	4	0.78	0.51–1.05	0.86	0.56–1.14	1.16	0.67–1.53	0.53	0.37–0.75
	–	7	1.01	0.92–1.29	1.11	1.01–1.43	1.36	1.08–1.92	0.72	0.62–0.87

[a] Only 3 bacteria examined at this point because of sparse growth.
[b] Only 2 bacteria examined at this point because of sparse growth.
All values are expressed in μm. Mean and range calculated from values of nine organisms unless otherwise specified. Diameters of equivalent spheres were calculated regarding the rod-shaped bacteria as cylinders.

Brossmann (1966) calculated the intensity of the light scattered by spherical particles under various conditions. He found that for measuring angles of $\theta \approx 0°$, the intensity of the scattered light rises with decreasing refractive indices. Practically, for the whole near forward measuring range, as used in all commercially available laser counters, there is only a slight dependence of the results on the refractive index of the particles to be analysed (Leschonski, 1987). As the refractive index of bacteria is certainly lower than that of the latex microspheres used for calibration ($n = 1.58$), this discrepancy cannot explain our results.

Rather, we attribute the weak signals to the fact that the rod-shaped water bacteria do not meet the requirements of Mie's theory (1908) which is valid only for homogeneous spherical particles. Apart from being mostly not spherical, the bacteria are certainly not homogeneous. The optical properties of the cell wall and other cell structures differ from those of the cytoplasm. Actually, the refractive index of the latter is very similar to that of water. In yeast cells, for instance, $n_{\text{cytoplasm}} \approx 1.38$ (Beyer, 1965).

Furthermore, the bacteria are enclosed in a shell of polysaccharides called the glycocalyx (Costerton et al., 1981) which gives them a ragged surface instead of the smooth ones Mie dealt with.

Bacterial size changes as a function of the growth phase and metabolic activity (Thimann, 1963). This probably also results in changing optical properties. We believe that the increasing difference between particle and bacterial counts during growth is due to these effects.

This means that the sensitivity needed to detect any bacterium in any given phase of growth defies all calculations, since not even the amount of light scattered by irregularly shaped particles can be predicted (Brossmann, 1966).

Conclusions

As has been shown (März et al., loc. cit.), there is no correlation of microbial retention with salt retention in several RO cartridges. Moreover, as 5% of monovalent ions slip through the membrane anyway, the measurement of electrical conductiv-

ity is insensitive to minor damage of the membrane.

Therefore, laser diffraction particle counting in the submicron range surpasses conductivity measurements by far as a means of monitoring the integrity of RO membranes. However, due to its susceptibility to distortion by particles released from the product side, this method is limited to long-term surveillance after some washout time and to continuously operating systems only.

Above the detection limit, which depends on the level of feedwater contamination and the amount of particles released from the product side of the cartridge, the microbial retention capacity of RO cartridges can be inferred from the retention of particulate matter. Given a feedwater contamination level of about 5×10^3 particles > 0.5 μm, a minute flaw which allows only 0.05% of feedwater particle contamination including bacteria to pass the membrane would have been detected in our experiments.

As bacteria themselves are not recognized, microbial growth in the cartridges goes undetected. Apparently, bacteria escape detection only narrowly as in most experiments with bottled water we observed slightly increasing particle counts. Thus, bacteria may be detectable in the future when new counters with a lower detection limit become available. However, as the number of other particles rises exponentially with their negative size, small numbers of bacteria will then be blanketed by other particulate contamination. According to the FDA's current GMP regulations for large-volume parenterals (1976), water for injection must have less than 10 CFU/100 ml. Therefore, we do not believe it will be possible to detect microbial growth at such an early stage by particle counting.

Thus, further research is required in order to devise a rapid test indicating microbial growth in RO water.

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