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Microbiological aspects in the production of water for injection by reverse osmosis

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Summary

Bacterial retention capacity and susceptibility to microbial growth in several reverse osmosis (RO) cartridges with membranes made of differing materials were determined. Operation of the RO cartridges with both dechlorinated tapwater and water artificially contaminated with large numbers of water-borne bacteria yielded reduction factors ranging from 10^2 to more than 10^6. There it was found that membranes with good salt removal properties may show poor rejection of microorganisms and vice versa. Therefore, despite widespread practice, conductivity measurements are of limited value in membrane monitoring when removal of bacteria is of paramount importance. In continuous runs of the RO apparatus (no overnight shutdowns), microbial growth on the product side of cellulose-triacetate cartridges started 4–5 days after complete sanitization. In cases where sanitization was not complete, no lag time of bacterial growth was observed. Polyamide and polysulfone cartridges showed little or no signs of bacterial growth. Several strains of *Pseudomonas fluorescens* were identified as the main growing organisms.

Introduction

Since 1974, the USP (19th edn.) has allowed water for injection (WFI) to be produced both by distillation and by reverse osmosis (RO). In Europe however, the European Pharmacopeia Commission turned down several proposals to allow RO water for use in parenteral formulations, considering RO not yet to be safe enough to produce water of the highest purity at a constant level.

To avoid bacterial production of pyrogens, particular attention must be paid to the microbiological purity of the product water, since bacteria can multiply rapidly even in pure water (Botzenhart and Kufferath, 1976).

The literature reports differing numbers of bacteria usually present in RO permeate (Frith et al., 1976; Juberg et al., 1977; Jacobs, 1981; Setz, 1985; Parise et al., 1985). This might be due to:

(i) different methods for the detection of microorganisms, such as growth media and temperature of incubation;
(ii) incomplete sanitization of RO cartridges;
(iii) varying type and composition of the employed RO cartridges;
(iv) variable quality of the membranes in the RO cartridges.

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Theoretically, RO water should be sterile, but imperfections of the membrane offer a way for microorganisms to enter the product (Frith et al., 1976). Moreover, no heat is applied during the process, so that bacterial growth may occur on both sides of the usually large membrane surface. There are several reports on the ability of RO membranes to produce WFI (Frith et al., 1976; Juberg et al., 1977; Jacobs, 1981; Parise et al., 1985), but little is known about the microbial retention capacity achievable by RO and about the speed of colonization of the product side by bacteria. This problem is aggravated by the availability of several membrane materials. Membranes can also be arranged differently in a cartridge (see Kunz, 1973 and Hentze, 1983 for details). In pharmaceutical applications, the spiral wound type is more commonly found than the hollow fiber configuration today (Flindt, 1984).

The purpose of this investigation was therefore:

(i) to obtain data on the microbial retention capacity of spiral wound RO membranes consisting of different materials;
(ii) to assess the kinetics of microbial growth and within the RO cartridges;
(iii) to identify the organisms involved in such growth and
(iv) to evaluate the use of conductivity measurements as a means to monitor membrane performance in applications where removal of bacteria is of paramount importance.

Materials and Methods

RO apparatus

For the experiments, two identical Hemo-RO systems (Millipore Corp., Eschborn, F.R.G.) were used. A schematic diagram of the RO assembly is shown in Fig. 1. Feedwater for the RO apparatus consisted of local tapwater from Lake Constance, which was pretreated by pH adjustment to 7.1 ± 0.1 by injection of dilute hydrochloric acid. Water for experiments with the polysulfone cartridge was softened (two softeners: Millipore ZWSO 095 FO) in order to remove calcium. Finally, the feed was passed through a carbon filter (Millipore CDFC01204) to remove residual chlorine and particles larger than 3 μm. To prevent bacteria from entering the system from behind, the ends of the waste and product lines were positioned 10 cm above sink level.

The feedwater inlet was equipped with an ESP Sanitary Sampling Valve (Millipore), the product and reject outlets being equipped with diaphragm sampling valves (type 671 (product) and 601 (reject); Schico, Munich). These sampling valves were mounted directly to the pressure vessel containing the RO cartridge to minimize distortion of the results. The sampling valves were validated by the procedure described by März et al. (1989).

Sampling procedures

The valves were sanitized with isopropanol 70% (ESP) or steam (Schico) (März et al., 1989). After rinsing, two samples were taken at a 10 min interval. Samples were processed within 30 min.

Enumeration of bacteria and taxonomic identification

Depending on the expected number of bacteria, appropriate volumes of the sample were either filtered through cellulose nitrate membrane filters (0.45 μm, Sartorius, Göttingen), or serial dilutions were made and spread in duplicate on low-nutrient agar plates lacking NaCl (nutrient agar, E. Merck, Darmstadt). The plates were incubated for 7 days at 25 °C.

This method was chosen because it yields considerably higher numbers of counts than soybean-casein digest agar medium specified by Ph. Eur. (1978) and USP XXI (1984). The results are similar to those obtained with R2A medium (Scheer, 1986).

After enumeration of the colonies, the most frequent ones were selected on the basis of their morphology. Characteristic colonies were transferred to the same medium for identification. Gram-negative, rod-shaped bacteria were identified using the API-20 non-enterics identification system (API-Biomérieux, Nürtingen, F.R.G.). Flagellation of the organisms was determined according to Bailey and Scott (1974) to cross-check the API results where possible. For information on flagellation and other properties of the isolated organisms, Bergey's Manual (Krieg and Holt, 1984) was consulted.

RO cartridges

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

	CA1	CA2	PA	PS
Membrane	cellulose	cellulose	polyamide	polysulfone
Material	triacetate	triacetate		
Manufacturer	Millipore	Millipore	Millipore	Millipore
Type	CDROO25SH	CDROO25H	CDRCO25SH	CDRSO25SH
Lot no.	C5P0649832	C3A7758403	119373	C7B1480550

Note that both CA cartridges are of the same type. All cartridges measured 63 cm in length with a diameter of 8.5 cm.

Salt rejection in terms of Na^+ removal was determined with a flame photometer (Dr. Lange M6D, Berlin, Germany).

Sanitization procedure and operation of RO unit

The RO unit was sanitized by circulating formaldehyde solution (3%) through the assembly for 1 h followed by an 8 h stand-by period. This cycle was repeated several times. The unit was flushed with sterile filtered water for 6 h until the CH_2O level was below the detection limit (0.1 ppm). Feeding sterile water, samples were taken to verify that sanitization was complete. The feed was switched back to tapwater and the unit was operated continuously except for minor shutdowns (< 5 min) for technical reasons.

Product and waste flux were determined using a measuring cylinder. The system % recovery rate was calculated as

$$C_r = \frac{V_{\text{Product}}}{V_{\text{Product}} + V_{\text{Reject}}} \times 100(\%).$$

Stress tests on reference microorganisms

Reference strains were obtained from DSM (German Collection of Microorganisms, Braunschweig) or ATCC (American Type Culture Collection, Rockville, MD) respectively. A suspension of the microorganisms under test (see legend to Fig. 3) was spread on a sufficient number of agar plates and incubated for 48 h at 25 °C. The resulting growth was scraped off and suspended in 10 l of sterile water. After sanitization and flushing of the RO unit, samples were taken to verify sterility of the system. The inoculum was then continuously injected into the sterile filtered feedwater by means of a triple piston pump (Lewa

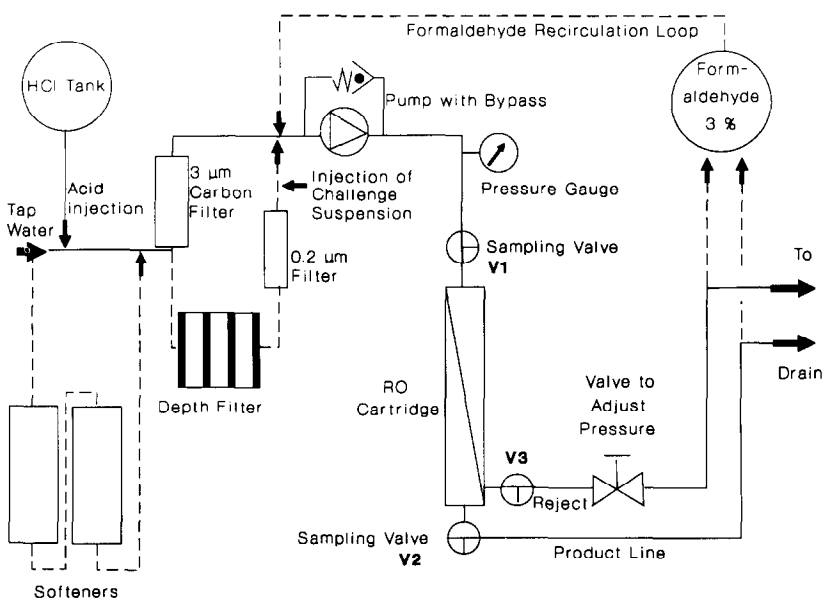

Fig. 1. Flow diagram of RO apparatus.

FL3, Leonberg, F.R.G.). Ten samples at valves V1 and V2 (see Fig. 1) were taken at intervals of 30 min. The system was operated at pressures of 12 ± 2 bar and recovery rate was adjusted to 30%.

Data analysis

To normalize data to the varying microbial load of the feedwater, the reduction factor was calculated for each sample by dividing the feedwater count by the product count. When more than 50% of the product samples of a given experiment showed no growth at all, mean counts of all samples were determined, dividing the sum of all counted bacteria by the sum of all sample volumes. The overall reduction factor was then calculated as above.

Results

Initial retention of microorganisms by the RO unit

Right from the outset of the experiments, bacteria could often be found in the product waters of the CA and PS cartridges, although at a highly

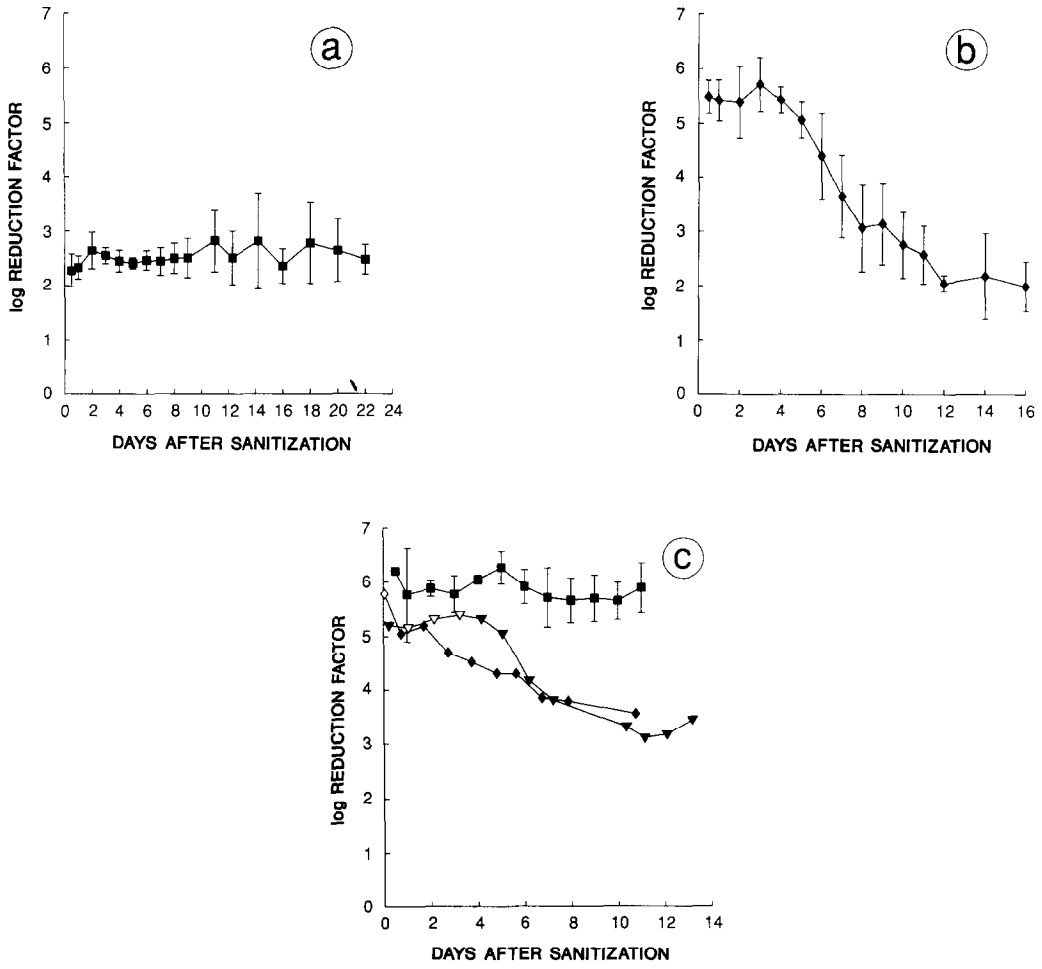

Fig. 2. Logarithmic microbial reduction factors of several RO cartridges vs. time of operation. (a) CA1 cartridge (cellulose-triacetate). Mean values of five runs numbered 1 to 5 in Table 1 showing run conditions. (b) CA2 cartridge (cellulose-triacetate). Mean values of 6 experiments (nos. 1–6, Table 1). (c) PS cartridge (polysulfone). Mean of experiment nos. 1–4 conducted at high product flow rates (■) and values of experiment nos. 5 (▼) and 6 (◆) when product flow was only about 30 l/h. Open symbols represent limit of detection (i.e. no counts in the particular sample). Error bars denote standard deviation.

reduced level. Those bacteria must have penetrated the cartridge because no microbes were detectable in the test after sanitization. Initial reduction levels were fairly constant in all assays of a given cartridge (cf. Fig. 2). Calculation of the median logarithmic reduction factors of samples taken before onset of microbial growth (see below) revealed a wide range of performance of the individual cartridges though all tested cartridges comply with the specifications of the manufacturer (Millipore data sheet EU194/G, 1986) stating 99% bacterial reduction. So in six runs the CA1 cartridge yielded median logarithmic reduction factors ranging from 2.2 to 2.8 whereas those calculated from the first 5 days of seven experiments with the identically structured CA2 cartridge ranged from 4.8 to 6.1. Similar reduction was achieved with the PS cartridge producing median log reduction factors between 5.3 and 6.1, respectively. In the product waters of the PA module bacteria could be detected only rarely during the first 10 days. Therefore, in all five experiments the logarithmic reduction factors which had to be calculated from the cumulated samples (see Data analysis) amounted to values between 6.0 and 7.8.

Neither conversion rate nor operating pressure nor microbial count of the feedwater seemed to influence reduction factors significantly (cf. Table 1 for operational data). Comparatively large variation and varying reduction factors observed with the PA cartridge are most likely due to contamination during sampling, as only few bacteria were found.

Stress tests

The results above are substantiated by those obtained feeding the membranes with suspensions

TABLE 1

Operational parameters of RO experiments

Cartridge	Experiment	Date of start	Feed count (median) [a] (CFU/ml)	Feed temperature (°C)	Product flow (l/h)	System percent recovery	Operating pressure (bar)	Pretreatment of feed
CA1	1	Dec. 27	3.8×10^2	8	55	25	17	HCl
	2	Jan. 20	1.3×10^2	8	49	22	17	HCl
	3	Feb. 4	5.0×10^2	8	54	25	17	HCl
	4	Mar. 23	1.8×10^2	8	52	23	16.5	HCl
	5	Apr. 13	4×10^4	8	55	25	17	HCl
CA2	1	July 6	1.7×10^3	9.5	47	31	13	HCl
	2	Aug. 3	2.0×10^3	10	36	20	10	HCl
	3	Aug. 20	6.3×10^2	10	57	40	14	HCl
	4	Sept. 15	9.0×10^1	10	83	31	17	HCl
	5	Oct. 3	4.0×10^2	9	41	30	13	HCl
	6	Feb. 6	8.0×10^2	8	37	16	10	softening
	7	Jan. 27	1.3×10^2	8	35	15	10	HCl
PS	1	June 11	2.0×10^4	8	90	31	15	softening
	2	July 6	2.6×10^3	9.5	125	30	19	softening
	3	Aug. 3	4.0×10^3	10	63	20	11	softening
	4	Aug. 20	1.0×10^3	10	123	40	19	softening
	5	Jan. 27	1.3×10^2	8.5	30	20	7	softening
	6	Feb. 6	2.1×10^2	8	32	21	7.5	softening
PA	1	Feb. 4	5.8×10^2	8	47	36	12	HCl
	2	Mar. 24	1.3×10^2	8	55	39	12.5	HCl
	3	Apr. 13	2.0×10^4	8	50	35	12	HCl
	4	Sept. 15	7.9×10^1	10	41	30	7.5	HCl
	5	Oct. 23	1.1×10^3	9	36	27	7.5	HCl

[a] Median of the first 10 days after sanitization.

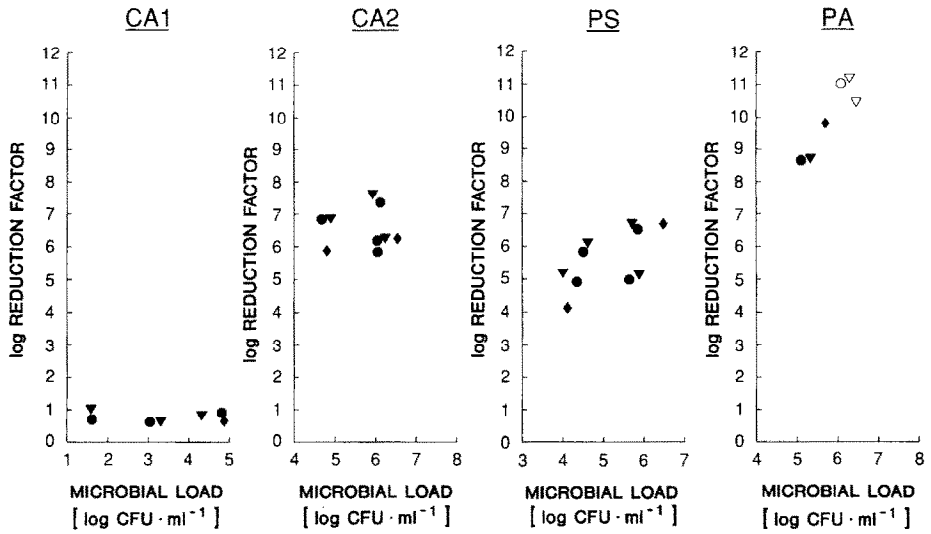

Fig. 3. Reduction factors for various bacteria and RO membrane elements at high levels of feedwater contamination. The symbols represent median logarithmic reduction factors of 10 samples. In the graph of the PA cartridge, closed symbols represent cumulative reduction factors calculated from all 10 samples because bacteria were found in less than half of the product samples only. Open symbols indicate that all product samples turned out to be sterile. Therefore, the limit of detection is given. *Flavobacterium capsulatum* DSM 30196 (◆); *Pseudomonas fluorescens* (isolated from RO water) (●); *Chromobacterium violaceum* DSM 30191 (▼). Experiments with *Ps. diminuta* DSM 1635, *Ps. paucimobilis* (isolated from RO water) and a *Xanthobacter* sp. isolated from distilled water yielded very similar results which are not shown for clarity.

of various water-borne bacteria (Fig. 3). Obviously, there is no significant difference in reduction factors of the tested bacteria. The number of bacteria the membrane was loaded with also did not significantly influence the reduction factors.

Compared to the tapwater experiments, retention rates of the CA1 cartridge were significantly lower with the artificially cultured bacteria. This cannot be due to sudden membrane failure, since tapwater-reduction was verified afterwards and found again to be greater than 10^2. The loss of extracellular polysaccharides in vitro (Costerton et al., 1981) possibly may cause this behavior, facilitating penetration through the membrane by alteration of the size and surface shape of the bacteria.

For the PA and PS cartridges, variation for most strains between single experiments is quite large, even at the same level of feedwater load. Because removal of the cartridges from the pressure vessel and subsequent reassembly seemed to influence the reduction rate, we suspect leakage past the O-rings of the connector between cartridge and housing to be the cause for additional contamination of product water.

Microbial growth on product side

Cellulose-triacetate membranes. During the first 4 days after complete sanitization the counts of colonies in the product of the CA2-cartridge remained fairly constant at a level of 0–1 CFU/1000 ml. Between the fifth and seventh day a rapid rise at a rate of about one order of magnitude per day was observed (Fig. 4a). Equilibrium count was reached in the range of 10^1–10^2 CFU/ml. This corresponds to a reduction rate declining from about $10^{5.5}$ to 10^2, as shown in Fig. 2b. The slope and onset of the growth curve seem to be dependent on the flow rate, although this is not statistically significant. Seasonal changes of temperature and TOC content of the feedwater could also be important. This, however, was not investigated further.

As any rise of microbial growth is blanketed due to the low bacterial retention capacity of the

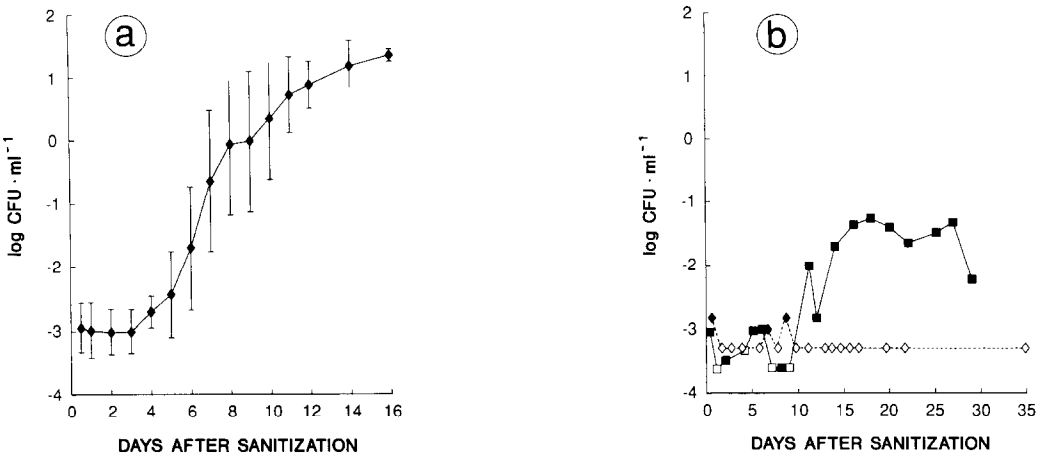

Fig. 4. Increase of microbial counts in product water of thoroughly sanitized cartridges with time. (a) CA2 cartridge (cellulose-triacetate). Mean values of experiment nos. 1–6. Bars denote standard deviation. (b) PA cartridge (polyamide). Experiment nos. 1 (■) and 4 (◆). Curves for experiment nos. 2 (16 days) and 3 (22 days) have been omitted for the sake of legibility, but are very similar to that of no. 4. Experiment numbers are those in Table 1 where run conditions are shown. Open symbols represent the limit of detection when no bacteria were found in the particular sample volumes.

CA1 cartridge, no significant change of the reduction rate could be detected during these tests. A shifting bacterial flora (see below) in the product of this cartridge after 10 days of operation, however, indicates growth of microorganisms.

Polyamide membrane. Data on the PA cartridge are variable. During some runs no increase in microbial count could be detected for periods as long as 35 days, whereas scarce growth to a level of 1 to 5 CFU per 100 ml was detected in experiment no. 1 after 10 days of operation (Fig. 4b). In a long-term experiment, run no. 5 which also showed slight growth at the 12th day was checked again after 50 days of continuous operation. At that point, counts had risen further to almost 1 CFU per ml.

The variability of the growth patterns perhaps is due to the excellent retention capacity of the cartridge under test, so that there is little chance of bacteria penetrating the membrane and multiplying on the product side.

Polysulfone cartridge. At normal operation pressures, i.e. $\Delta p > 14$ bar, no microbial growth was detectable. Because of the higher flux across the polysulfone membrane, product flow rate at this pressure is 50% higher than in PA and CA cartridges. For comparison of the susceptibility to bacterial growth, experiments were started adjusting the pressure to 7 bar, which corresponds to a product flow rate of about 30 l/h. Under these conditions, actually microbial counts rose by two log units, starting the fifth day after sanitization. This indicated a significant decline of reduction factors (Fig. 2c).

Growth after incomplete sanitization. When sanitization was not complete and viable bacteria were detected in the sterility test, the microorganisms started to proliferate immediately. If any, only short lags were observed. This was shown for the CA2 cartridge as well as for the PA membrane.

Microbial flora

Rise of microbial counts in the CA2 cartridge was found to be solely caused by several strains of *Pseudomonas fluorescens*. In the feed as well as in the product water of the first few days, fluorescent pseudomonads were found sporadically only, other pseudomonads and members of the genus *Flavobacterium* being the prevailing organisms. CA1 product water initially also reflected the feed flora exactly, but *Ps. fluorescens* could be detected occasionally about 10 days after sanitization.

Two *Ps. fluorescens* strains and, at a 10-fold lower level, a yellow pigmented organism identified as *Ps. paucimobilis*, colonized the polysulfone

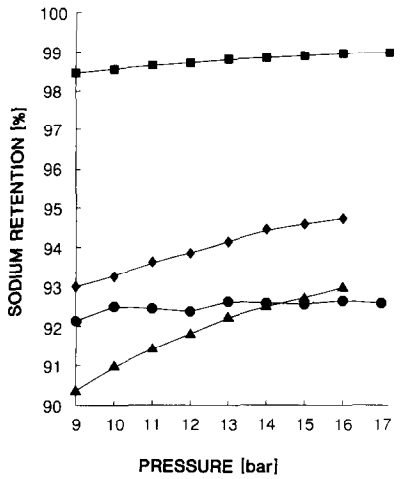

Fig. 5. Na^+ retention of RO cartridges at various operating pressures (system percent recovery, 30%; concentration of Na^+ in feedwater, 82 mg/l). (■) PA cartridge (polyamide), (◆) PS cartridge (polysulfone), (●) CA1 cartridge (cellulose-triacetate), (▲) CA2 cartridge (cellulose-triacetate).

cartridge. Two strains of *Ps. alcaligenes*, however, were found to grow predominantly in the PA cartridge. Starting after 20 days of operation, growing numbers of *Flavobacterium* sp. were also detected.

Salt rejection

A plot of Na^+ rejection of the membranes vs. operating pressure is shown in Fig. 5. Retention of Na^+ rises with the pressure applied. For the CA1 membrane, however, only a very slight increase in salt rejection with increasing pressure was found. At pressures below 14.3 bar, the microbiologically superior CA2 cartridge showed lower Na^+ retention capacity than the CA1 cartridge.

Discussion

Although our results are not directly comparable to those reported by Wallhäusser (1983b) due to the different experimental conditions, it can be stated that RO yields bacterial rejection factors at least equivalent to those of most ultrafilters.

Because of the small number of cartridges tested, it is not safe to draw inference as to the superiority of particular membrane materials in removing bacteria. In a preliminary study (Graf et al., 1986), for instance, a polyamide membrane obtained from another manufacturer yielded bacterial reduction rates averaging 2–3 logarithmic units only. Our results thus indicate that retention of bacteria varies widely in commercially available modules of *all* types. This may explain the differing microbial counts for RO water reported in literature.

However, we have shown in this investigation that some of the RO membranes on the market are clearly capable of reducing microbial contamination by more than 5 orders of magnitude. This applies to the pure polysulfone membranes, developed only recently, as well as to cellulose triacetate and polyamide. Given a typical feedwater contamination of about 10^4 CFU/ml at most (cf. Table 1), those membranes would easily produce water within the 10 CFU/100 ml limit set forth in cGMP/LVP (Food and Drug Administration, 1976). Because of inevitable salt passage, for most feedwater qualities the installation of product staged plants is necessary (Parise et al., 1985). This configuration would provide double safety in bacteriological respects.

What remains is to identify bacteriologically efficient cartridges, because as we have shown determination of salt rejection is of little value. It is obvious that each single cartridge and its fitting inserted in a RO plant for the production of WFI must be thoroughly tested prior to use. Bacterial retention capacity of a given membrane can be determined by loading it with large numbers of a suitable indicator organism. For this purpose, Jacobs (1981) used an apathogenic *E. coli* strain. Since this organism dies rapidly in pure water (Botzenhart and Kufferath, 1976) and thus may not live long enough to be detected in the product water, we do not consider this method to be safe. Rather, we would recommend the use of *Flavobacterium capsulatum* DSM 30196, since this organism showed the lowest reduction factors. There is only slight variation in retention rates between particular organisms, so that other water-borne strains may also be used for determination of reduction rates. It should be noted however, that even water-borne bacteria often

cannot survive in extremely pure water (Botzenhart and Kufferath, 1976). *Ps. diminuta* DSM 1635 is such an example as we found the number of viable cells of this organism to decline in RO-water at a rate of one logarithmic unit in 7 h. Despite this problem, we also conducted stress tests on this organism because of its importance in the testing of membrane filters. However, the results did not differ significantly from those of the other bacteria.

We did not observe any sudden presence of large numbers of microorganisms in the product water indicating membrane failure. Furthermore, at the end of the experiments retention capacity of every cartridge had not changed as against when it was used for the first time. Yet it is clear that RO membranes are subject to deterioration both chemically and mechanically (Jacobs, 1981; Ridgway et al., 1984). Therefore, the performance of the membranes should be monitored continuously. For this purpose, electroconductivity measurements are widely used (Jacobs, 1981; Parise et al., 1985). The usefulness of this method for testing membrane integrity has been questioned by Jacobs (1981), pointing out that conductivity measurements in pure water are very susceptible to distortion by varying pH values. Nevertheless, even if the concentration of dissolved salts is measured more accurately, comparison of salt rejection and bacterial retention shows clearly that microbial retention cannot be determined by inference from ion removal. This is due to the fact that a minor hole in the membrane allowing 1% of feedwater contaminants to enter the product side would lower microbial retention factors from maybe 10^6 to 10^2 while reducing ion removal by 1% only. On the other hand, from the different behaviour of the CA1 and CA2 cartridges we conclude that different types of membrane imperfections may occur: if the membrane has many holes smaller than bacteria only the retention of ions is affected, whereas in the case of one or more flaws in the micrometer range mainly microbial retention would be lowered. In the latter case, salt passage via viscous flow according to Hagen-Poiseuille's law is blanketed by ions that permeate the membrane by diffusion (about 5% for monovalent ions).

The CA cartridges proved to be the most susceptible to microbial growth, but the others are also affected, albeit to a lesser extent. Further work is necessary to determine the reasons for those differing growth patterns as well as the location of microbial growth in the cartridge.

It is not surprising that microbial growth in the cartridges is caused mainly by pseudomonads, since members of this genus only have been detected by Wallhäusser (1983a) who observed them to be involved in the contamination of sterile filtered water. On the other hand, such growth constitutes a severe problem since pseudomonads are mostly pyrogenic (Krüger et al., 1976). Moreover, fluorescent pseudomonads such as *Ps. fluorescens* or *Ps. cepacia* (Gold Sheet, 1986) have been repeatedly reported to cause contamination of pharmaceutical products. Determination of colony counts takes several days and a quick test would be most desirable to find out when sanitization is necessary.

We are currently working on the evaluation of particle counters as potential means to overcome the problems of membrane monitoring. Results of another approach consisting in the use of the LAL-test to detect gram-negative bacteria in RO water will be published elsewhere.

Acknowledgments

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