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PALL

Validation of endotoxin removal capabilities of Pall positively charged filters

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A Pall Scientific and Technical Report

Table of Contents

Introduction

Pyrogen contamination of water used in the production of pharmaceutical products is a major concern of pharmaceutical manufacturers and regulatory authorities. Both chemical and biological pyrogenicity are well documented, but by far the most significant pyrogen within the pharmaceutical industry is bacterial endotoxin.

Pyrogenic substances produce an increase in body temperature after intravenous injection into man and most animals. Bacterial endotoxin intravenously injected results in the development of fever by inducement of synthesis and release of endogenous pyrogens from host bone marrow derived phagocytic leukocytes [1, 2]. These in turn induce a wide range of chemically harmful events which are manifested in the febrile response [3].

Methods currently applied in the preparation of high purity water, and the only ones approved by the majority of regulatory authorities for the production of water for injection (WFI), involve distillation or reverse osmosis (RO). These effectively remove endotoxin by liquid/vapour phase separation, and by solute rejection, respectively. By their very nature, these methods cannot be applied to parenterals.

There are other methods for the removal of endotoxins in high purity pharmaceutical water systems such as ultrafiltration [4], but of all the methods currently used positively charged filter media are unique in offering systems of low capital cost, high efficiency and ease of use.

There are also methods for controlling endotoxin levels which involve inactivation of the pyrogenic properties of the endotoxin rather than its physical removal [4]. Such methods may involve moist heat, acid-base treatment, alkylation, oxidising agents, *Limulus amoebocyte lysate* and polymyxin B. These methods all have limitations in their effectiveness, and in their use in the depyrogenation of water, parenterals and biological fluids since many solution components are sensitive to the chemical extremes imposed. The subsequent removal of the deactivating agent may also cause problems. The inactivated toxic part of the endotoxin (lipid A) has also been shown to combine with proteins such as bovine serum albumin to regain its toxicity and pyrogenic nature [5].

Bacterial cell associated endotoxins are high molecular weight lipopolysaccharide - protein complexes within the outer membrane of Gram negative bacteria [6] (Figure 1). Endotoxins are ubiquitous, and are found in any liquid where Gram negative bacteria are present. They are continually shed into the surrounding environment by the bacteria during cell growth, division and on cell death, when the bacteria will fragment.

Traditionally, the presence of bacterial endotoxin has been detected by the USP rabbit pyrogenicity test [7] ie by monitoring any increase in body temperature following injection with potentially pyrogenic substances. FDA approval of the more sensitive *Limulus amoebocyte lysate* (LAL) test [8] has led to its rapid and wide acceptance as the current bacterial endotoxin detection method within health care industries. The test utilises the extract of lysed cells from the blood of the horseshoe crab *Limulus polyphemus,* forming a clotted gel in the presence of bacterial endotoxin.

In their purest form, in the presence of strong surface active agents and in the absence of divalent cations, bacterial endotoxins consist of 10,000 - 20,000 dalton molecules made up of a lipid part (lipid A - which is responsible for the toxic properties of the molecule [5]), a core polysaccharide, and an O-antigenic polysaccharide side chain (specific to the bacterial serotype) (Figure 2) [9]. Purified endotoxin is generally referred to as lipopolysaccharide (LPS) to distinguish it from its more natural protein complexed cell membrane associated form.

In the presence of divalent cation sequestering agents (eg EDTA), and in the absence of surface active agents, lipopolysaccharide is believed to arrange itself into a micellar structure of molecular weight around 1×10^6 daltons.

Practically speaking, this is the smallest form of bacterial endotoxin that is likely to exist in aqueous liquids. In the presence of divalent cations such as Ca^{2+} and Mg^{2+}, a bilayer structure appears to exist which will pass through a 0.2 µm rated membrane, but which is excluded from a 0.025 µm rated membrane. Vesicles of LPS up to 0.1 µm diameter may also be formed in water in the presence of these divalent cations [10, 11].

The self aggregation of LPS is generally a function of the toxic lipid A component of the molecule [12], which also confers on the LPS the ability to bind to hydrophobic surfaces. These aggregated structures form with the hydrophobic lipid A on the inside and the hydrophilic O-antigenic side chains on the outside. To interact with hydrophobic surfaces shear forces are required to break up these structures and expose the lipid A [13] moiety. Hence the efficiency of hydrophobic filters to remove endotoxins is usually low.

LPS contains exposed phosphate groups [13, 14]. Generally, at pH values above pH 2, these phosphate groups are strongly negatively charged (pI ~ 1.83). Most liquids used in the pharmaceutical industry have a pH value above this and positively charged filters therefore provide the opportunity for removal of the negatively charged endotoxins.

PALL 'N_{66}' 'Posidyne' is a hydrophilic nylon 66 filter medium containing a high concentration of quaternary ammonium groups throughout the membrane structure. In the presence of aqueous liquids these groups provide a positive zeta potential, which is maintained across a wide range of pH values (pH 3 - 10). This filter medium and other PALL proprietary charge-modified filter media used in prefilters are capable of effectively and efficiently removing negatively charged contaminants such as endotoxin structures and endotoxin containing cell fragments, even though they are smaller than the filters' absolute removal rating [15]. The density of the charge is such that these filter media perform far better than many other methods of endotoxin control.

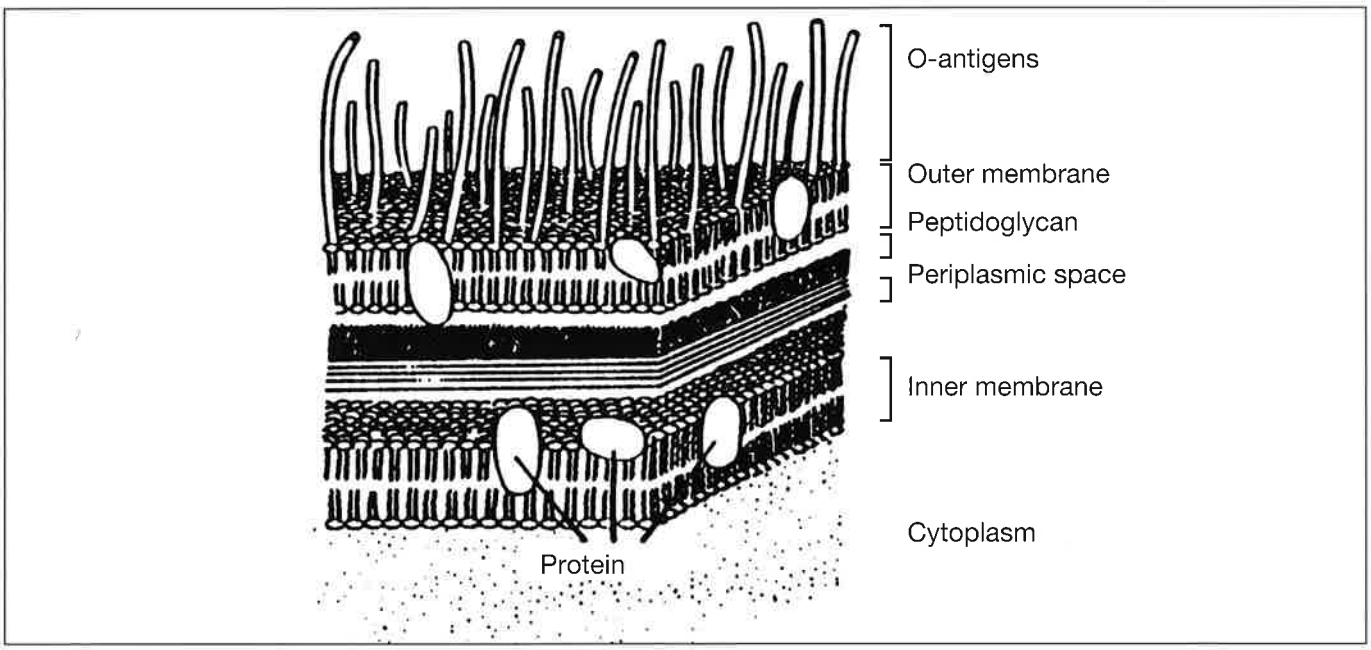


FIGURE 1:
Schematic representation of gram-negative bacterial cell membrane with LPS in outer membrane.

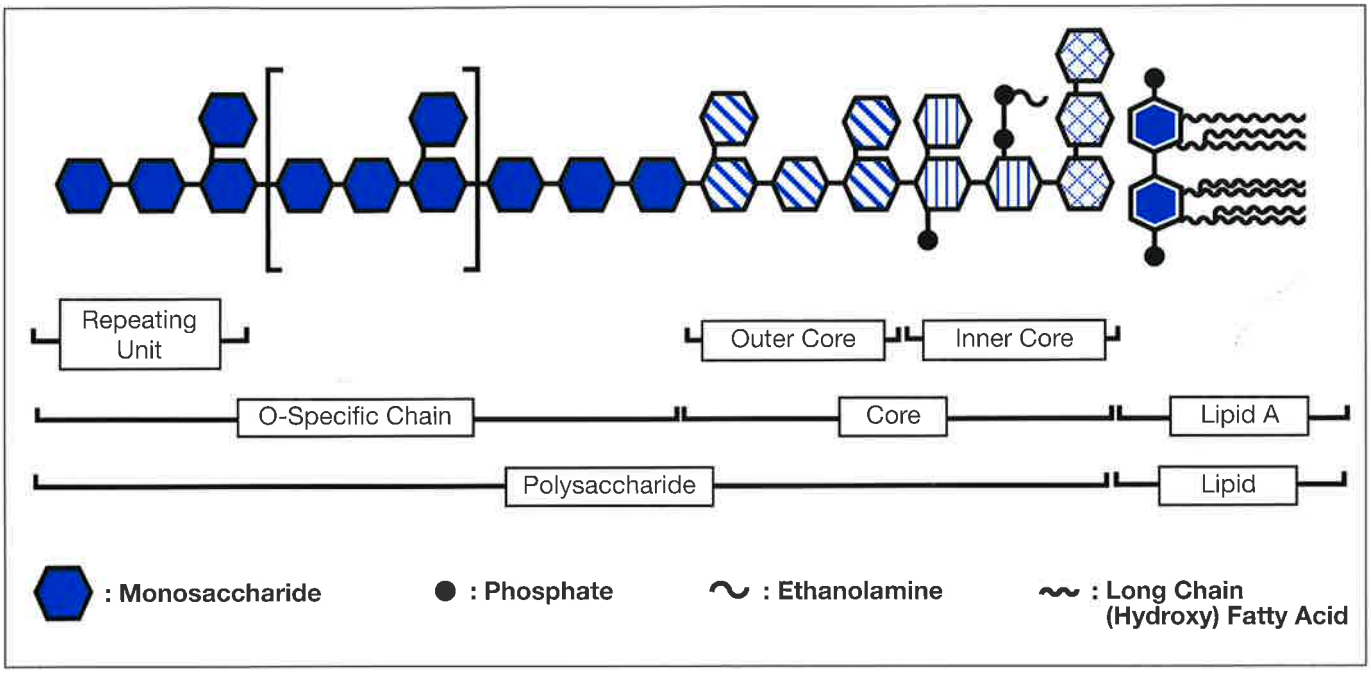


FIGURE 2:
Schematic representation of bacterial Lipopolysaccharide.

The effectiveness of positively charged filter media in endotoxin removal has been shown in a number of studies. For instance, Holmes *et al* [16], showed that intravenous (IV) filters made of various polymeric membranes, while retaining high levels of Gram negative bacteria, shed endotoxin into the sterile effluent. Following this, Baumgartner *et al* [17] showed that PALL positively charge modified IV filters retained this endotoxin, providing sterile effluent, free of detectable endotoxin by the LAL test.

More recently, Horibe *et al* [18] and Richards *et al* [19] have shown similar results with PALL positive charge-modified IV filters in a range of parenteral solutions. Wallhauser *et al* [20] have also attempted to determine the effectiveness of removal of endotoxin by positive charge-modified filter media, and to provide indications as to the retention capacity of such media. Hou *et al* [21], have also shown the efficiency of positive charge based removal of endotoxins from raw water systems, while at the same time demonstrating the limitations of using nominally rated depth media as the supporting matrix.

Control of microbial bioburden in the pharmaceutical manufacturing industry by utilising filtration technology, is well established. Questions within the industry about phenomena such as bacterial "grow through" during long term use of filters, and penetration by small water borne organisms have been answered [22].

Effective control of endotoxin levels by positive charge-modified filters, both bacterially retentive and coarser particulate grades (prefilters), has been the subject of much discussion by those directly concerned.

We present here data on the endotoxin removal capabilities of a range of PALL positive charge-modified filters intended for use in pharmaceutical water systems, in an attempt to answer questions raised about general performance expectations, including performance across a range of physical process conditions.

1. Observed capacities of PALL 254 mm long double and single layer 'N66' filters when challenged with bacterial cell associated endotoxin

Results

The observed level of removal of bacterial cell associated endotoxin from the various Nylon 66 positive charge - modified 'N_{66}' 'Posidyne' filters and control 'Ultipor' 'N_{66}' filters is shown in the log scaled chart of Figure 3. It should be remembered that all breakthrough points were determined at a detection limit of 0.125 EU/ml in the effluent.

Discussion

The capacity of the filter to retain this form of endotoxin is governed by two mechanisms:

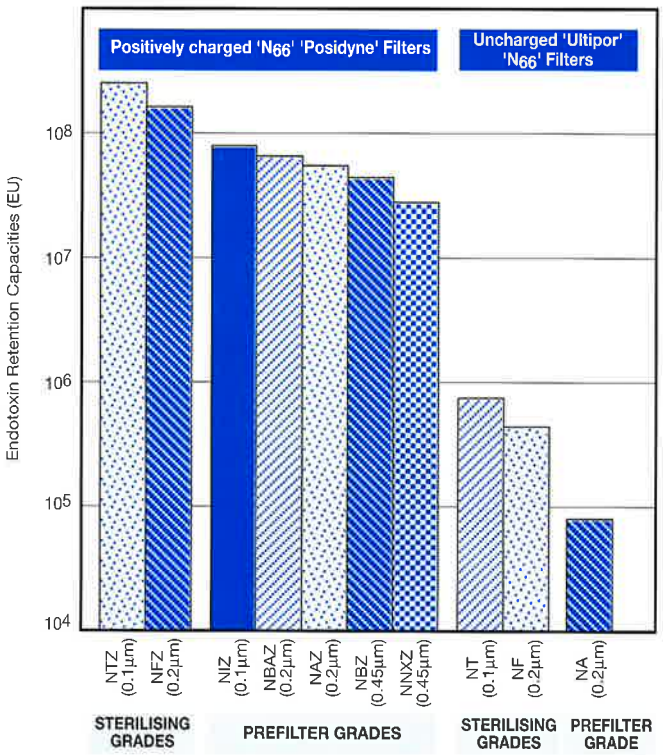
a) The primary removal mechanism is by charge adsorption by the positive zeta potential of the 'N_{66}' 'Posidyne' filter medium.

b) The secondary mechanism is mechanical retention of the larger endotoxin vesicles and bacterial cell wall fragments contained in the challenge suspension. This effect increases with decreasing pore size. The age of the cell culture is also important since there is a higher proportion of smaller more penetrating fragments in older cultures.

The relative contribution from the secondary filtration effect may be more significant at lower endotoxin concentration challenges since uncharged 'Ultipor' 'N_{66}' filters show pore size dependent endotoxin removal of cell associated challenges of up to 8 x 10^5 EU in this study. In addition, at finer pore sizes, filtration effects may help reduce the challenge to the adsorptive charge sites by removing material by direct interception which might otherwise compete for adsorptive sites. Such material may consist of cells, cell fragments and vesicles (compare NT and NA grade filter removal capacities in Figure 3).

The efficacy of the double layer 'N_{66}' 'Posidyne' sterilising grade (NTZ and NFZ) filters in endotoxin removal can be seen in Figure 3. The efficacy of other 'N_{66}' 'Posidyne' filters is also clearly demonstrated.

FIGURE 3:
Endotoxin retention capacities of PALL 254mm length 'N66' filters.



2. Observed capacities of PALL 'Profile II Plus' and 'Ultipor GF Plus' pre-filters when challenged with bacterial cell associated endotoxin

Results

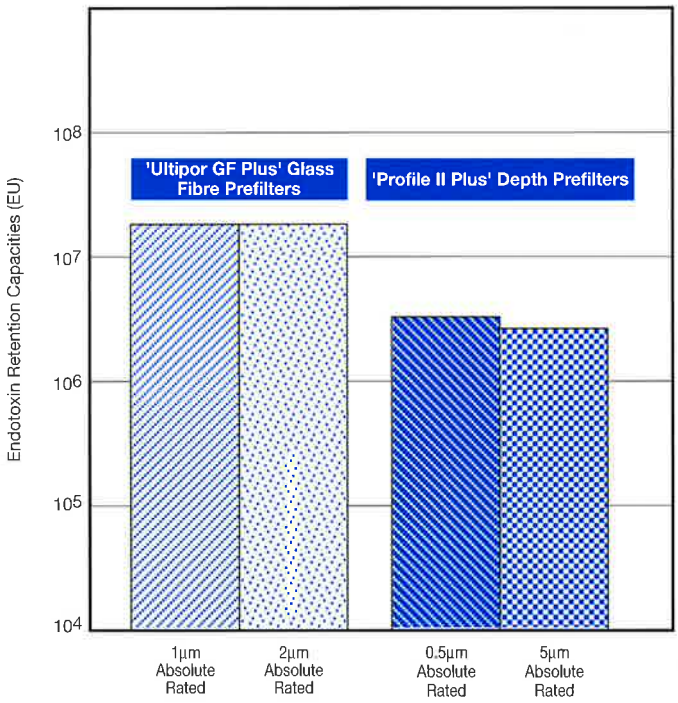
Figure 4 shows the observed removal capacities for some of the pre-filter cartridges available in positive charge-modified filter media. 'Ultipor GF Plus' absolute rated glass fibre and 'Profile II Plus' absolute rated depth pre-filters were tested. As in Figure 3 capacity is determined by breakthrough at a detection limit of 0.125 EU/m1.

Discussion

The filters show a high endotoxin removal capacity, even though their absolute removal ratings are in the coarser range from 0.5µm to 5µm, compared to the 0.1µm, and 0.2µm absolute rated sterilising grade filters shown in Figure 3.

PALL positive charge-modified pre-filters can, therefore, provide substantial removal capacities for endotoxin, in addition to protection of 'N_{66}' 'Posidyne' final filters in a total process system producing sterile effluent. This offers systems with very high total capacities for use in endotoxin control.

FIGURE 4:
Endotoxin retention capacities of PALL pre-filters.



3. Observed capacities of PALL 0.1µm and 0.2µm absolute rated 'N_{66}' 'Sealkleen' style filters when challenged with purified (Lipopolysaccharide) endotoxin.

Results

Figures 5a and 5b show high endotoxin removal capacities of up to 10^8 EU for 0.1 µm and 0.2 µm rated prefilter and sterilising grade filters in the smaller 'Sealkleen' cartridge style, when challenged with purified *E.coli* 055.B5 endotoxin. Capacity is determined by breakthrough at a detection limit 0.125 Eu/ml. In contrast, uncharged 'Ultipor' 'N_{66}' filters showed substantially lower capacities.

Discussion

The 'N_{66}' 'Posidyne' filters remove purified LPS to essentially the same levels as cell associated endotoxin, whether EDTA is present or not, (see figures 5a and 5b) since the major removal mechanism is by charge adsorption.

However, the results for the uncharged 'Ultipor' 'N_{66}' filter challenges in Figure 5a and 5b show how the proportion of LPS aggregates removed by 0.2µm rated filters is higher when EDTA is not present to remove the divalent cations which facilitate formation of such complexes. There is, however, only a slightly lower level of LPS aggregates removed by 0.1µm rated and (NT) filters when EDTA is present indicating larger LPS aggregates are still present.

Comparison of the two figures demonstrates the effect of minimising the tendency of the purified LPS to self-aggregate into larger structures, by the addition of EDTA.

These observations illustrate the caution needed when interpreting endotoxin removal data from non-adsorptive systems. Changes in transport solution conditions may cause disaggregation and subsequent downstream contamination.

FIGURE 5a:
Retention of purified (LPS) endotoxin challenge by PALL 'Sealkleen' SLK 7001 style filters.

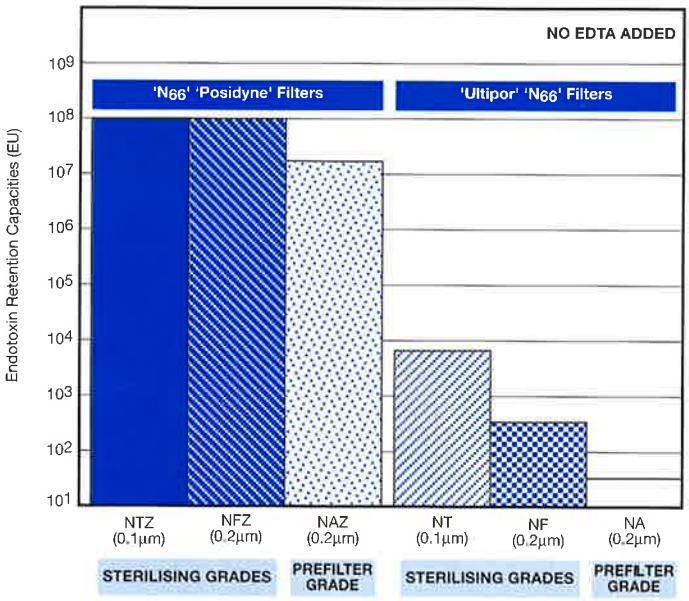
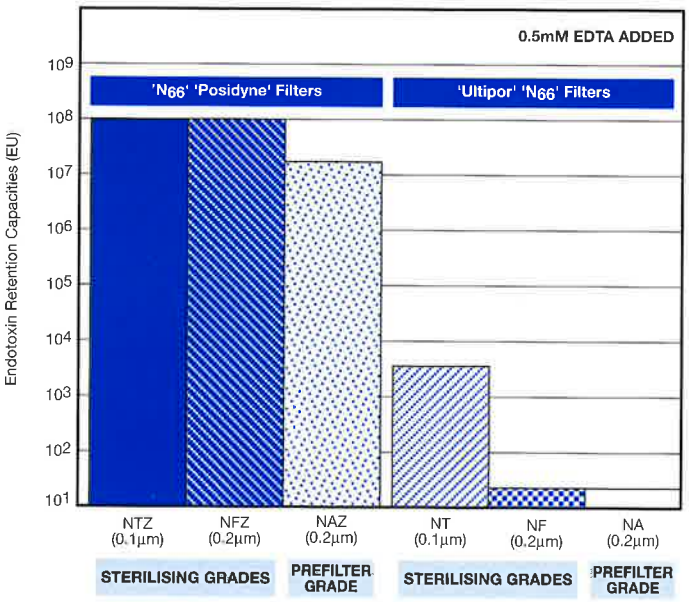


FIGURE 5b:
Retention of purified (LPS) endotoxin challenge by PALL 'Sealkleen' SLK 7001 style filters.



4. Studies into the effects of changing physical conditions on the endotoxin removal capacities of PALL double layer 0.2µm absolute rated (NFZ Grade) 'N_{66}' 'Posidyne' filters.

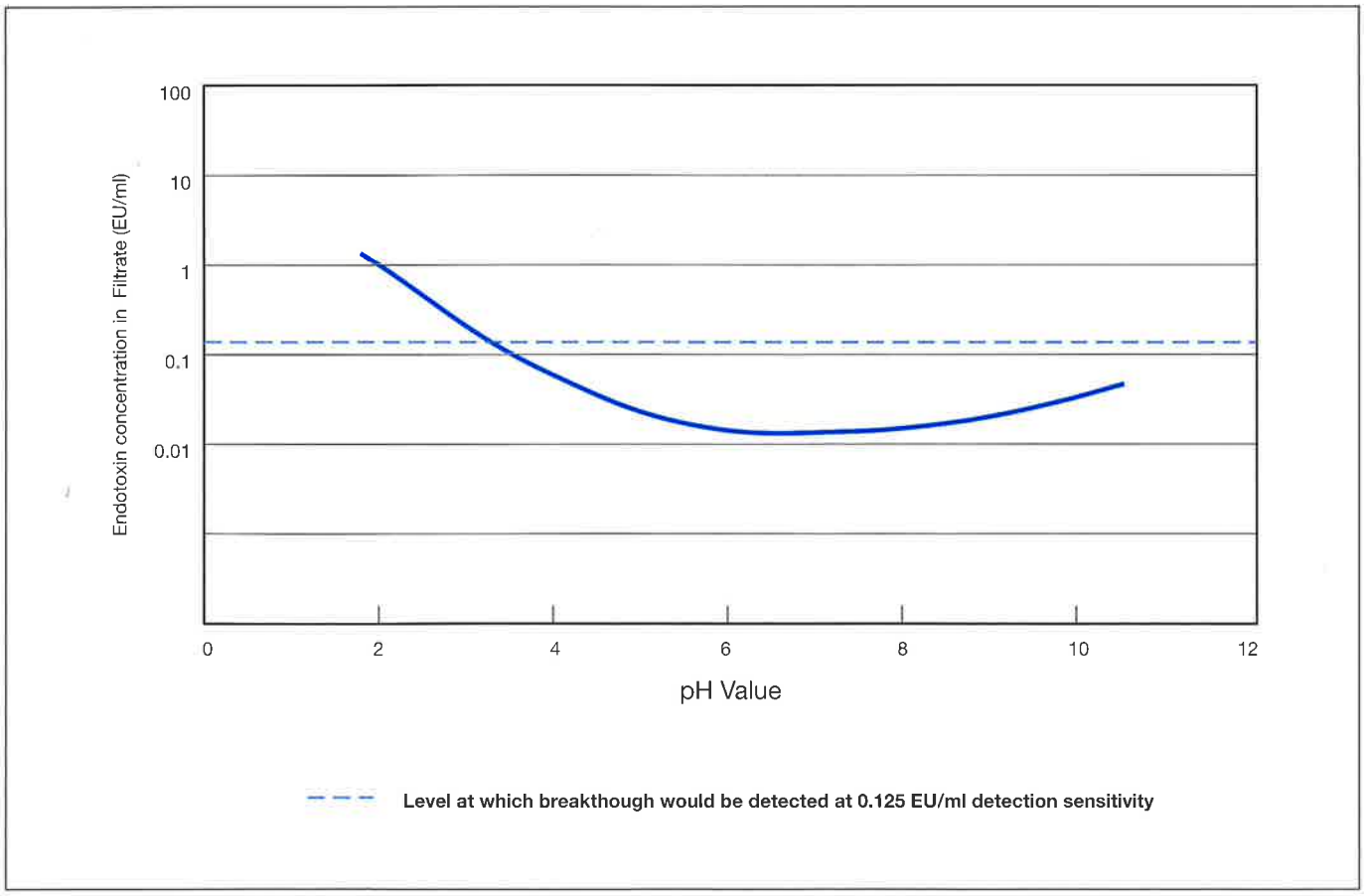
All comparative tests in these studies were performed on PALL 'Sealkleen' style NFZ grade 'N_{66}' 'Posidyne' filters. The endotoxin challenge presented was in all cases *E.coli* 055.B5 purified (LPS) endotoxin in the presence of 0.5 mM EDTA, to maximise the challenge to the filters' adsorptive mechanism. All tests were conducted with an equivalent total challenge level of approximately 8 x 10^7 EU (below the observed breakthrough limit in high purity water for a 254 mm cartridge), so that any significant reduction in removal capacity due to the physical changes imposed could be observed.

4.1. Effect of Challenge Solution pH Changes

Results

Figure 6a shows the effect of pH change on the endotoxin removal properties of the NFZ grade 'N_{66}' 'Posidyne' filters when challenged as described. Changes in detectable levels of endotoxin were insignificant for most of the pH range investigated.

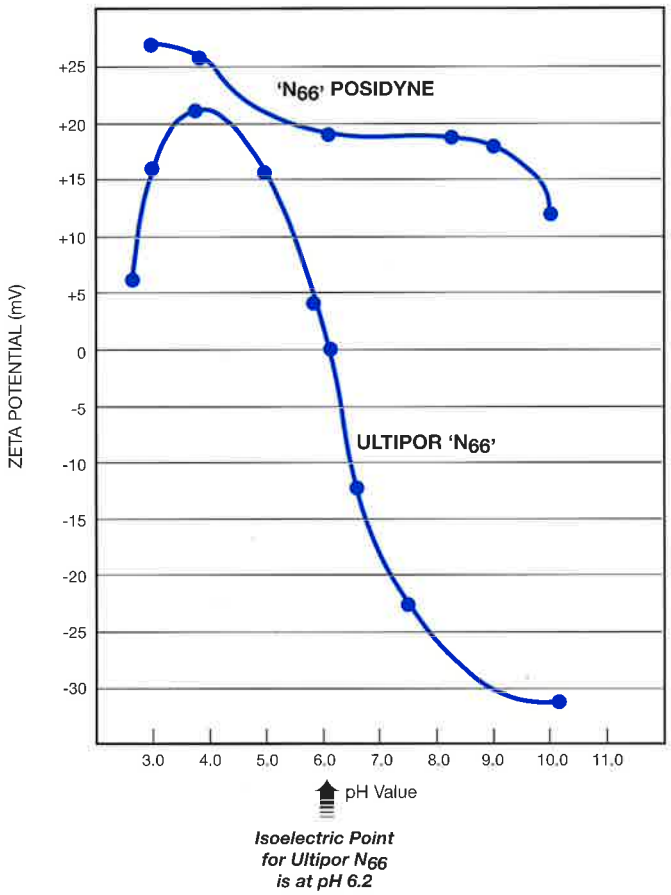
FIGURE 6a:
Effect of pH on purified (LPS) endotoxin removal capacity of PALL 0.2µm rated (NFZ grade) 'N_{66}' Posidyne' Filters.



Discussion

PALL 'N_{66}' 'Posidyne' medium retains its positive charge over a wide range of pH values as shown in Figure 6b. Change in pH over the range pH value 4-10 has little detectable effect on the filters capacity for endotoxin retention. It is only below pH 4 that reduction in endotoxin capacity becomes significant. This may be explained by the fact that the charged phosphate groups on the LPS, ascribed as the most probable sites for charge attraction, have an isoelectric point generally in the pH region 1.8 - 2.0. As the pH of the solution is lowered and this value approached, the negative charge, and thus the binding attraction, becomes less strong and consequently more endotoxin is detected in the effluent. This effect becomes more pronounced below pH 4, yet adsorptive removal capacity is still substantial to pH 3.

FIGURE 6b:
Zeta potential vs pH value of 'Ultipor' 'N_{66}' & 'N_{66}' 'Posidyne' media.



4.2. Effect of Flow Rate

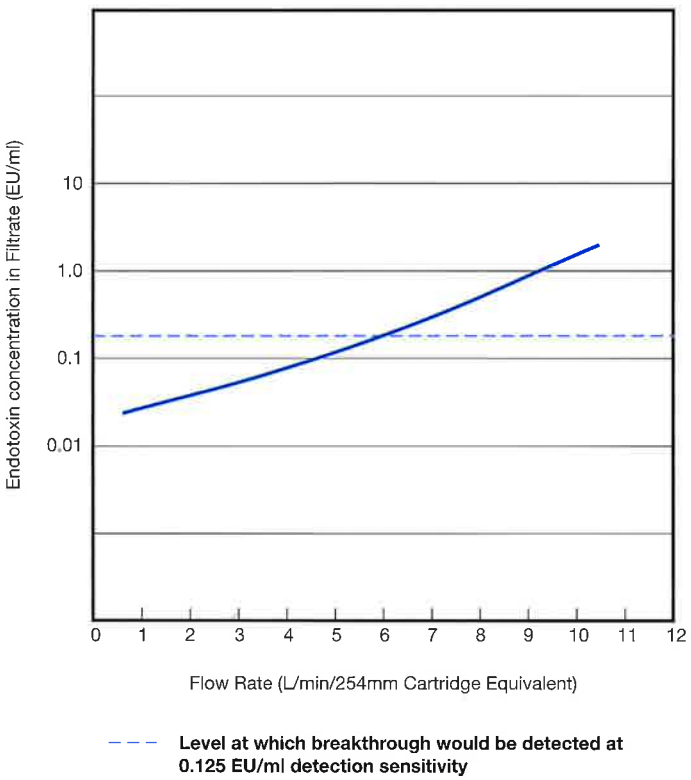
Results

Figure 7 shows the effect of flow rate on endotoxin removal capacity of PALL 0.2µm rated (NFZ grade) 'N_{66}' 'Posidyne' filters. PALL 'Sealkleen' pleated cartridge filters (0.14m² surface area) were used for the study but to assist in comparative assessment flow rates have been expressed as the equivalent flow rate (L/min) through a standard 254mm cartridge (0.79m² surface area). As flow rate increases, adsorptive removal capacity tends to decline, passing the 0.125 EU/ml effluent detection limit at around 6 L/min, though again, there is still substantial endotoxin removal at 10 L/min.

Discussion

This effect is probably due to the shortening transition time of the endotoxin solution through the filtration medium and consequently less chance of the endotoxin encountering a suitable adsorptive charge group on the inner surfaces of the medium. This observation demonstrates the need to take into account the flux (flow rate/unit area) through the cartridge when sizing systems, to optimise the effectiveness of the filter for endotoxin removal, and so produce consistent effluent quality. A similar effect has been shown to occur during use of charge-modified depth media [24].

FIGURE 7:
Effect of flow rate on purified (LPS) endotoxin removal capacity of PALL 0.2µm rated (NFZ grade) 'N_{66}' 'Posidyne' filters



4.3. Effect of Change in Influent Temperature

Results

Figure 8 shows the lack of any detectable effect of influent temperature on the removal capacity of PALL 0.2µm rated (NFZ grade) 'N_{66}' 'Posidyne' medium, from 4° C to pasteurising temperatures exceeding 60° C. In addition, this serves to illustrate the stability of the chemistry of the positive charge modification and the strength of the bond formed between the endotoxin and filter medium.

4.4. Effect of Steam Sterilisation Procedures

Results

Figure 9 is presented to show that pre-use steam sterilisation or autoclaving does not have a detectable effect on the stability of the charge modification or on endotoxin binding, and hence the filters' total endotoxin removal capacity. It also shows that a significant bacterial challenge (such as would result from bacterial contamination of a previous batch when the filter is used for cold sterilisation of multiple batches) has no effect on the subsequent endotoxin retention capacity of the filter medium when challenged under these conditions, and does not result in unloading or 'leaching' of endotoxin on re-use.

4.5. Effect of 'Bolus' Application of Endotoxin Challenge

Results

Figure 10 shows that when a significant "bolus" (high concentration over a short period of time) challenge level of purified endotoxin is presented to a PALL 0.2µm rated (NFZ grade) 'N_{66}' 'Posidyne' filter, rather than as a gradually increasing continuous challenge as in previous tests, the capability of the filter to retain such a total endotoxin challenge is not compromised. "Bolus" challenges may, for instance, occur through accidental contamination, or may have accumulated in a dead leg in a system.

FIGURE 8:
Effect of temperature on purified (LPS) endotoxin removal capacity of PALL 0.2µm rated (NFZ grade) 'N_{66}' 'Posidyne' filters.

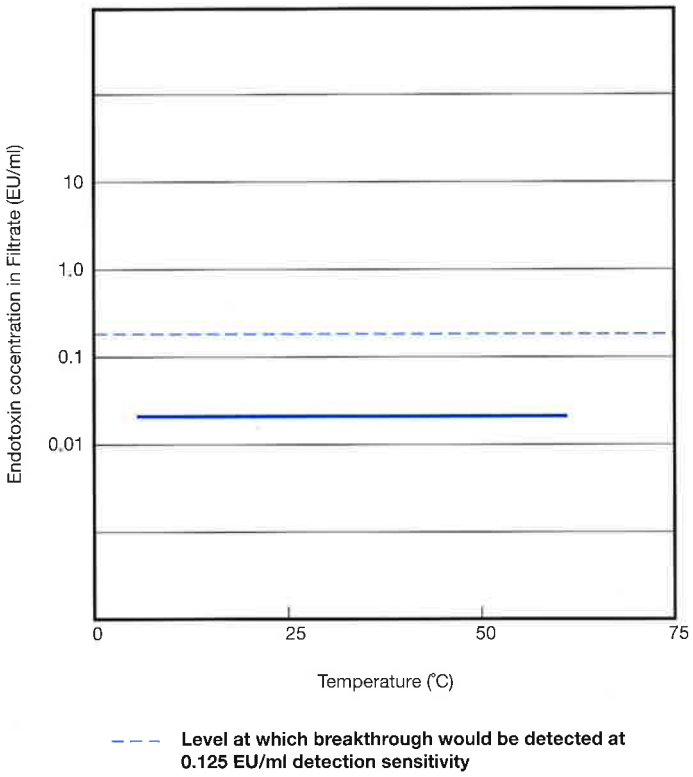


FIGURE 9:
Effect of steaming and pre-challenge with bacteria on purified (LPS) Endotoxin removal capacity of PALL 0.2μm rated (NFZ grade) 'N66' 'Posidyne' filters.

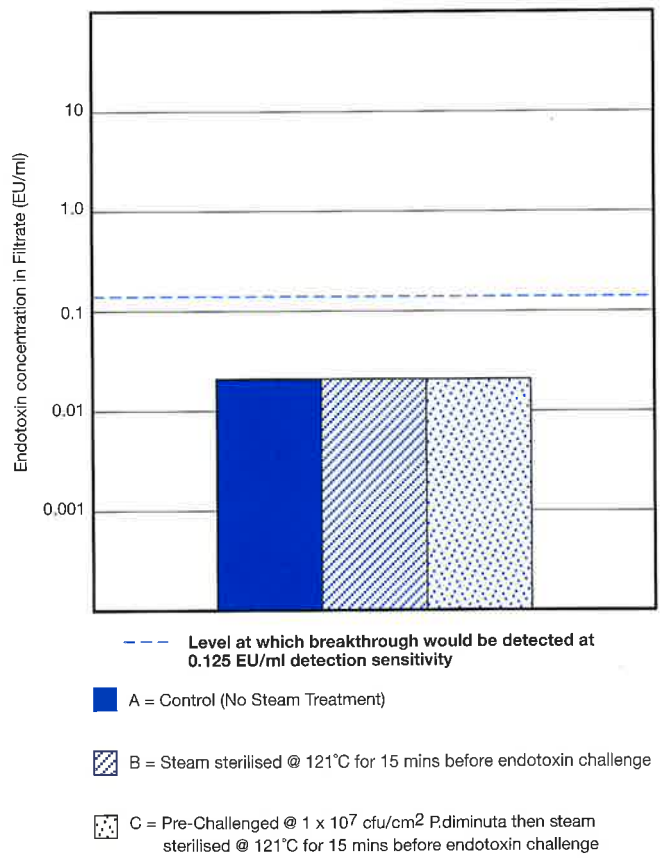
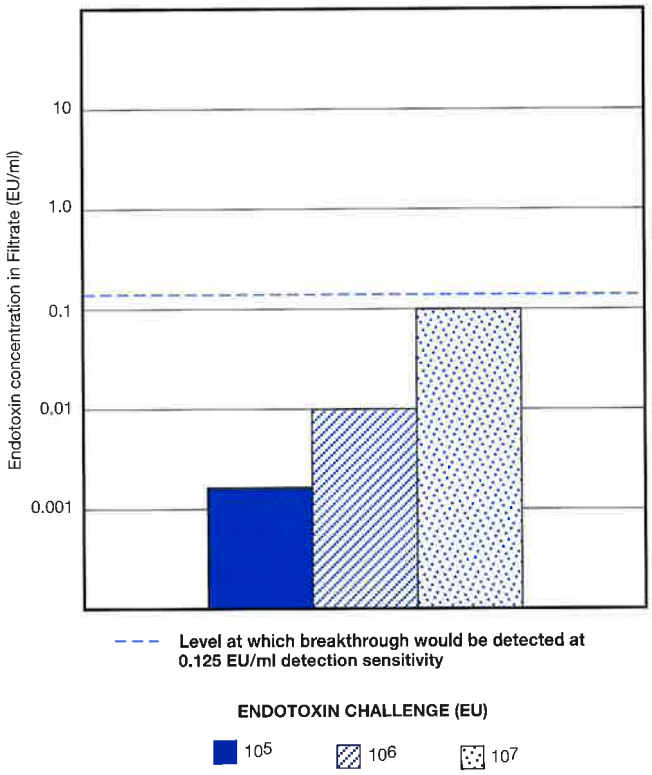


FIGURE 10:
Effect of initial purified (LPS) Endotoxin challenge concentration on Endotoxin removal capacity of PALL 0.2μm rate (NFZ grade) 'N66' 'Posidyne' filters.



Conclusions

The previous test programmes of James et al [22, 25], using 0.2 µm, 0.1 µm and 0.04 µm, rated PALL 'N_{66}' 'Posidyne' filters, showed that these filters could retain *Pseudomonas diminuta* (ATCC 19146), and the system associated bioburden for test periods of up to five months in a simulated high purity water system. No grow-through or bacterial cell associated endotoxin penetration occurred.

This complementary programme of tests was instigated to establish endotoxin removal properties and typical endotoxin retention capacities of such filters by testing to actual breakthrough under a wide range of process conditions typically found in water systems.

The high level of endotoxin removal achievable by a single sterilising grade 'N_{66}' 'Posidyne' cartridge is clearly demonstrated in the results. The level of protection afforded by larger pore and pre-filter grades of positive charge-modified membrane media approaches that of sterilising filter grades, and thus provides extremely high levels of protection to such final filters in the total system.

A high endotoxin capacity and consistent performance are essential in long term use where process conditions or the quality of influent raw water may change (perhaps daily and certainly seasonally) and chance contaminations may occur.

The data presented here also show that the clearance levels achievable by PALL charge-modified filters are not compromised across a wide range of different physical system conditions which may be imposed.

These studies on PALL 'N_{66}' 'Posidyne' membrane filters, 'Profile II Plus' absolutely rated depth pre-filters and 'Ultipor GF Plus' glass fibre membrane pre-filters show that PALL positive charge-modified filters can provide high and consistent levels of endotoxin clearance when subjected to challenge with both purified (lipopolysaccharide) endotoxin and simulated environmental endotoxin derived from autoclaved high titre bacterial cell cultures. Removal performance is not significantly affected across a wide range of pH, temperature, flow rate, initial challenge concentration, or by filter steam sterilisation procedures.

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Appendix - Material and methods

Tests to determine endotoxin retention capacities

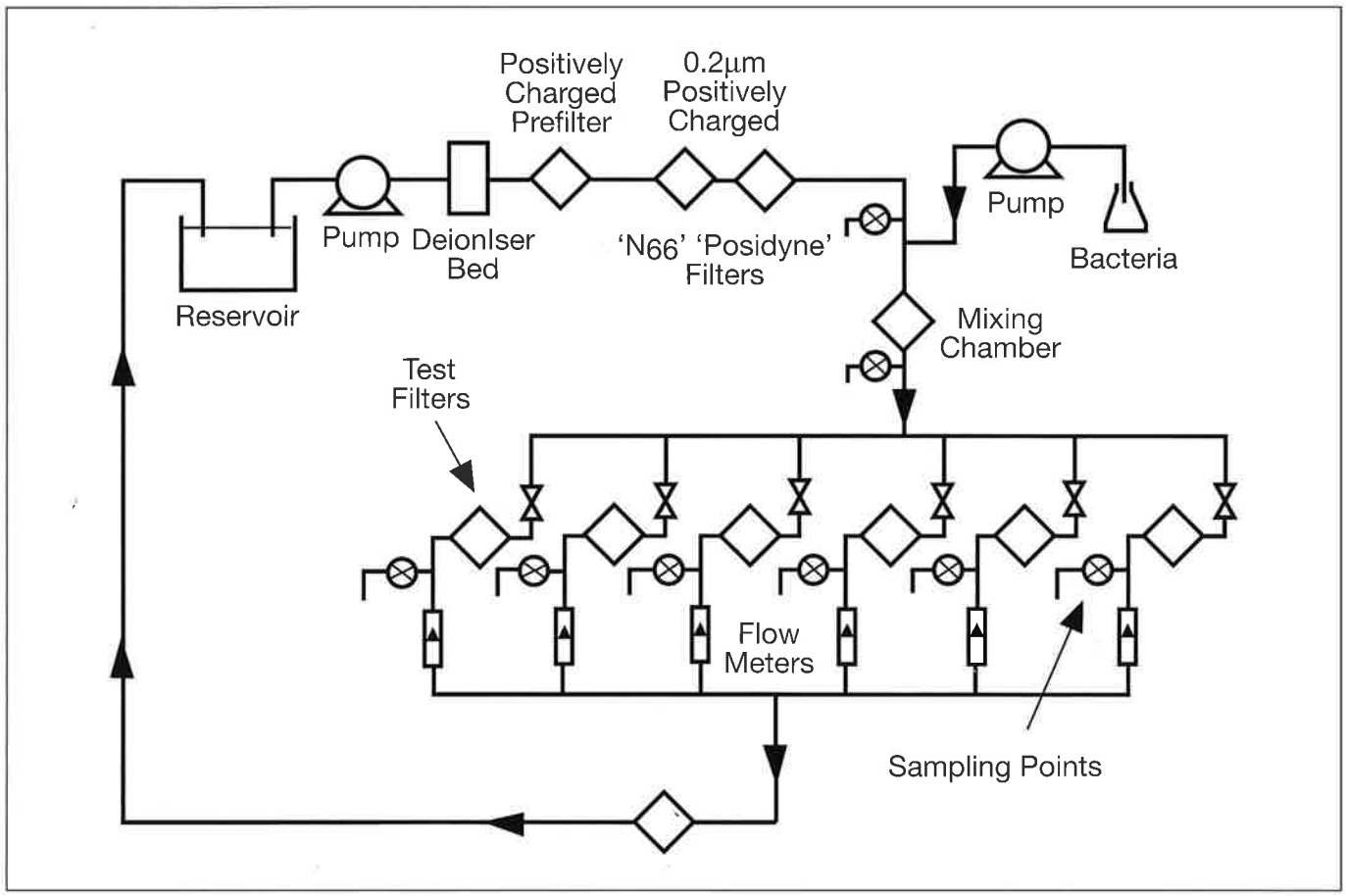
A range of single and double layer positive charged-modified PALL 'N_{66}' 'Posidyne' and uncharged 'Ultipor 'N_{66}' filters of absolute rating 0.45 µm to 0.1 µm were tested, together with positive charge-modified PALL 'Profile II Plus' absolutely rated polypropylene depth prefilters (0.5 µm and 5 µm) and PALL 'Ultipor GF Plus' glass fibre absolutely rated membrane prefilters (1 µm and 2 µm).

The tests, to endotoxin breakthrough, were performed using endotoxin challenges consisting of:

1. Bacterial cell associated endotoxin from autoclaved high titre cultures of logarithmically growing *Pseudomonas diminuta* ATCC 19146, incorporating a range of bacterial cell derived endotoxin structures.

2. Purified lipopolysaccharide (LPS) obtained from *E.coli* type 055.B5 and treated with 0.5 mM EDTA to help provide a micellular/bilayer size based endotoxin challenge, the smallest endotoxin structure which would be practically encountered.

FIGURE 11:
Test Rig for Endotoxin Challenges.



Detection of endotoxin was by the kinetic turbidimetric LAL test using the Associates of Cape Cod LAL 5000 system. Under ideal conditions, this method can give a resolution of 0.001 EU/ml detectable over the range 0.001 EU/ml to 100 EU/ml. The system is based on the principle that the optical density (turbidity) of a solution containing LAL and endotoxin increases as gelation occurs [23]. The rate of change of optical density is measured to a threshold limit, and this time value is proportional to the concentration of endotoxin present in the sample, when measured against test standards. All measurements were ultimately referenced to reference standard endotoxin (RSE) lot EC-5. Breakthrough was deemed to have occurred when an endotoxin level of 0.125 EU/ml was detectable in the effluent from the filters.

Bacterial Cell Associated Endotoxin Challenges

Bacterial cell associated endotoxin challenges of 254 mm cartridges were carried out using a recirculating rig as depicted in Figure 11. The system incorporating all housings and guard filters was first sterilised by recirculating 4% hydrogen peroxide with no test filters in place for two hours. The rig was then pumped dry and flushed twice with filtered deionised water. Following this sterilisation procedure, the guard filters were changed and test filters inserted. Filtered mains water was then pumped from the holding tank and passed through a mixed bed deioniser. Water exiting from the deioniser bed then passed through a prefilter (1 µm absolute rated 'Ultipor GF Plus' filter) followed by the two serially located sterilising grade 0.2 µm 'N_{66}' 'Posidyne' guard filters.

The water was tested immediately downstream of these guard filters for conductivity, temperature, pH, sterility and endotoxin level. The filtrate from these guard filters then passed through a mixing chamber to a six position manifold which distributed flow to the six filters under parallel test. Equal flow to each test filter (1 - 1.5 L/min) was ensured by a tap and calibrated rotameter flowmeter on each line. Effluent from each of the six test filters then passed into the return line to the holding tank. Effluent sampling was achieved by taking aliquots of effluent which had passed through continuously flowing slip-stream sample lines located immediately downstream of each test filter.

The rig was finally depyrogenated by flushing-up by recirculation with no challenge applied for at least one hour, and until all upstream and downstream sampling points gave no detectable endotoxin as judged by the threshold limit (ie <0.125 EU/ml) and a resistivity = > 10 MΩ/cm. Following this flush up, bacterial cell derived endotoxin from the autoclaved high titre cultures of *Pseudomonas diminuta* ATCC 19146 was injected via the contaminant injection point downstream of the guard filters, upstream of the mixing chamber.

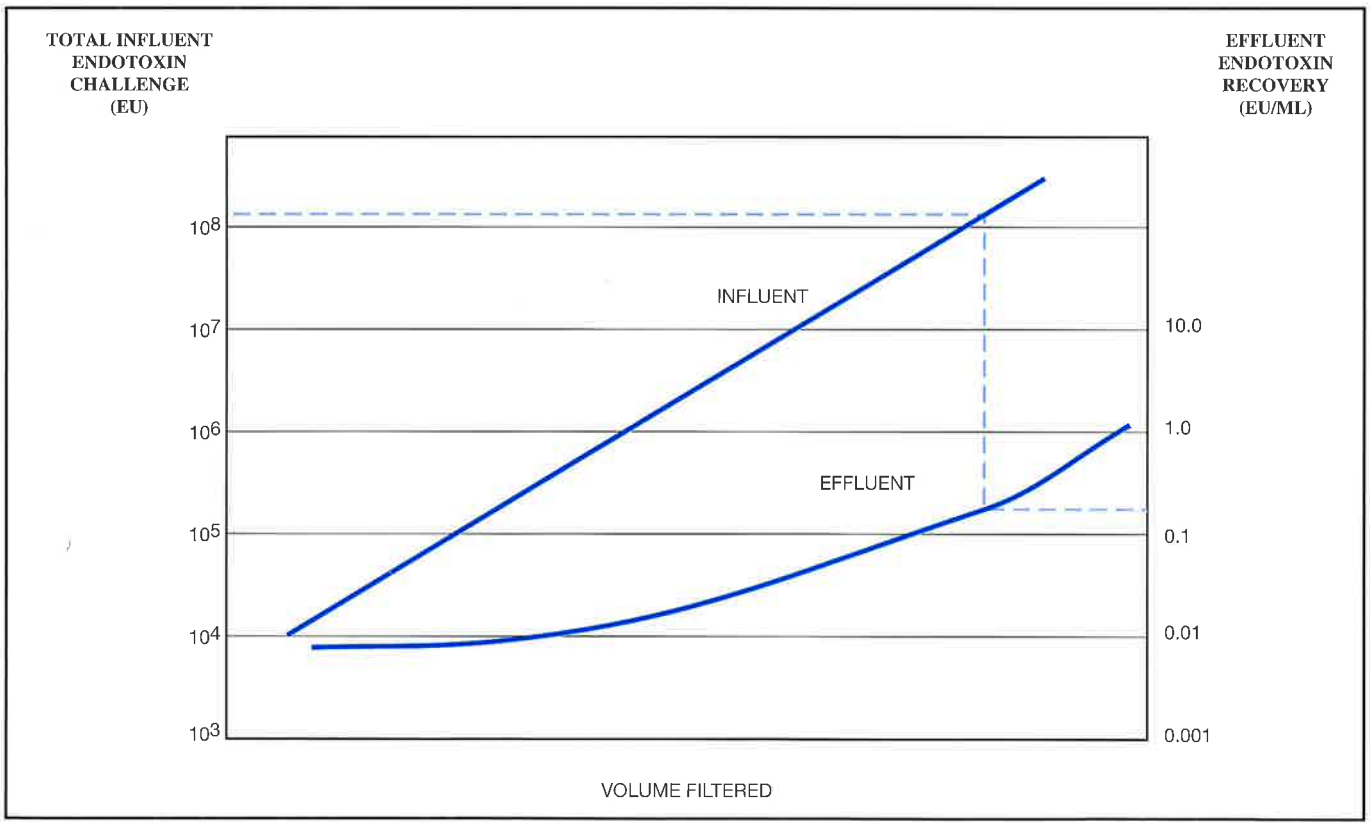
The contaminant injection rate was varied to obtain a logarithmic increase of endotoxin contaminant loading using diluted and undiluted quantities of the autoclaved culture. Contaminant injection was performed over a six hour period. Challenge tests were performed at ambient temperature.

Upstream samples to determine the influent challenge to all test filters at all sampling times were taken via a continuously flowing sample line located downstream of the mixing chamber but upstream of the six position manifold. Control sampling of influent water quality to the system was made at the continuously flowing sampling point immediately downstream of the guard filters, but upstream of the contaminant injection point.

Concurrent sampling was carried out downstream of each test filter at regular intervals during the course of the injection of the increasing levels of endotoxin, and for one hour after completion of contaminant injection. All samples of both influent endotoxin challenge and corresponding downstream contaminant monitoring were tested for endotoxin level as described, and regularly for sterility.

A plot (Figure 12) was then produced of endotoxin recovery downstream of each test filter with increasing upstream challenge level, and the level of challenge at which endotoxin was detected at 0.125 EU/ml in the effluent was determined.

FIGURE 12:
Example plot of Endotoxin recovery downstream of test filter with increasing total challenge to filter.



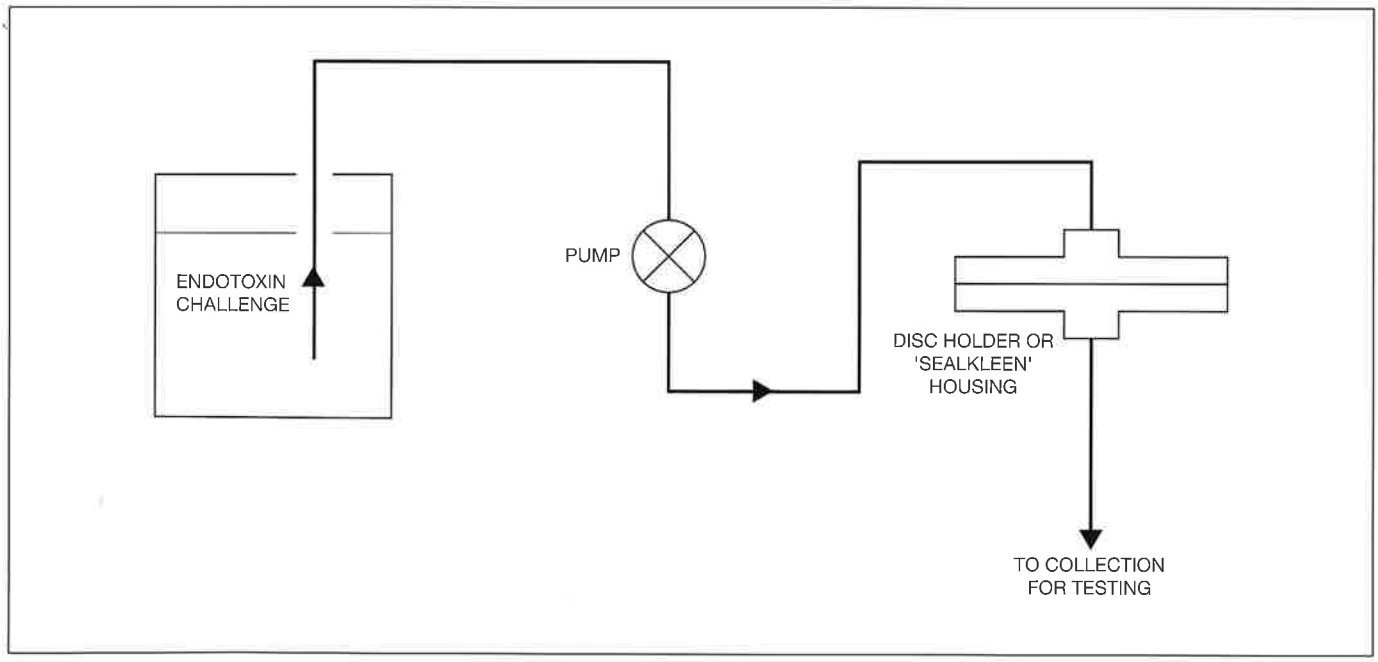
Purified endotoxin (lipopolysaccharide) challenges

For purified endotoxin challenges, a simple rig was employed (Figure 13) and challenges performed serially. Housings and associated fittings /pipework were depyrogenated using dry heat (280° C for three hours) and heat sensitive equipment (tubing, O-rings etc) was flushed continuously in depyrogenated water until pyrogen free (less than 0.125 EU/ml detectable in flush water) by LAL testing. Smaller media area PALL 'Sealkleen' 7001 style cartridges were used in these purified endotoxin tests due to the large quantities of purified endotoxin required.

After assembling the depyrogenated rig, challenges were performed by first flushing the cartridge with 500 ml of pyrogen free water (which was tested for endotoxin level), followed by application of a logarithmic challenge of lipopolysaccharide reconstituted in pyrogen free water with 0.5 mM EDTA added.

Downstream sampling was carried out regularly throughout the application of the challenge. After sample testing for the presence of endotoxin, a plot of endotoxin recovery versus total challenge was similarly produced as above.

FIGURE 13:
Basic challenge rig used in purified endotoxin tests.



Determination of the distribution of endotoxin activity with respect to bacterial cell fragment size in bacterial cell derived endotoxin challenges

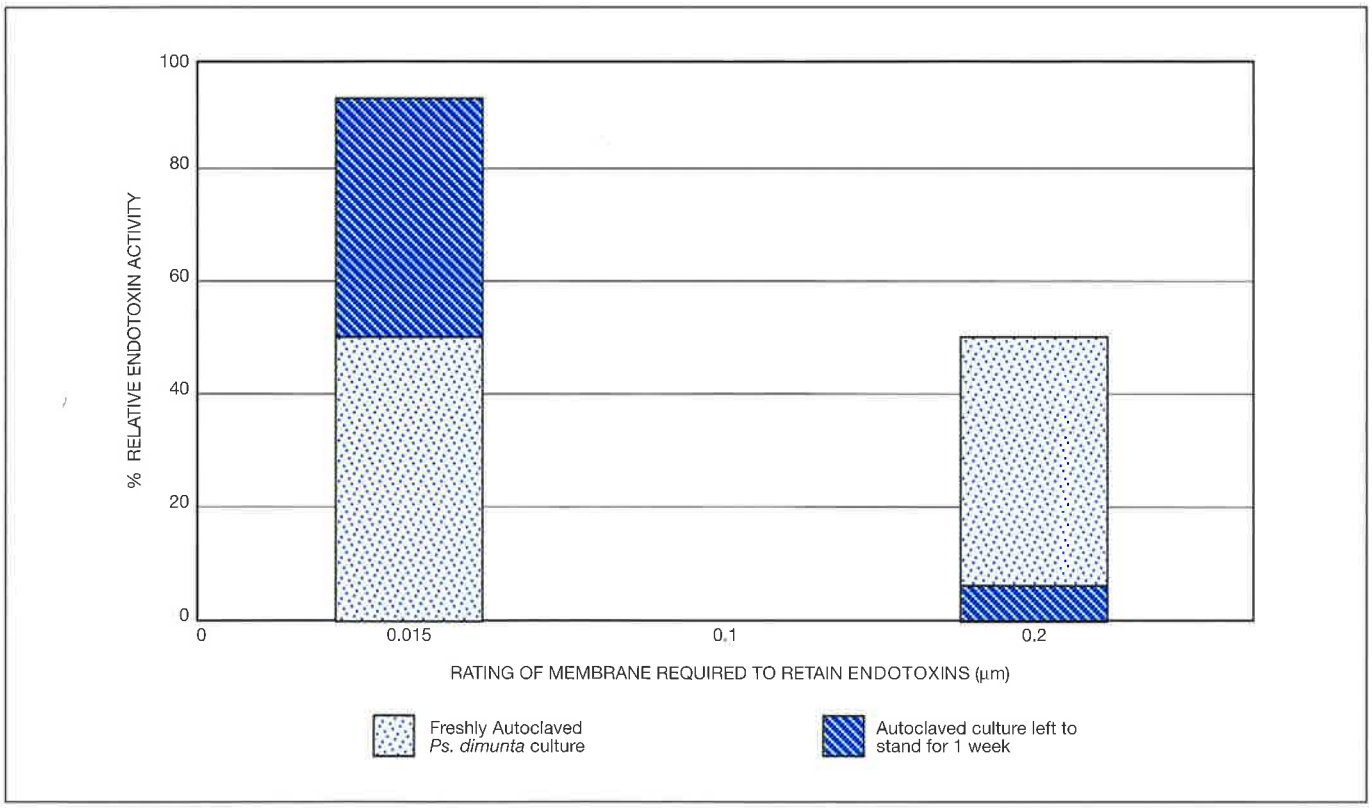
Endotoxin activity was determined in filtrates of suspensions of autoclaved *P.diminuta* from Nuclepore membrane and Amicon nominal molecular weight cut-off disc filters. The autoclaved culture solution was passed through Nucleopore 0.2 µm, 0.1 µm and 0.015 µm rated discs and Amicon 1 x 10^6, 1 x 10^4 dalton NMW cut-off filters. Filtrates were analysed for the presence of endotoxin by the LAL turbidimetric assay.

Results (Figure 14) show that freshly autoclaved cultures (containing an average 1 x 10^{12} organisms) have approximately 50% of the endotoxin activity of the solution in material which was retained by a 0.2 µm filter. Less than 1% of the remaining 50% of detectable endotoxin was retained by a 0.1 µm filter, but 49% was retained by a 0.015 µm filter.

If an autoclaved culture was left to stand for a number of days, the quantity of detectable endotoxin retained by the 0.2 µm filter fell, while that retained by the 0.015 µm filter rose (Figure 14). Again, there was little retention of endotoxin structures by the 0.1 µm rated filter, ie the bacterial cell membranes containing the endotoxin were either essentially intact, or disintegrated into small fragments not generally retained by the 0.1 µm filter.

Based on these results, to maximise the severity of the challenge to the adsorptive mechanism of removal of the test filter (reducing filterable effects), one week old autoclaved cultures were used in cell associated endotoxin challenges.

FIGURE 14:
Change of bacterial cell fragment size distribution with age of culture.



Effect of the presence of Ethylenediamine tetraacetic acid (EDTA) on purified (LPS) endotoxin aggregate size when suspended in pyrogen free water

Purified (LPS) *E.coli* 055.B5 (2.5 mg/vial Whittaker Bioproducts Inc.) endotoxin was reconstituted, according to the manufacturers' instructions, in high purity pyrogen free water, either in the presence or absence of the chelating agent EDTA. This agent sequesters divalent cations present in the transport medium (in this case, water) which facilitate and stabilise formation of larger endotoxin structures [10]. Solutions (50-100 EU/ml) from both EDTA treated and untreated reconstitutes were passed through pre-filtered Nucleopore 0.1 µm and 0.05 µm rated membrane filters and Amicon 100,000 nominal molecular weight cut-off membrane filters. The filtrates from each filter stage were then tested for endotoxin level by the LAL test. The results are shown below.

The data shows the effect of the added 0.1 mM EDTA on filterability, particularly through the Amicon 1 x 10^5 dalton NMW cut-off membrane which represents penetration by the smallest structures. This data supports observations made during purified (LPS) endotoxin challenges (with and without EDTA added) of non-charged 0.1 µm rated 'Sealkleen' cartridges (Figures 5a and 5b).

The data therefore provides evidence that the removal mechanism, under these conditions, is essentially due to adsorptive effects and that filtration effects are absent or minimal even with a 0.1 µm absolute rated filter.

EDTA at a concentration of 0.5 mM was used in the final purified (LPS) endotoxin preparations, to maximise the removal of divalent cations without appreciably affecting the LAL reaction. Some inhibition of the LAL reaction was seen to occur at a concentration of 1 mM EDTA.

The detergent Tween or chaotrope sodium deoxycholate were not incorporated in these tests to further reduce LPS aggregate size, due to their surface active properties and potential enhancing effect when determining endotoxin removal capacities. Such solution conditions are unlikely to be encountered in a water system in practice.

HIGH PURITY PYROGEN FREE WATER

With 0.1 mM EDTA added			With no added EDTA		
Filter Type	**Rating**	**Endotoxin Penetration**	**Filter Type**	**Rating**	**Endotoxin Penetration**
Nuclepore	0.1 µm	100%	Nuclepore	0.1µm	100%
Nuclepore	0.05 µm	100%	Nuclepore	0.05µm	75-100%
Amicon	1 x 10^5D	15-20%	Amicon	1 x 10^5D	1-2%

Experiments to determine the effect of physical changes in filtration conditions on endotoxin removal by positive charged modified filters

These experiments were performed using purified (LPS) endotoxin for two reasons:

1. To minimise the interfering effects from complex bacterial culture medium components associated with using cell derived endotoxin challenges

2. To obtain the required endotoxin challenge levels in as small a volume as possible.

The endotoxin challenge solutions were prepared, as before, in high purity water in the presence of 0.5 mM EDTA.

Testing was, unless otherwise stated, performed using the test rig and protocol outlined in the materials section describing purified endotoxin challenges, (Figure 13). 'Sealkleen' SLK7001NFZP style cartridges were used as the test filter medium in these investigations.

ALTERATION OF pH

Using appropriate quantities of either HCl or NaOH, an endotoxin challenge solution, prepared as above, was brought to the required pH and the challenge to the filter performed. Samples of the filtrate, taken at appropriate intervals, were returned to neutral pH before testing for the presence of endotoxin using the turbidimetric LAL method.

FLOW RATE VARIATION

Control of experiment flow rate was achieved by using a peristaltic pump at lower flow rates, and by use of displacement by regulated sterile filtered air pressure at higher flow rates. A calibrated measuring cylinder and stopwatch were used to determine effluent flow rate.

CONTROL OF TEMPERATURE

For lower temperature experiments, cooling of influent challenge solution was achieved using an ice bath. Temperature was measured using a calibrated mercury thermometer and both filter influent and effluent temperatures were monitored. For higher temperature experiments, a calibrated hot water bath was used to maintain the required test temperature.

APPLICATION OF 'BOLUS' ENDOTOXIN CHALLENGE

Purified (LPS) endotoxin was applied to 'Sealkleen' SLK7001NFZP cartridges in small volumes (10 ml - 50 ml) of solutions of the appropriate endotoxin concentration. Solutions of purified *E.Coli* 055.B5 endotoxin of concentrations 10^4 EU/ml, 10^5 EU/ml and 10^6 EU/ml were applied after pre-flushing each cartridge and housing with pyrogen free water, and then draining to remove excess water. The challenges were applied essentially as previously described (Figure 13), and downstream samples taken throughout the 'bolus' application and afterwards.

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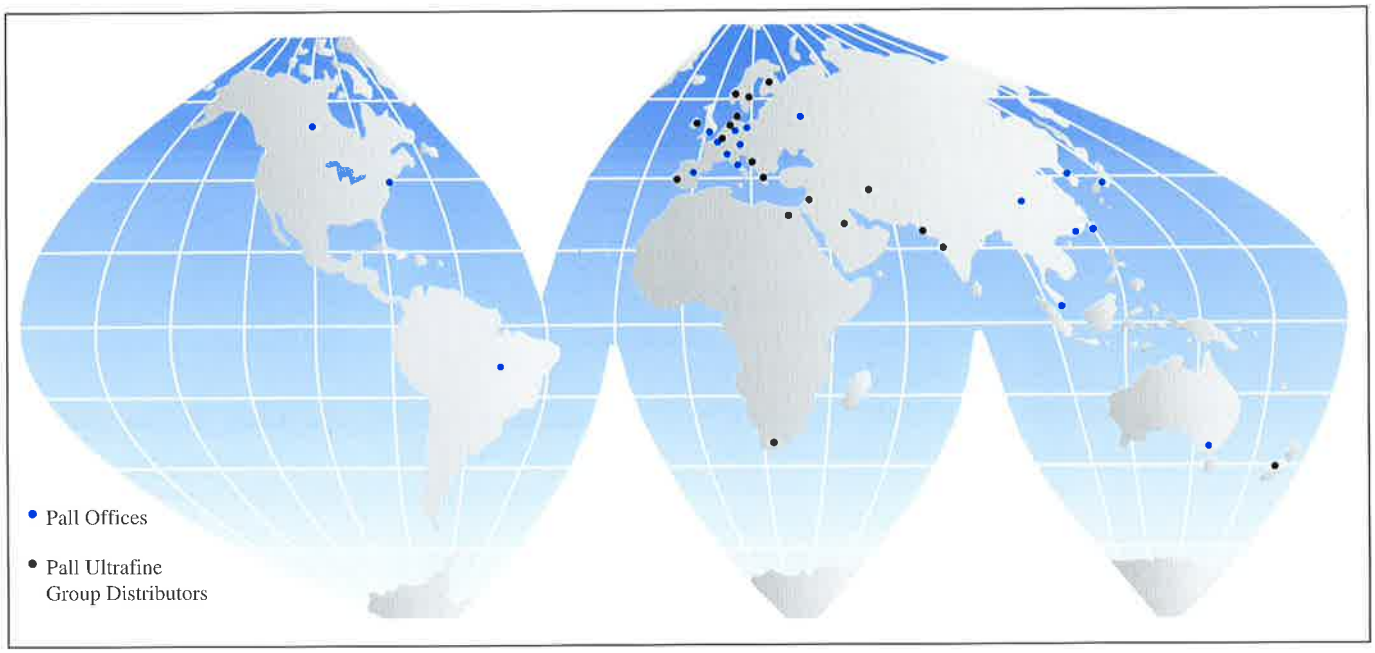

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