A new approach to produce water free of bacteria, viruses and halogen in a recyclable system.

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The antimicrobial activity of a new cross-linked N-halamine polymer was evaluated against bacteria and viruses. The polymer achieved a 9 $\log_{10}$ reduction in 1.5 hours against bacteria (both *Escherichia coli* and *Staphylococcus aureus)* and a 5 $\log_{10}$ reduction against PRD1 bacteriophage in three hours. At the same time, the ability of the non-halogenated polymer to trap halide ions was examined. The polymer was incorporated into a multi-filtration system to study the ability to produce water free of bacteria, viruses and halide ions. Antimicrobial activity,

useful life-time, halide ion level and recycling possibilities of the system were quantified on laboratory scale. A design for a large scale multi-filtration system based on this polymer is proposed.

The latest report from the WHO and UNICEF Joint Monitoring Programme shows that the world is on-track to meet Goal 7, Target 7c of the Millennium Development Goals (MDGs) (1) with respect to access to improved water supplies. Nevertheless, despite the progress that has been made during the last 10 years over 850 million people do not have access to improved sources of drinking water; almost all of them living in developing regions. In Europe most people are connected to a piped water supply managed by one of the water utilities. The remainder, around 10% of the population, receive their water from small, or very small, private water supplies (2). A recent survey of the microbiological quality of water from private water supplies in the UK has shown that just over one third show evidence of faecal contamination and, by projection, that 54% of all such supplies are likely to be unsatisfactory (2).

Unsafe water from all sources contributes significantly to the global burden of disease (3) principally through the waterborne transmission of gastrointestinal infections, such as cholera, typhoid, hepatitis, and a wide range of agents that cause diarrhoea. Although estimates vary of the reduction in burden of disease that can be brought about by water treatment, there is a consensus that water treatment is beneficial (3). Thus, cheap and effective water treatment systems that can be used at different scales, from

single point water sources to small community water supplies, can make a valuable contribution to reducing the burden of disease by improving access to safe water.

N-halamine polymers have been shown to have significant antimicrobial properties (4) and their ability to exchange halogen with microorganisms has raised the possibility of incorporating the polymer into water filters (4, 5, 6, 7). Several mechanisms have been proposed to explain the halogen exchange between the N-halamine polymer and microorganism. A widely held explanation is that protons of the amide functional groups of the protein sheet in the cell wall are oxidised by the halogen that has been gained from the polymer (17). But we have shown in a previous study that the N-halamine polymers can modify some of the bacterial metabolites which may result in the cell's death (17).

Prepared by constructing amide, imide or amino containing heterocyclic rings on polymer backbones followed by halogenation, (8, 9, 10, 11) their production costs are one of the main problems that restrict the application of N-halamine polymers on a large scale (13, 14, 15, 16). We demonstrated, in a previous study, the preparation of a commercial polymer, **2** (Figure 1), with low cost (17). The aim of this study was to evaluate the biological activity of the halogenated form of this polymer, **3** (Figure 1), against bacteria and viruses and to study the possibility of producing the water also free of halide ions by trapping these ions on the non-halogenated form of the polymer. Trapping halogen is important to avoid the production of potentially hazardous disinfection by-products such as organic halo-compounds (trihalomethane) (18). The antibacterial properties of the polymer were tested using *Staphylococcus aureus* (*S. aureus*), a Gram-positive cell, and *Escherichia coli* (*E. coli*), a Gram-negative cell, and a key indicator of water quality.

Bacteriophage PRD1 was used to test the viricidal activities of the polymer. PRD1 has been used as a model for waterborne, human pathogenic viruses, in particular the larger icosahedral viruses such as Adenovirus and Rotavirus, in a number of studies of the fate and transport of viruses in the environment (19, 20).

The biological activity and recycling possibilities of this polymer were evaluated using three methods; stirred flasks; columns and in a multi-filtration system on a laboratory scale. This latter system consisted of three columns containing; sand, halogenated and non-halogenated polymer. The halogenated polymer was expected to remove microorganisms from the water while its non-halogenated form was used to remove halide ions. Based on this system, a large scale design suitable for treating small community water supplies (for example, village scale) has been proposed that could be developed in a future project.

The results of this work provide encouragement for transferring such technology to large scale applications as the production cost of the polymer would be reduced. Moreover, applying such system on wide scale may reduce the toxic effect of the action of the free chlorine currently used in water disinfection; producing water free of bacteria, viruses and halide ions.

METHODS AND MATERIALS

Materials. *M*-phenylenediamine, cyanuric acid were supplied by Sigma Aldrich Chemicals, UK. Polyepichlorohydrin, sodium hydrogen carbonate, starch, sodium thiosulfate, potassium iodide, N,N-dimethylformamide (99.99%) and methanol were supplied by Fisher Chemicals, UK. Tryptone soya agar, bacteriological agar no.1,

tryptone soya broth, Ringer's solution, nutrient broth and nutrient agar were supplied by Oxoid, Ltd, UK. Bacteriological media were prepared according to the manufacturer's instructions. Ringer's solution was prepared at a quarter-strength. All other chemicals were used as obtained from suppliers without extra purification.

Growth and maintenance of stock cultures. *Staphylococcus. aureus* and *Escherichia coli* K12 were obtained from the University of Surrey culture collection. Primary cultures were maintained on nutrient agar slopes stored at 4^{o}C. Experimental stocks of the bacteria were prepared by making subcultures of the primary culture on nutrient agar plates. Subcultures were grown at 37^{o}C for 24 hours and then stored at 4^{o}C.

Bacterial suspensions were prepared by inoculating a single colony of the test strain from the experimental stock plates into 10ml of nutrient broth and incubating for 17 hours at 37^{o}C.

PRD1 (ATCC-BAA-769-B1) and host (*Escherichia coli* ATCC-BAA-769) were sourced from the American Type Culture Collection and were grown / assayed using tryptone soya agar and broth (19). The titre of the stock culture was 2 x 10^{8} pfu/ml

Preparation of cross-linked polyepicyanuriohydrin 2. Polyepichlorohydrin (2 g, 0.02 mol) was dissolved in N,N-dimethylformamide (DMF, 30 ml). *M*-phenylenediamine (0.2 g, 10% w/w) and sodium hydrogen carbonate (0.3 g) were added and the reaction was heated at 120^{o}C for 24hr. Cyanuric acid (3.2 g, 0.025 mol) and sodium hydrogen carbonate (1.9 g) were added and the heating was continued for 24hr. The resulting gel was washed with hot water (80^{o}C, 100 ml) to remove any cyanuric acid contaminating the polymer, Figure 1 (17).

Analysis: FTIR (KBr): ν_{max} (cm^{-1}), 1739, 1702, 1646 (C=O, heterocyclic ring), 1589 (C=N), 1260 (C-N and C-O), 2842, 2992 (CH aliphatic), 3210 (NH), 3434 (OH). Solid state ^{13}C NMR, 30-40 (aliphatic part), 155, 162 (C=O, heterocyclic ring) (17).

Polymer **2** was chlorinated using sodium hypochlorite (10% w/w). The polymer (1.0 g) was soaked in sodium hypochlorite (10% w/w, 15 ml) and 5 ml distilled water overnight. The product was filtered, washed with 100 ml distilled water and dried (17). The amount of halogen loaded on the polymer was determined using iodometric titration, 115ppm ±20 (4, 12).

Antimicrobial activity of the chlorinated cross-linked polyepicyanuriohydrin 3. The antimicrobial activity of the halogenated polymer **3** was evaluated against PRD1, *E. coli* and *S. aureus*.

Bacteria: polymer **3** (1 g) was stirred with 10 ml bacterial suspension. A sample of the suspension (0.1ml) was taken immediately after the addition of the polymer (time 0) and then at the intervals described in the results. Serial 10-fold dilutions of the samples were made in physiological saline solution and the number of viable bacteria in each dilution was determined using the method of "Miles and Misra" (21).

Viruses: polymer **3** (3 g) was stirred with 30 ml water containing PRD1 (2 $X10^8$ pfu/ml). A sample of the suspension (0.1ml) was taken immediately after the addition of the polymer (time 0) and then at the intervals described in the results. Serial 10-fold dilutions of the samples were made in quarter-strength Ringer's solution and the number of viable PRD1 in each dilution was determined using the double-layer agar method (22).

In both evaluations two controls were used; non-halogenated polymer and an equal amount of non-treated suspension which was retained as bacterial/viral control.

The swelling behaviour of polymer 2. Polymer **2** (0.05 g) was soaked in tap-water, distilled water, or saline solution (1% w/w) in three different universal bottles for 24 hours. The weight of the polymer was determined after the soaking period and the swelling was calculated as in equation 1 (7).

Equation 1: Swelling ratio determination.

% Swelling ratio= [(Polymer weight after soaking – Polymer weight before soaking)/ Polymer weight before soaking] X 100

Polymer 3 Re-cycling. Polymer **2** (1 g) was soaked in 20 ml sodium hypochlorite (7.5% w/w) overnight. The polymer was filtered, washed with distilled water (100 ml) and dried at 45^{o}C for 24 hours. The amount of halogen on the polymer was determined using iodometric titration (4, 12). The polymer was heated in 20 ml sodium thiosulphate (0.01M) at 45^{o}C for one hour then filtered, washed with halogen-free water and dried at 45^{o}C for 24 hours. The polymer was re-halogenated using sodium hypochlorite then the halogen was removed with sodium thiosulphate (the process was repeated four times; charging with halogen then discharging with sodium thiosulphate) and in each case the amount of halogen loaded on the polymer was determined. After the fourth cycle the biological activity of the polymer was studied against bacteria (*E. coli* and *S. aureus*) using the method described above.

Evaluation of the halogenated polymer 3 in water filters on a laboratory scale. Polymer **3** (10 g) (1-2 mm diameter granules) was packed into a 20 ml glass syringe as a model column. The column was closed, distilled water was added and the column was left overnight to allow the particles to swell. Excess water was removed and

the column washed three times with distilled water; 10 ml each time. Bacterial suspension (*E. coli* or *S. aureus*) (10 ml) was perfused through the column. The reduction in bacterial numbers was followed by measuring the viable count before and after perfusion. The suspension was re-perfused again (for 10 cycles) through the column and bacterial viability was determined after each cycle. A column containing the non-halogenated polymer **2** was used as a control (5). Bacterial numbers were quantified using the Miles and Misra method (21); a small amount (0.1ml) of each sample of the outlet was taken and kept on ice to determine the bacterial viability.

Determination of the halogenated polymer 3 life time in water filters. The previous method was repeated. Bacterial suspension recycling through the column was increased to 12 times. Each 12 cycles considered as one run. Each run was performed with a fresh bacterial suspension. Three runs were performed for *E. coli* and four runs for *S. aureus* in two separated columns; one for *E. coli* and the other for *S. aureus.* Viable counts were determined using the method described above (21).

Non-halogenated polymer 2 columns as a reversing column to the halogenated polymer column 3. Halogenated **3** and non-halogenated polymers **2** (10 g each) were packed into two different columns. Both columns were allowed to swell by treating with distilled water as described earlier. Bacterial suspension (*E. coli*) was perfused through the halogenated polymer column followed by the non-halogenated one. Bacterial viability was followed before and after perfusion through each column. The experiment (perfusion) was repeated 4 times using fresh bacterial suspensions each time.

Sand as regulator. Sterilized sand was packed into three different glass columns (30 g each) (20 ml glass syringes were used as model column). The columns were challenged with different bacterial suspension concentrations of *E. coli* (1.6×10^4, 1.6×10^7 and 1.6×10^{10}cfu/ml, one concentration per column) and the viability determined before and after perfusion through each column using the Miles and Misra method (21) as described above.

Determination of the quality of a water purification station on a laboratory scale. A station (on a laboratory scale) was designed from three columns; sand (30 g), N-halamine biocidal polymer **3** (15 g) and non-halogenated polymer **2** (15 g) column, and challenged with five different runs of bacterial suspensions (*E. coli*). Each run (10 cycles) was performed using a fresh bacterial suspension with a bacterial concentration up to 10^3 cfu/ml. The same columns were used for each run to determine the maximum number of bacteria removed by the station. Viable counts were performed before and after perfusing through each column as described previously. Columns containing polymers were treated with distilled water before starting the experiment.

Station recycling. The previous experiment was repeated twice to investigate the recycling possibilities of the station. The station was washed with 100 ml sodium hypochlorite 5% (w/w) per column to kill any bacteria from the first experiment followed by washing with sterile halogen-free water (100 ml water per column). The water used in washing was added in parts; 10 ml each time. The level of halogen in the washing water at the outlet of each column was measured until no halogen content was recorded. The halogenated polymer **3** column was refreshed by filling it with sodium hypochlorite 10% (w/w) overnight to reload the polymer with halogen. The halogenated polymer **3** column

was rewashed with distilled water. The water was added in portions (10 ml each) and the chlorine content in the outlet was measured using iodometric titration. Flushing was continued until a constant concentration of halogen was recorded (corresponding to that usually released by this polymer in water, 2.4ppm ± 0.5). After cleaning and washing, the second experiment was performed with fresh bacterial suspension. The same precautions were followed in each recycling stage.

RESULTS AND DISCUSSION

A new synthetic pathway was developed to prepare a low cost N-halamine biocidal polymer (17). The polymer was prepared by cross-linking polyepichlorohydrin with *m*-phenylenediamine (10%, w/w ratio) in the presence of sodium hydrogen carbonate (17). Cyanuric acid was added with an additional amount of sodium hydrogen carbonate to produce a new cross-linked heterocyclic polymer **2** (17). Attaching the nitrogen atom of the heterocyclic ring to the CH_2 group of polyepichlorohydrin stabilizes the halogen on the heterocyclic ring as an electron donating group. Polymer **2** was halogenated by soaking in sodium hypochlorite overnight to give the polymer the required time to swell and react with the hypochlorite.

The antimicrobial activity of halogenated polymer **3** was studied by determining its effect on bacteria (*E. coli* and *S. aureus*) and viruses (PRD1). The halogenated polymer **3** achieved a 9 $\log_{10}$ reduction in 1.5 hour for both *E. coli* and *S. aureus*, while no antimicrobial activity was observed with the non-halogenated polymer (Figure 2). By comparison, the halogenated polymer **3** achieved a 5 $\log_{10}$ reduction against PRD1 bacteriophage in 3 hours, Figure 3.

The swelling behaviour of the polymer was determined in different media (distilled water, tap water and saline solution) to investigate the range of granule-size occurring during different, potential applications. Tap water was used to investigate the swelling behaviour in conditions representative of normal domestic use. Distilled water was used as a control and the saline solution was used to investigate the level of swelling in the presence of elevated ion concentrations. This is especially important for their use in water filters as the swelling ratio of the polymer can affect the water flow-rate. The results showed that the swelling in distilled water, tap water and saline solution was 26.3, 21.6 and 20.9% respectively. The swelling behaviour in saline was lower than that in distilled and tap water. The swelling ratio was not more than 27% which is very important; reducing the spaces between the particles to ensure good contact between the polymer particles and water.

To increase the economic value of N-halamine polymer **3**, the potential for the polymer to be recycled was investigated. N-halamine polymers can be recycled by re-halogenation. The polymer was loaded with halogen and unloaded four times before testing its antimicrobial activity. The amount of chlorine was determined using iodometric titrations (in triplicates) after each cycle of charging the polymer with halogen. Five cycles were performed and the halogen load was 115 ppm (± 2.0) after the first cycle, 101 ppm (± 2.3) after the second cycle, 102 ppm (± 3.3) after the fourth cycle and 74 ppm (± 5.3) after the fifth cycle (± signifies the range in each experiment). The results show that the polymer lost some of its ability to be recharged with halogen during the second loading process, but retained a similar amount of halogen during the third cycle. During the fourth recharging process, the polymer lost a significant amount of its

potential for retaining the halogen. The antimicrobial activity of the polymer was tested again after the fourth charging cycle to determine its efficacy with lower halogen content (Figure 4). After four cycles of polymer recharge the halogenated polymer **3** achieved a 9 $\log_{10}$ reduction of *E. coli* numbers and an 8 $\log_{10}$ reduction *S. aureus* numbers in five hours. These results indicate that the polymer retained its antimicrobial activity for at least four recycling processes, although its efficacy was slightly reduced, Figure 4.

The potential for using the halogenated polymer **3** in a water filter was tested in a laboratory scale filter system. The column was packed with the polymer, and the polymer was allowed to swell overnight by soaking in an excess of distilled water. A 10 ml suspension of *E. coli* (4.3 X 10^9 cfu/ml) was perfused through the halogenated polymer column for 10 cycles and the viability was determined before and after each cycle. No viable bacteria were detected after 10 cycles. By comparison, the non-halogenated polymer **2** column succeeded in performing 1 $\log_{10}$ reduction; in all probability due to a filtration effect.

As Polymer **3** showed antimicrobial activity in water filters, the life time of this column was investigated to determine the maximum number of bacteria that can be removed by this column. Different runs were performed with the column after the normal treatment with distilled water to obtain maximum swelling of the particles; each run with fresh bacterial suspension and 12 cycles per run. The runs were stopped as soon as the antimicrobial activity of the column decreased.

E. coli results showed that column activity starts to decline after the first run. It has a low activity during the second run while in the third no significant effect was

noticed. Similar results were obtained for *S. aureus* but the column was still showing some antimicrobial activity up to the fourth run

Halogenated and non-halogenated polymers in combination. Analysis of the eluent from the halogenated column has shown a low level of free halogen (4.8ppm +/- 0.7) emerging from the column In this experiment a column of the non-halogenated polymer **2** was set up in sequence with the column of halogenated polymer to determine its effectiveness at capturing the free halogen. The bacterial suspensions were perfused through the halogenated polymer **3** column followed by the non-halogenated **2**. Both columns were equilibrated with distilled water before starting the experiment.

However, it was observed that although the first column (halogenated polymer column **3**) was deactivated, the non-halogenated polymer **2** did not gain any antimicrobial activity. This may be explained on the basis that the halogenated polymer may lose its activity before all the halogen in it was removed. Therefore, the halogen passing to the second column may not be enough to activate it. At the same time, not all halogen reaches the non-halogenated polymer as most of the halogen will be delivered to the bacterial cells (17). These results restrict the idea of reversing the columns. However, no halogen was detected in the water after passing through the second column. This supports the design of a system employing the non-halogenated column in series after the halogenated column to produce halogen-free water. Removing halogen may reduce the amount of disinfection by-products but it may also remove any residual disinfectant from the water that could prevent recontamination during distribution or storage. This was taken inconsideration in the suggested design of a large scale station.

A laboratory scale water purification system, formed from three columns; sand, halogenated polymer and non-halogenated polymer was created. The sand was used as a primary filter to regulate the number of bacterial cells passing to the main column (halogenated polymer column) and at the same time to stop any residue or solid contents reaching the second column. When sand was applied alone in three different filters against three different concentrations of *E. coli*; 1.6×10^{10}, 1.6×10^{7} and 1.6×10^{4}, the filtration effect succeeded in removing 8.8×10^{6}, 3.5×10^{3} and below the detection level respectively demonstrating the ability of the sand to reduce the number of bacterial cells passing to the main column.

The bacterial concentrations used in evaluating the system were reduced (1.5×10^{3} cfu/ml) to reflect those more commonly encountered in nature (23). Different runs were performed through the station using fresh bacterial suspensions each time. Break-through of viable bacterial cells was detected after the third run. In the fourth run there was some disinfection effect while from the fifth cycle the biocidal activity of the station had decreased.

No halogen was detected in the eluent samples after perfusing through the station. These results indicated that the system succeeded in performing significant disinfection without halogen release into the outlet (the non-halogenated polymer**, 2,** had succeeded in removing the halogen released from the halogenated polymer).

The recycling possibilities of the station were further investigated to evaluate the economics of the proposed system. The station was first cleaned by washing the columns with sodium hypochlorite (10% w/w) followed by washing with sterile distilled water. The halogenated column was charged with halogen by filling the column with sodium

hypochlorite and keeping it closed overnight, followed by washing with water. This cleaning and charging procedure was followed to recycle the station and the system was shown to be fully effective, after repeating the recycling process 3 times (up to 5 runs each). It was noticed that the biological activity after recycling was greater that at the beginning. This may be due to the fact that the polymer was not taken out of the column after the halogenation to dry it but it was used directly. Drying the polymer would result in some loss of the halogen. Moreover, no halogen was detected in the outlet at any stage of the regeneration demonstrating that the station can be regenerated and used several times without any halogen release into the outlet. It is worth noting that washing the third column (non-halogenated polymer column) with hypochlorite is not enough to fully charge it with halogen but it is enough to act on any cells attached to it. Fully charging this polymer with halogen required soaking overnight to achieve the maximum biological effect.

These results show that the halogenated polymer **3** is able to disinfect a bacterial suspension in a laboratory scale water purification station; that the polymer and the station can be recycled for use; and that no halogen was detected in the outlet water.

Based on this laboratory scale station, a suggested large-scale design for a water purification station based on multi-filtration technology is suggested for future work, Figure 5. The station is formed from three main columns; sand (unit 1, Figure 5), halogenated polymer (unit 2, Figure 5) and non-halogenated polymer (unit 3, Figure 5). It is supported with a sodium hypochlorite source for washing the columns during recycling, to kill any retained bacterial cells in the columns (unit 4, Figure 5). The same tank will be used to re-halogenate the halogenated polymer in unit 2. Sodium

hypochlorite waste (unit 5, Figure 5) can be recycled by warming to separate the chlorine from the water. Chlorine (unit 6, Figure 5) can be reacted again with sodium hydroxide to form sodium hypochlorite which can be used again in unit 5. In addition, the water freed from the chlorine can be neutralized (unit 7, Figure 5) and used again for washing proposes or dissolving sodium hydroxide (unit 8, Figure 5), after filtration from any possible bacterial residue, Figure 5. Tubes connecting different units could be prepared from N-halamine polymers to keep the system disinfected. This system must be monitored in order to detect any bacterial contamination in the water outlet in addition to the recycled water. The level of halogen must also be monitored, to be sure that the water is completely free of halogen. This suggested scheme would introduce a complete system providing potable halogen-free water on a large scale; and transferring the water to customers in tubes manufactured from bioactive polymers would introduce another business opportunity and will keep the water clean and safe with the lowest possible level of halogen ion. The addition of extra columns for removing metal ions that may contaminate the water would enable the construction of a complete water purification system. Trials will be conducted in future work to achieve this goal by applying and quantifying this suggested system.

CONCLUSION

A new cross-linked bioactive N-halamine polymer was prepared and shown to have good antimicrobial activity using indicator viruses and bacteria. The polymer was used successfully in water filters on a laboratory scale and recycled up to four times. The biological life-time of the polymer was determined. The polymer was used successfully

in a water purification station on a laboratory scale. The station life-time and regenerability were determined; the station being recycled three times. The station was specifically designed to have a column containing the non-halogenated polymer to act as a trap for halogen-released from the main column; no halogen was therefore detected in the water outlet from the station. Sand was used to regulate the number of bacteria delivered to the main column. Using such a station may support the production of water free from bacteria and halogen. A water purification system based on multi-filtration technology was suggested to be applied on a large scale using this type of polymer.

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Fig 1 - Preparation of cross-linked polyepicyanuriohydrin and its halogenation.

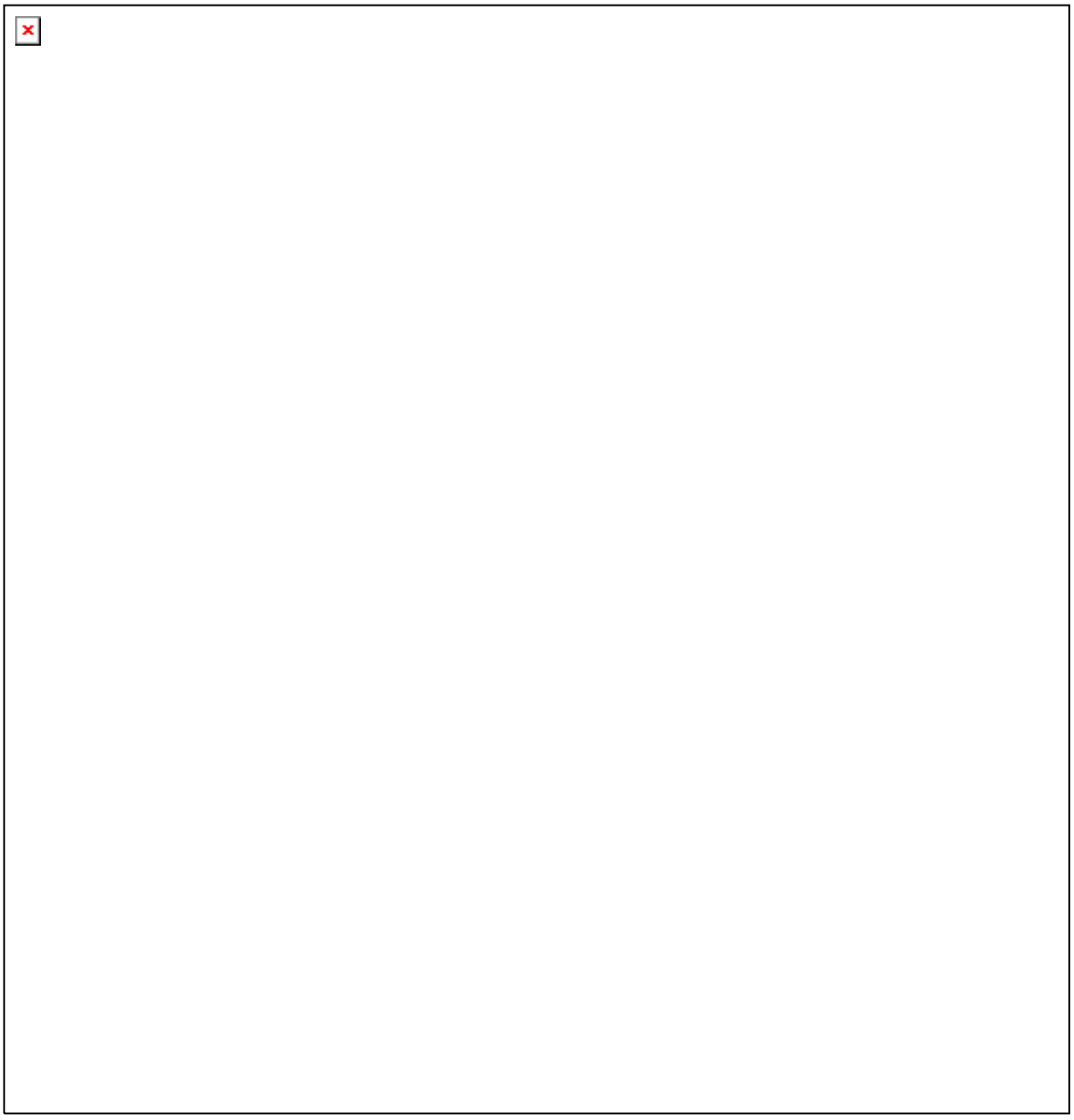
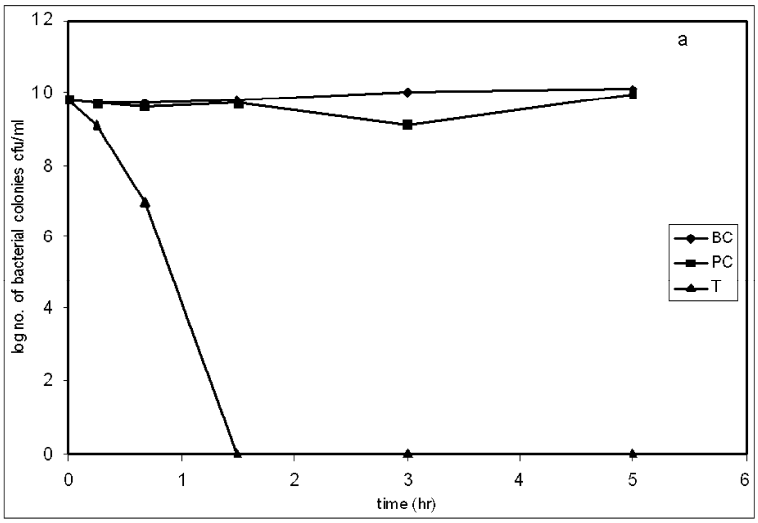


Fig 2 - Effect of halogenated polymer (**3)** on (a) *E. coli* and (b) *S. aureus* viability. BC is the bacterial control, PC is the non-halogenated polymer (polymer control) and T is the halogenated polymer **(3)**. The error bars have been removed as the error is too small to display. The lowest level of detection was 8 cfu/ml.

a)



b)

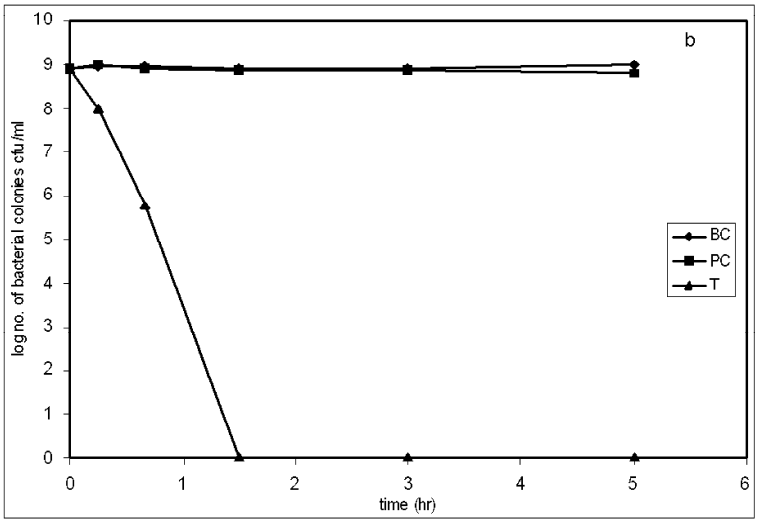
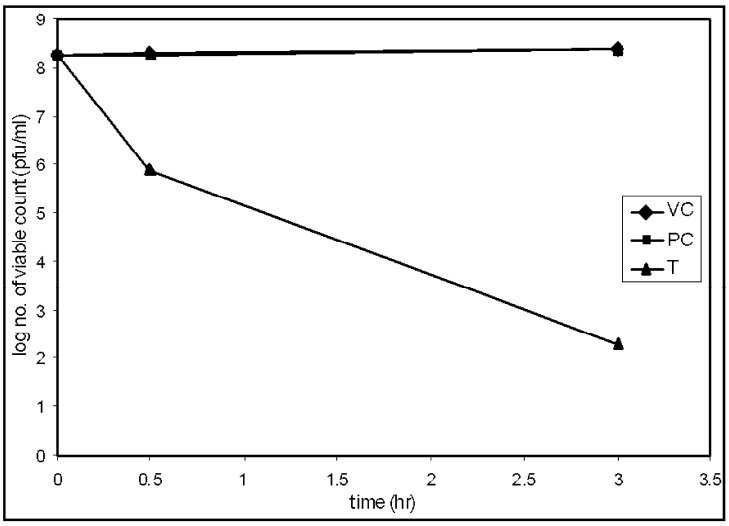
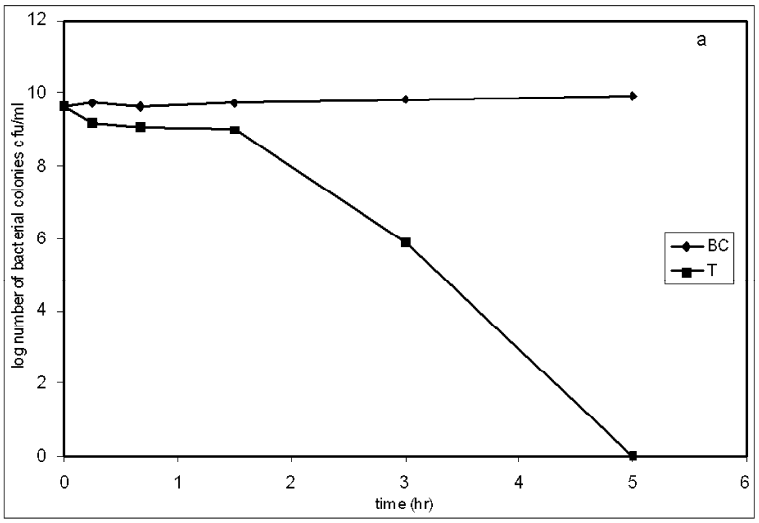


Fig 3 - Effect of halogenated polymer (**3)** on PRD1 bacteriophage viability. VC is the virus control (no treatment), PC is the polymer control (viruses treated with non-halogenated polymer) and T: N-halamine polymer (viruses treated with halogenated polymer). The error bars have been removed as the error is too small to display. The lowest level of detection was 8 cfu/ml.



1 Fig 4 - Effect of halogenated polymer (**3)** (after the fourth cycle of charging with
2 halogen) on the bacterial viability of (a) *E. coli* and (b) *S. aureus* respectively. BC is the
3 bacterial control while T is the chlorinated polymer **(3)**. The error bars have been
4 removed as the error is too small to display. The lowest level of detection was 8 cfu/ml.

5 a)



6

7 b)

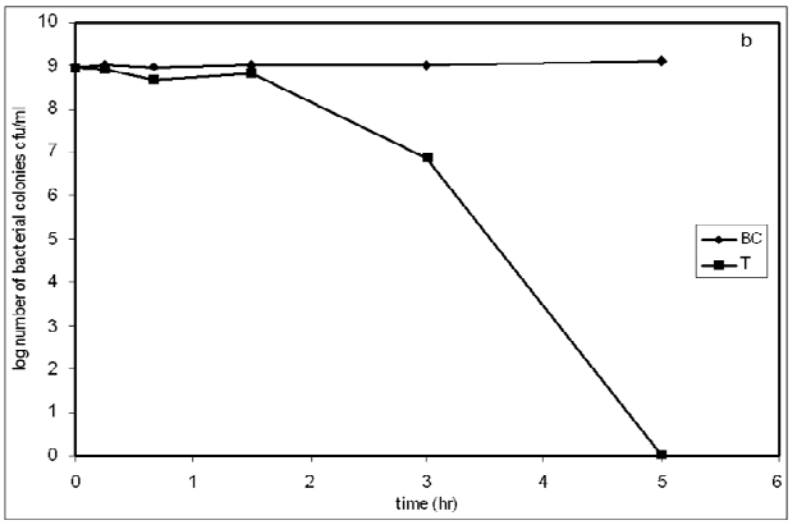


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Fig 5 - A suggested design for a large scale water purification system based on multi-filtration technology.