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EVALUATION OF THE MEDICAL EFFICACY OF THE PRE-MAC MODEL FWP INDIVIDUAL WATER PURIFIER FOR TREATING MICROBIOLOGICAL CONTAMINANTS IN WATER

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Tests were conducted inhouse to determine the microbiological treatment efficacy of the Pre-Mac Model FWP hand-held water purifiers. Test waters represented various physical/chemical challenge conditions. Microbial challenges consisted of enteric bacteria (Klebsiella terrigena), enteroviruses (Echovirus 1 and Poliovirus 1), protozoan oocysts (Cryptosporidium parvum), and a cyst simulant (latex beads). Additional tests were conducted at the University of Arizona (UA) to determine the viability of Cryptosporidium parvum oocysts after exposure to the Pre-Mac iodine, and to evaluate Pre-Mac water purifiers fitted with second generation 1.0 micron fine mesh filters for physical removals of cysts from product water. Studies used the U.S. Environmental Protection Agency's interim "Guide Standard and Protocol for Testing Microbiological Water Purifiers" for guidance. Results indicated that bacteria and viruses were readily removed by the Pre-Mac filter units. However, test results from inhouse studies and UA studies indicated that the Pre-Mac water purifiers were not adequate to physically remove or reduce infectious levels of protozoan cysts to meet the 3-log reduction criteria.

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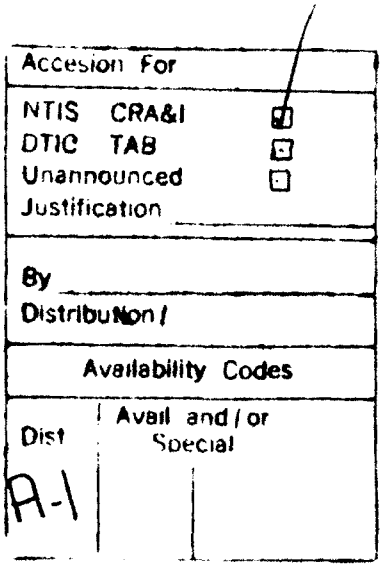

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INTRODUCTION

The United States Army and Marine Corps are interested in enhancing their capability to provide individuals and small groups of combat soldiers with a capability to purify water for drinking when they cannot be provided with bulk water supplies from normal Quartermaster logistics sources. Under most circumstances the military will provide treated, disinfected water from reverse osmosis water purification units (ROWPU) to soldiers; however, there are circumstances or occasions when small units may be separated from such drinking water supplies. Examples of these are military operations with long range patrols, special forces and special operating forces, and scenarios where logistics is unable to provide water because of geographical or militarily constrained limitations.

Typical operational requirements for an individual microbiological field water purifier in a military setting may include the following. It should be capable of treating all microbiological contaminants at the anticipated worst case levels likely to be found around the world; it would produce a quantity of water, without replacement, to insure that users could fully conduct a field mission (typically 10-30 days); it would be easy to use and would rapidly produce at least a liter of drinkable water within several minutes; and it would be rugged, durable, small, and lightweight. At this time such a unit would not be required to provide treatment of chemical toxicants or nuclear, biological, and chemical warfare contaminants, but such would be a desirable additional capability and may become an additional requirement for a second generation individual purifier of the future.

This report describes the results of research conducted by the U.S. Army Biomedical Research and Development Laboratory (USABRDL) to determine the microbiological treatment efficacy of the Pre-Mac Model FWP hand-held water purifier to meet military requirements for individual soldier water production from contaminated surface or ground water sources. It is manufactured by Pre-Mac (Kent) Limited, 103 Goods Station Road, Tunbridge Wells, Kent TN1 2DP, England. The requirement to conduct the study was provided by the U.S. Army Quartermaster Center and School which also provided partial funding for the project.

The Pre-Mac Model FWP water purifier is designed to provide 1,000 liters of microbiologically safe drinking water, free of bacterial, viral and parasitic contaminants. The treatment cartridge component can be separated from the pump and hoses and replaced when it becomes clogged or the iodine resin becomes depleted. The FWP can be directly attached to the appropriately modified canteen, thus significantly reducing post treatment contamination of the water. Typically, the flow rate is 500 ml

per minute but vigorous pumping can increase this rate. The FWP weighs 900 g, and is 70 mm in diameter and 210 mm long. The purifier has a 2-foot long water inlet hose and is operated by a hand pump with a low profile handle (Figure 1). The FWP incorporates several stages of filtration and purification (disinfection):

1. Prefiltration using silver impregnated activated carbon materials to reduce organic and chemical contamination.

2. Primary disinfection, using a uniquely formulated iodine-resin complex which acts as a contact microbicide. The resin also releases small but controlled amounts of iodine into the product water, which further disinfects recalcitrant organisms and serves to maintain the treated water in a sterile condition.

3. Fine mesh filtration removes particulate material down to either a 1.0 or 3.0 μm diameter size before water exits from the purifier.

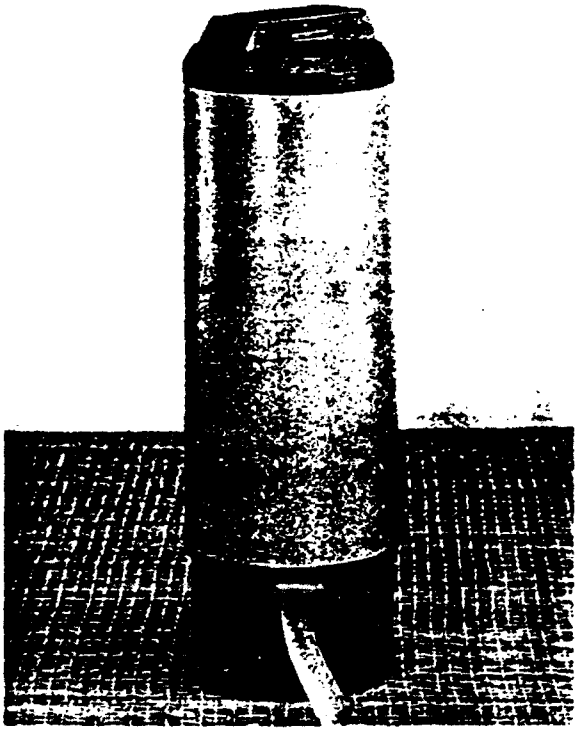
In-house studies with the Pre-Mac FWP purifiers were designed to evaluate their disinfection and physical removal capability for enteric bacteria and viruses, and the efficiency of physical removal for protozoan cysts and a cyst simulant.

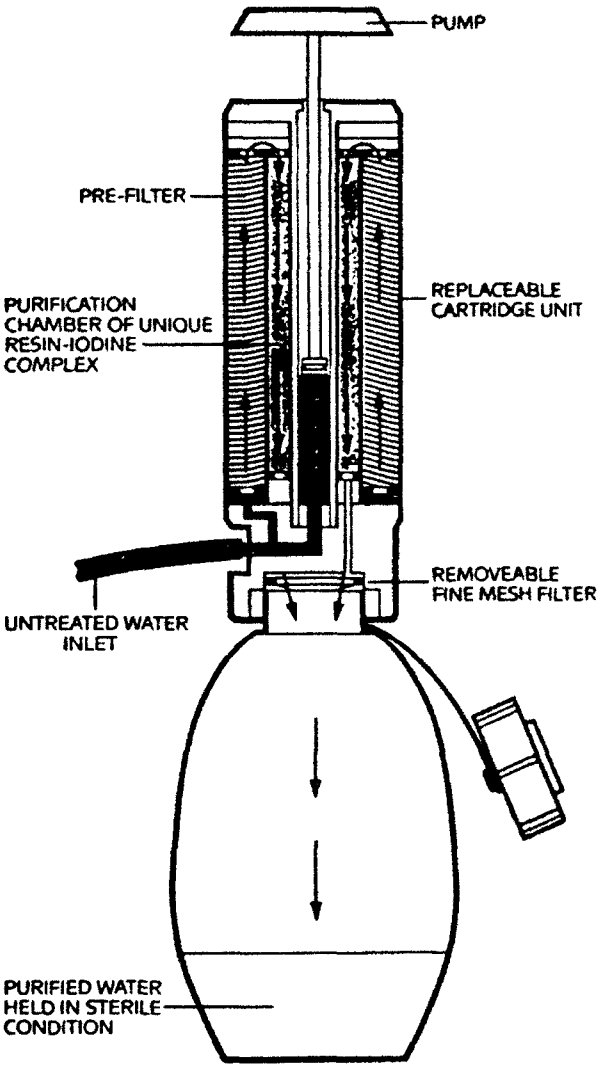
Collaborative research efforts between the USABRDL and the University of Arizona (UA), Department of Veterinary Science, Tucson, AZ, were also conducted. These studies were established to determine the effectiveness of Pre-Mac FWP purifiers containing a second generation final filter and to evaluate the associated iodine resin material for filtration/disinfection of Cryptosporidium parvum oocysts, utilizing microscopic analysis and animal infectivity as the primary measures to quantify its effectiveness. These studies were necessary when it became apparent from USABRDL in-house efforts that the oocysts were not effectively removed physically by the Pre-Mac treatment process. Two types of studies were conducted at the UA: Phase I - evaluation of the viability of oocysts solely after disinfection using the iodine resin provided in the Pre-Mac FWP purification units (without filtration); and phase II - evaluation of the effectiveness of the complete Pre-Mac FWP water purification unit processes including the newly developed final 1.0 μm filter for the total removal of oocysts. All phase II Pre-Mac purifier operational and testing procedures were essentially identical to those used during USABRDL in-house tests, except that the strain of Cryptosporidium oocysts used was that provided by the UA.

The tests conducted in this study followed the general guidelines of the U.S. Environmental Protection Agency's (USEPA) interim "Guide Standard and Protocol for Testing Microbiological Water Purifiers".[1]

SPECIFICATION

Cartridge Capacity	1,000 litres
Flow Rate	½ litre per min
Weight	900 gms
Size	70 mm dia × 210 mm





PRE-MAC (KENT) LIMITED, 103 Goods Station Road, Tunbridge Wells, Kent TN1 2DP England
Tel: (0892) 34361 Fax: (0892) 515770

Figure 1. Pre-Mac™ (Model FWP) water purifier

USABRDL MATERIALS AND METHODS

IN-HOUSE STUDIES

1. Microbiological Challenge--The selection of the microbiological challenges was modified somewhat from the USEPA's guide standard and protocol. Table 1 shows the waterborne microbiological challenges and their removal endpoint requirements. Rotavirus was excluded because of limitations in the time and availability of the appropriate cell cultures. Also, the Giardia sp. cyst challenge was replaced in the test protocol by Cryptosporidium parvum oocysts because it was felt that the Cryptosporidium represented a worse challenge due to their smaller size and resistance to common water disinfectants. Military exposures to Cryptosporidium were also thought to be of worldwide significance.

Table 1. Microbiological Challenges and Removal Endpoints

Test Organisms	Challenge Levels/L	Minimum Removal in Log_{10} (%)
Bacteria - Klebsiella terrigena (overnight culture)	10^8 CFU[a]	6 (99.9999)
Enteric viruses - Echovirus 1 & Poliovirus 1	10^7 PFU[b]	4 (99.99)
Protozoan cysts - Cryptosporidium parvum	10^6 cysts	3 (99.9)
Latex bead cyst simulant - 3.7 µm AccuBeads™*	10^7 beads	3 (99.9)

* FASTEK, 7120 Henry Clay Blvd, Liverpool, NY 13008
[a] CFU = colony forming units
[b] PFU = plaque forming units

a. Bacteria Preparation--Klebsiella terrigena (#33257) was obtained from the American Type Culture Collection, grown in nutrient broth for 18 hours at 35 °C, and frozen in 1 ml aliquots for test seed stock. For testing purposes, a 24-hour culture of Klebsiella was centrifuged at 8,000 rpm for 10 minutes using a Sorvall GSA rotor, washed three times in demand free phosphate buffered saline (PBS), and filtered through a Whatman # 2 filter pad to remove bacterial clumps. The filtered Klebsiella bacteria were diluted in PBS and adjusted to a final concentration of approximately 1.0 X 10^8 CFU/ml using a Klett-Summerson photoelectric colorimeter. Bacterial cells were added to the challenge waters to provide 1.0 X 10^8 CFU/liter of test water.

b. Virus Production, Purification and Separation--Echovirus 1, strain V239, and Poliovirus 1, strain LSc, were obtained from Dr. Mark Sobsey, University of North Carolina, and replicated in Buffalo Green Monkey Kidney (BGMK) cells (obtained from Whittaker Bioproducts, Inc., Walkersville, Md. 21793-0127) for test seed stock.

(1) Echovirus 1--Two 150 cm^2 flasks of BGMK monolayers were washed two times in Hanks' Balanced Salt Solution (HBSS) minus calcium and magnesium and then infected with Echovirus 1 at a multiplicity of infection (MOI) of 10 PFU/cell. The virus was absorbed for 60 minutes at 36 °C in a 5 percent CO_2 in air incubator, after which time a volume of 45 ml of Minimum Essential Medium (MEM) Eagle in Earle's Balanced Salts Solution (EBSS) with 2 percent fetal calf serum was added to each culture. The cultures were further incubated at 36 °C for 8 hours at which time cytopathic effect (CPE) was 90 percent with minimal cell detachment. The detached cells were concentrated in a tube by centrifugation at 10,000 X g for 30 minutes. After the liquid portion was discarded from the centrifuge tube, the cell pellet was resuspended in 4 ml of pH 7.2 PBS and saved. Twenty ml of PBS were added to each flask of infected BGMK cells, and the cell monolayers were removed with a cell scraper. These cells were then combined in the centrifuge tube with the 4 ml of cells from above. The combined cell suspension was "freeze-thawed" three times and then extracted three times in a Waring blender for 1 minute with 1,1,2-trichlorotrifluoroethane at a ratio of 4 ml per 6 ml virus suspension. After each extraction, the mixtures were separated by centrifugation at 800 x g for 10 minutes; and the aqueous phases containing the virus were collected and combined. The virus particles were concentrated in a final volume of 20 ml in an Amicon Centriprep-30 tube by centrifugation at 1500 X g in an IEC refrigerated centrifuge. Ten ml of the echovirus suspension were layered onto sucrose gradients (10 to 30 percent w/v prepared in 0.05M PO_4 demand-free phosphate-buffer, pH 7.2), and the virus particles were separated by rate-zonal centrifugation at 90,000 X g for 2 hours and 15 minutes in a Beckman SW28 rotor.[2] The gradients were collected in 2 ml fractions and assayed on BGMK cells by the plaque assay titration method. Test seed stock was prepared by combining fractions F-9 through F-14 and F-19 to provide a proportional number of small, medium and large aggregates. The echovirus seed stock yielded 6.32 X 10^9 PFU/ml when assayed on BGMK cell monolayers and was stored at 4 °C with no significant decrease in titer. Seed virus was prepared from the stock suspension on each test day and added to the challenge test water.

(2) Poliovirus 1--Challenge virus was prepared using the same procedure as Echovirus 1 above. The purified, pooled poliovirus fractions, which yielded 8.0 X 10^8 PFU/ml of stock virus, was stored at 4 °C without a significant decrease in

titer. The virus was diluted on the day of testing and added to the challenge test water.

c. Cryptosporidium Parvum Oocyst Purification--Calf feces (50 percent in 2.5 percent potassium dichromate) containing Cryptosporidium parvum oocysts were obtained from the University of Idaho, Department of Veterinary Science, Caldwell, ID, and purified using the PBS-ether sedimentation and Percoll™ discontinuous density gradient technique described by E. Waldman et al.[3] Fifteen ml volumes of the calf feces suspension were dispensed into 50 ml polypropylene conical centrifuge tubes, and an equal volume of PBS (pH 7.0) with 0.1 percent Tween 20 was added to each tube. The suspensions in the tubes were mixed and centrifuged at 500 X g for 10 minutes. The liquid portions were discarded, and the pellets were resuspended in 15 ml PBS-Tween 20. Five ml of anhydrous ether was added and mixed with the suspension for 1 minute. The tubes were then centrifuged at 500 X g for 10 minutes. The top three layers (ether, debris plug and PBS-Tween 20) were removed and discarded. The pellets containing the cysts were resuspended in 10 ml PBS with 0.01 percent Tween 20 and pooled. The pooled oocyst suspension was again centrifuged, and all but a small volume of the liquid was discarded. Oocysts were further purified using Percoll™ (Sigma # P-1644) discontinuous density gradients. A 1.04 density stock was prepared by adding 3 ml of Percoll™ to 7.0 ml of 0.15M NaCl, and a 1.08 density stock was prepared by adding 6.5 ml of Percoll™ to 3.5 ml of 0.15M NaCl. Percoll™ gradients were prepared in 10 ml centrifuge tubes by layering 3 ml of the 1.04 density on top of 3 ml of the 1.08 density. A volume of 0.5 ml of PBS-oocyst sediment was layered on top of the Percoll gradient in the centrifuge tubes, and the tubes were centrifuged at 250 X g for 10 minutes at room temperature. The upper bands, lower bands, and pellets from the bottoms of the tubes were collected separately, diluted in ten volumes of PBS, and centrifuged at 500 X g for 10 minutes. The PBS-Percoll fluids were discarded, and the pellets were resuspended in a small volume of PBS-Tween 20 and examined microscopically for oocysts. The pellet from the lower bands, which contained the oocysts, was further diluted in PBS-Tween 20 to contain approximately 5.14×10^6 oocysts per ml.

d. Protozoan Simulant (Latex Beads) Preparation--A suspension of 3.7-4.0 μm AccuBead™ particles (latex bead particles) obtained from FASTEK (A Kodak Company, Liverpool, NY) was diluted to contain $1.5\text{-}2.0 \times 10^7$ latex beads per ml in sterile deionized distilled water (ddH_2O) supplemented with 50 μg/ml of sodium dodecyl sulfate (SDS) to reduce bead clumping caused by electrostatic attraction. The beads were added to the test water containers to provide a final challenge concentration of 2.0×10^7/liter.

2. Test Water Characteristics--Test waters were prepared according to the U.S. Environmental Protection Agency's interim

"Guide Standard and Protocol for Testing Microbiological Water Purifiers." During the general water tests, the procedures for preparing the test waters had to be modified when it became apparent that the tapwater collected from the laboratory water supply contained a "toxic" chemical substance which killed or altered the ability of the bacteria to replicate after a 2-hour exposure period (equivalent to a typical purifier testing day).

a. Preparation of Test Waters

(1) General (Tapwater) Test Waters--Hot tapwater was collected in containers, stirred for 24-48 hours at room temperature to dechlorinate, and tested for residual chlorine before use.

(2) Modified General Test Waters--Distilled water from the laboratory still was amended to simulate general tapwater conditions as follows: TOC-1 mg of humics per liter; TDS-250 mg of sea salt per liter; turbidity-1 mg of AC fine test dust per liter (Part No. 1543094 obtained from AC Spark Plug Division, General Motors Corporation, Flint, MI); and 2 ml of 0.1M Na_2HPO_4 per liter of water to obtain a pH of 7.0.

(3) Modified Worst Case Waters--Distilled water was prepared to the required volume, the pH was adjusted to 7.0, and the following components were added for each liter of water: TOC-10 mg of humics; TDS-1500 mg of sea salt; turbidity-150 mg of AC fine test dust.

b. Test Water Challenge Seed--Three polypropylene tanks were set up for each test water challenge. One large tank contained 45 liters of test water, and two smaller vessels each contained 6 liters of challenge test water. All three containers with test waters were seeded with Klebsiella terrigena bacterium (10^8/liter) and the latex bead cyst simulant (10^7/liter). One of the two 6-liter vessels containing bacteria and latex beads was seeded with Cryptosporidium oocysts (10^6/liter) and Echovirus 1 (10^7/liter), and the other was seeded with oocysts (10^6/liter) and Poliovirus 1 (10^7/liter). The smaller, separate containers of challenge material were necessary to conserve the cyst material and to reduce the volume of waste waters that require a more stringent decontamination process for cysts.

3. Testing Procedures

a. Test set up and operation-- Three Pre-Mac, Model FWP, Water Purifiers were designated as Units 1, 2, and 3 respectively, for the laboratory tests. During general challenge water testing, the filter units were equipped with 3.0 μm fine mesh filters, but were changed to 1.0 μm fine mesh filters for worst case water tests (Figure 2). For testing, the Pre-Mac water inlet hoses were placed inside the 45-liter prepared

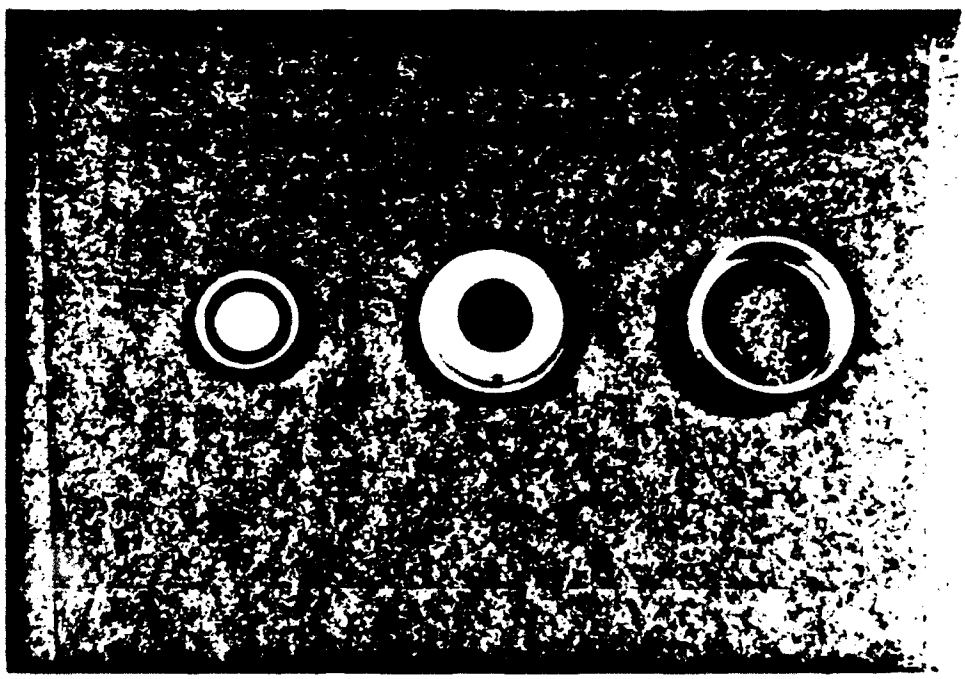


Figure 2. Disassembled Fine Mesh Filter

test-water tank, and the water was manually pumped through the filters into clean graduated polypropylene tanks for measurement of throughput volumes.

b. Sampling--On operational days 1-5 and days 6-10, 15 liters of test water were pumped through each Pre-Mac filter unit daily. On day 1, the first liter and the last 1.5 liters of product water from the 15-liter daily throughput were collected for microbiological analyses. On days 3 and 5 of the general challenge water tests and days 6, 8, and 10 of the worst case challenge water tests, the final 1.5 liters of the 15-liter daily throughput were collected for analyses. After pumping on days 5 and 10, the residual water was left in the Pre-Mac units for a 48-hour stagnation period, after which the first liter of water pumped through each purifier was sampled for micro-organism residuals. Except for the 48-hour stagnation samples, sampling at the designated sample point for analyses was performed by removing the purifier inlet hose from the large tank, expelling the residual water from the filter unit, and then placing the inlet hose in the challenge water in the first smaller vessel. The first 100-200 ml of water through the filter unit was discarded, and the next liter of filtered water was collected for analysis in a sterile graduated cylinder. Then the inlet hose was removed from the first vessel, the residual water in the filter unit was expelled, the inlet hose was placed in the second small vessel, and the sample was collected as before. Echovirus samples were collected first followed by poliovirus samples. Possible carryover of echovirus to the poliovirus assay was not considered to be a problem at the dilutions used since echovirus plaques could not be visualized and read until after 72 hours, whereas poliovirus plaques were quantified by 48 hours. For the sampling after the 48-hour stagnation period, the first 1.5 liters of product water from each purifier were collected for analyses, 750 ml from the echovirus vessel and 750 ml from the poliovirus vessel.

4. Sample Analyses--Sodium thiosulfate was added to each filtered product water sample immediately following removal of 10 ml for iodine analysis.

a. Iodine Analysis--Iodine levels were measured using the LaMotte Colorimeter. Iodine levels in the product water samples from the echovirus and the poliovirus vessels were determined immediately after filtration through each of the three Pre-Mac filter units.

b. Bacterial Analysis--m-Endo broth medium for membrane filtration quantification of Klebsiella terrigena was used, and analyses were made according to the USEPA protocol. Triplicate volumes of 1 ml, 10 ml, and 100 ml were filtered through Millipore 0.45 μm membrane filters for each sample. The membrane filters were placed on m-Endo medium pads in 50 mm

snap-cap petri dishes (Falcon # 2006). Colonies were counted 24 hours after incubation at 35 °C.

c. Viral Analyses--Echovirus and poliovirus samples were diluted 1:2 in 2X MEM containing 4 percent newborn calf serum (NCS). Subsequent serial 10-fold dilutions were made in 1X MEM containing 2 percent NCS. BGMK cells were grown to confluent monolayers in 60 X 15 mm tissue culture dishes. The growth medium was decanted from the dishes and a volume of 0.5 ml of each virus dilution was inoculated onto triplicate BGMK cell monolayers. After incubation for 1 hour in 5 percent CO_2 in air at 36 °C, the cells were overlayed with 5 ml of Medium 199 containing antibiotics, 2 percent NCS, 1.4 percent Difco purified agar, 0.125 percent sodium bicarbonate and 0.01 M HEPES buffer. Eight hours prior to counting plaques, 4 ml of HBSS containing 5 percent neutral red stain (GIBCO # 630-5330) were layered over the agar surface of each monolayer, adsorbed for 1 hour, and then poured off. Plaques were counted after an incubation period of 48 hours for poliovirus infected cells and after 72 hours for echovirus infected cells.

d. Protozoan and Cyst Simulant Analyses--A 1-liter volume of the collected product water from each Pre-Mac FWP filter unit was measured into a graduated cylinder, and 0.1 percent Tween 20 was added to each sample. The oocysts and latex beads were concentrated by collection on a 47 mm diameter, 1.0 μm pore size Nuclepore™ polycarbonate filter membrane. Each filter membrane was then cut into four sections and placed into a polypropylene centrifuge tube. Oocysts and beads were washed from the filter membrane with 10 ml of PBS containing 0.01 percent Tween 20. Filter membranes were washed five times with vigorous mixing on a vortex mixer during each wash. The wash solution materials were pooled, and the oocysts and beads were concentrated by centrifugation at 1500 X g for 15 minutes. The wash solution was aspirated from the tube except for approximately 2.0 ml which was used to resuspend the pellet. The samples were then stained for 20 minutes at room temperature with 0.5 percent Malachite Green and decolorized with 0.25 percent sulfuric acid prior to quantitation by the hemacytometer method. Oocysts and beads were distinguished by the absence of color against a green background. Beads and oocysts were differentiated by their morphologies and the refractory nature of the beads.

UA MATERIALS AND METHODS

COLLABORATIVE STUDIES

1. Microbiological Challenge--For this part of the study the USABRDL provided all necessary supplies, equipment, test protocol, and the Pre-Mac FWP water purifiers (and iodine resin) to perform the treatment procedures on waters containing the oocysts. USABRDL also performed the iodine resin disinfection portion of the oral neonatal mouse infectivity studies. The study was physically conducted at the UA Veterinary Department Laboratories. The UA collaborators provided the Cryptosporidium oocysts, neonatal test animals, animal per diem, test animal holding facilities, and reagent waters used in the study. The UA Veterinary research staff also determined the appropriate cyst dosages for the mouse infectivity studies, prepared the various disinfectant treated test waters, performed the tests, prepared and infected the animals with control and treated cysts, performed monoclonal antibody analysis of disinfected oocysts, and performed histological examinations of the gastrointestinal tract of the animals for evidence of Cryptosporidium infection.

2. Cryptosporidium Parvum Oocyst Production and Purification--Cryptosporidium oocysts were recovered from the feces of experimentally infected 2- to 5- day old Holstein bull calves by means of a previously developed method.[4] Briefly, Cryptosporidium parvum infection was induced in calves by feeding 1.0-2.0 X 10^8 infective oocysts suspended in 1 liter of reconstituted commercial milk replacer. The calves were isolated in vealer pens; and the feces excreted during the peak oocyst shedding period were collected, mixed with an equal volume of 5 percent potassium dichromate ($K_2Cr_2O_7$), and stored at 4 °C. The collected feces were sieved sequentially through stainless steel screens of decreasing aperture, ending with 63 μm pore size (230 mesh). Sequential discontinuous sucrose gradient (1.064/1.103 g/ml) centrifugation followed by isopycnic Percoll™ gradient (1.091 g/ml) centrifugation completed oocyst purification. The purified oocysts were stored in 2.5 percent potassium dichromate at 4 °C. Oocysts were withdrawn from storage as needed and washed with phosphate buffered saline (PBS, 0.025M, pH 7.4) by filtration through polycarbonate filters (1-3 μm pore size) or by centrifugation to remove the dichromate storage solution.

3. Mice--Late term pregnant female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Within 24 hours of birth, mouse pups were randomized and placed back with the mothers (6 to 7 pups/litter) to minimize maternal effects on experimental outcomes. Animals were maintained in micro-isolate cages throughout the experiments. Room temperature was maintained at 18 to 26 °C with a 12-hour light/dark cycle and

a relative humidity of 40 to 70 percent. Mice were fed Tekland Mouse/Rat Chow and sterile water ad libitum.

Previous experience with this mouse infectivity model indicated that neonatal mice can be routinely infected orally with 10^4 - 10^5 Cryptosporidium oocysts at 5 days of age. Gastrointestinal (GI) colonization and oocyst shedding develops 2 to 5 days later and resolves in about 5 to 7 days. BALB/c mice have been used for numerous experiments with Cryptosporidium.[5,6] Sterling's laboratory has just completed a susceptibility dynamics infection in relation to the BALB/c neonatal mouse.[6] They were able to infect 100 percent of the neonatal BALB/c mice through 9 days of age with 10^5 oocysts by oral intubation.

4. Phase I Test Procedures

a. Phase I Study Set-up and Operation--The USABRDL investigative staff prepared the designated 5 °C test waters (distilled deionized and worst case) with the various chemical and physical challenge characteristics, and dosed the waters with 1.0×10^8 oocysts per liter. The waters and iodine resin were mixed at a 1:1 ratio with a 20 ml volume of water and a 20 ml volume of resin. The reaction mixtures were mixed thoroughly and frequently. Whole reaction tube samples were taken at 1 and 5 minutes after contact by filtering the material through a nylon filter to hold back the resin, and residual iodine was immediately neutralized in the liquid phase with sodium thiosulfate. The neutralized samples were then processed by the UA investigative staff who concentrated the oocysts using the appropriate membrane filter or centrifugation techniques. They then physically enumerated and diluted the oocysts for infectivity studies and adjusted the oocysts to provide the ID_{50} (115) and high (1150) doses to the neonatal mice by gavage.

b. Phase I Sampling

(1) Cyst Recovery/Concentration From Water Samples--During phase I experiments Cryptosporidium inoculated control and Pre-Mac iodine resin treated samples were filtered through 1 μm pore size Nuclepore™ polycarbonate membrane filters. (Iodine resin treated samples were immediately neutralized with sodium thiosulfate at the appropriate time before filtration through the membrane filters.) Each filter was carefully removed and placed in a 50 ml tube (tube A) with 10 ml of washing solution (1 liter of nanopure water containing 10 μl of Tween 20); and the tube contents were mixed for 15 seconds. The filter was then removed, placed in another 50 ml tube (tube B) with an additional 10 ml of washing solution, and mixed. The contents of tube B was added to tube A, and the filter in tube B was washed again. The filter in tube B was removed, while the second wash from that tube was also combined with tube A. Tube B was washed twice with 7.5 ml of nanopure water and combined with tube A.

The entire contents of tube A was centrifuged at 3000 rpm in a Sorvall T-6000B centrifuge with a H1000B rotor for 10 minutes. The liquid portion was aspirated down to 1 ml, and the pellet was thoroughly mixed with the ml of residual wash water. Appropriate dilutions of this concentrated sample were used to measure residual cyst concentrations and were diluted for oral gavage of test animals.

c. Phase I Sample Analyses

(1) Neonatal Mouse Infectivity--Median infectious dose range determinations for the phase I studies with Pre-Mac iodine resin were conducted by infecting mice by gavage in which a range of Cryptosporidium oocysts were administered to groups of neonatal mice. The ID_{50} dose was determined by extrapolation from the dose range studies in which infection of intestinal villi was determined. Companion efforts to determine the baseline ID_{50} used an in vitro excystation method (Appendix 1).[7] Excystation was accomplished in triplicate, and the percent theoretical sporozoite yield was determined according to the following formula:

$$\% \text{ Theoretical Sporozoite Yield} = \frac{\text{Sporozoites counted}}{4(\text{Intact cysts} + \text{Shells})} \times 100$$

ID_{50} Excystation Data

Trial	Intact	Shells	Sporozoites
1	56	193	298
2	46	162	284
3	49	158	324
Mean	50.3	171	302

$$\% \text{ Theoretical Sporozoite Yield} = \frac{302}{4(50.3 + 171)} \times 100 = 34.12\%$$

The $\log_{10}$ of this value was used to determine the expected ID_{50} from the chart in Appendix 2. The regression line on the chart is based on the log change of sporozoite yield vs. the log change in ID_{50}. Therefore, the regression line equation was used to calculate the ID_{50}.

The protocol for experimental animal dosing utilized centrifuged final sample preparations from 50 ml centrifuge tubes. The volume was increased to 5 ml with nanopure water and vortexed to disperse and mix the oocysts. The dilutions needed to obtain the "high dose," "median dose," and "low dose" in 100 μl of inoculum were based on the previously determined ID_{50} and the recovery efficiencies as follows:

# of oocyst seeded per liter X (recovery efficiency) divided by (ID_{50} x 10^5) = # of ml of dilution needed for 10,000 X ID_{50} in 100 µl. This was the "high dose."

Dilute the above suspension 1:10 to obtain 1,000 X ID_{50} in 100 µl. This provided the "medium dose."

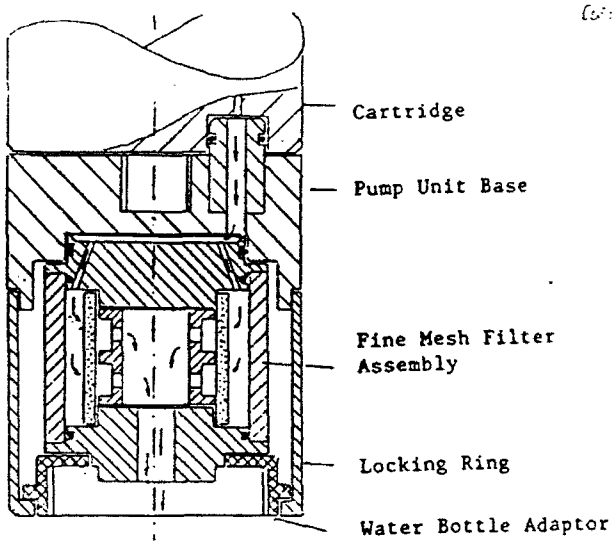
Dilute the "medium dose" suspension 1:10 to obtain 100 X ID_{50} in 100 µl. This gave the "low dose."

For experimental studies, Pre-Mac iodine resin treated oocysts (ID_{50} dose and sequential 10-fold higher dose levels) were administered by oral gavage to 5- to 7-day old neonatal BALB/c mice. This was accomplished with a blunted slightly bent, 1/2 inch, 25-26 gauge hypodermic needle fitted with a short piece of polyethylene tubing mounted on a 1 cc tuberculin syringe. The animals were sacrificed 7 days post inoculation; and approximately 3 cm of the terminal ileum was removed, fixed in 10 percent formalin, embedded in paraffin, and sectioned. Hematoxylin and eosin stained paraffin sections were examined microscopically for evidence of Cryptosporidium infection in the microvillus region of villous enterocytes. Specimens with parasitic stages present were scored as positive, those without were scored as negative. Positive specimens always showed numerous parasitic stages (at least 50-60 per 100X microscope field), while no parasites could be found on any sections taken from negative tissue samples. Infection was scored by the relative concentration of Cryptosporidium in the ileum ranging from a 0 to 4+ level of infection. Any level of infection was scored as a positive result.

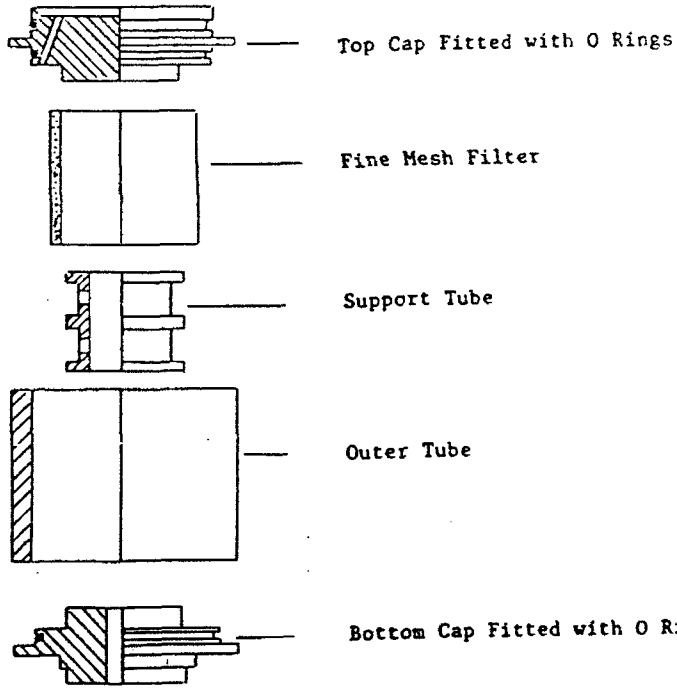
(2) Fluorescent Monoclonal Antibody Procedure--During phase I, companion tests using fluorescent monoclonal antibody (OW64 MAb) directed at the suture line of Cryptosporidium oocysts were utilized as a measure of infectivity and were compared against sample results from neonatal infectivity procedures. The suture line in oocysts only appears when the oocyst has experienced some degradation, and the antibody was prepared against this suture material. The number of oocysts and their infectivity were determined microscopically, using both a fluorescent monoclonal antibody for the cyst wall (total cysts) and one for the suture line. Because the antibodies were tagged with different color fluorescing dyes, the full cysts and cysts with suture lines could be discriminated; and each could be counted. Only damaged oocysts would show the fluorescence of the antibody prepared against the suture line proteins.

5. Phase II Test Procedure--These studies evaluated the physical removal of oocysts by the entire Pre-Mac FWP water purification units having a second generation improved final 1-micron fine mesh filter (Figure 3). Two Pre-Mac water

purifiers were tested in waters seeded with 1.0 X 10^8 oocysts per liter and 1.0 X 10^8 latex beads per liter. Twenty five liters each of distilled deionized water and worst case water were processed through the filter units as previously described for in-house USABRDL tests. The challenge water reservoirs were stirred constantly to keep the oocysts, latex beads, and particulates in suspension. Processed water was measured in large calibrated jars to keep track of the volume pumped. Liters 2, 12, and 25 of the product water were collected for oocyst and bead processing. Oocysts and beads were recovered from distilled deionized water samples by filtration through the 1 μm pore size polycarbonate filters, while samples from worst case water were concentrated by centrifugation. Oocyst viability was evaluated by both neonatal mouse infectivity and the use of the 50 μl OW64 MAb to detect suture lines as previously described. For the antibody technique, samples were incubated 20 minutes at room temperature, washed with PBS, and resuspended in 50 μl of fluorescein isothiocyanate (FITC) labeled secondary antibody for an additional 20 minutes. Samples were washed to remove residual antibody, and counts were made in a hemacytometer at 400X magnification by combined phase contrast and epifluorescent microscopy. Duplicate tests were performed on each water type, and hemacytometer counts were made in triplicate. Latex beads were enumerated by hemacytometer counts using phase contrast microscopy.



Section Showing Pump Fitted with Fine Mesh Filter



Exploded View of Fine Mesh Filter Assembly

Figure 3. Improved (second generation) fine mesh filter schematics

USABRDL RESULTS

IN-HOUSE STUDIES

1. Validation of the Pre-Mac FWP for Effectiveness of Bacterial Disinfection--During the initial Pre-Mac FWP filter tests with the general challenge water, Klebsiella terrigena bacterium levels in the seeded tapwater tanks decreased by several logs due to an unidentified non-halogen toxicant (could not be detected as a free or combined halogen residual). All of the tapwater prepared for each run-day had tested "negative" for free chlorine or total residual chlorine by the LaMotte DPD colorimeter method. The "toxic" contaminant did not appear to have any adverse effect for the challenge viruses and protozoan cysts. An experiment was performed to determine the level of bacterial "kill" over the typical daily 2-hour Pre-Mac operating interval. Three flasks of tapwater were collected and dechlorinated as follows: Flask # 1 contained cold tapwater dechlorinated by sunlight for 72 hours; flask # 2 contained hot tapwater dechlorinated by stirring at 27 °C for 48 hours; and flask # 3 contained hot tapwater cooled to room temperature and then dechlorinated with 10 percent sodium thiosulfate at 100 mg per liter. Water from each of the three flasks was tested for chlorine by the LaMotte DPD colorimeter and no free available chlorine or total combined chlorine was detected. The pH of flask 1 was 7.45 and the pH of flasks 2 and 3 was 6.8. Klebsiella terrigena was prepared as usual and added to each flask containing 1 liter of water at a concentration of approximately 1.0×10^8 CFU per liter. Samples were collected at 0 time, 1 hour, and 2 hours, diluted in PBS, and enumerated using the m-Endo method. Both the cold and the hot dechlorinated tapwater results show a >3-log decrease in bacteria over the two-hour period (Table 2). The hot tapwater dechlorinated with sodium thiosulfate did not appear to produce a significant decrease in colony numbers after two hours. It was not determined if chlorine had reacted with other materials in the water, or whether other elements in the tapwater may have interfered with the DPD color reactions; and attempts to identify the interfering substance in the tapwater were not pursued. Because of the contaminant problem, all challenge tests for the Pre-Mac FWP purifier were conducted using distilled water with ingredients added to simulate either the general or worst case waters (see composition in materials and methods section).

Table 2. Inactivation of Klebsiella terrigena in Tapwater

Tapwater Flask #	Klebsiella terrigena CFU/Liter Inoculum	0 Hour	1 Hour	2 Hours
1	1.20×10^8	6.35×10^7	8.50×10^5	$<1.00 \times 10^5$
2	1.20×10^8	1.09×10^8	$<1.00 \times 10^5$	$<1.00 \times 10^5$
3	1.20×10^8	1.15×10^8	1.40×10^8	9.50×10^7

The evaluation of the Pre-Mac FWP for microbiological treatment effectiveness validated the disinfection of Klebsiella from exposure to the iodine resin in the Pre-Mac filters using the modified waters described above. No significant decrease in bacterial challenge levels was found in the pre-filtration samples collected at the beginning and end of the purifier operations, which averaged 1.43×10^8 vs. 1.15×10^8 CFU/liter, respectively.

The iodine levels in the filtered product water from the Pre-Mac FWP units 1, 2 and 3 averaged 5.88 ppm, 5.99 ppm and 7.00 ppm, respectively (Table 3). It can be seen that the levels of disinfectant reaching the product water were consistently above 5.00 ppm during normal operations where 15 liters were pumped. However, following the 48-hour stagnation periods when the first 1.5 liter throughputs were collected, the iodine levels from units 1 and 2 were below 5.00 ppm, ranging from 3.70-4.55 ppm. Also, unit 2 only produced an iodine level of 3.79 ppm during the first liter throughput.

Table 3. Pre-Mac Filter Treated Water Iodine Levels

Liters Pumped	Water Type	Test Day	Averaged Iodine Levels (ppm) Unit 1	Unit 2	Unit 3
1	General	1	6.80	3.79	5.05
15	"	1	7.09	5.71	9.55
45	"	3	NA*	9.16	9.96
75	"	5	7.57	8.41	8.41
76.5	"	Stag.**	4.55	4.17	5.27
91.5	Worst Case	6	5.26	5.73	7.09
106.5	"	7	5.26	5.73	6.24
121.5	"	8	5.98	6.79	6.94
136.5	"	9	5.98	7.09	7.09
151.5	"	10	6.24	6.24	6.24
153	"	Stag.	4.03	3.17	5.18
Avg.			5.88	5.99	7.00

* Data Not Available (Sample Lost)
** 48-Hour Stagnation Sample

2. Evaluation of Bacterial Disinfection/Removal--For the bacterial removal evaluations, the iodine in the filtered water from each unit was neutralized with sodium thiosulfate immediately following collection. Sample water volumes of 100 ml, 10 ml, and 1 ml from each filter unit were assayed for Klebsiella by the m-Endo method. The results of this evaluation indicated that there was no survival of the Klebsiella after processing through the Pre-Mac purifiers at any time over the 150+ liters of water processed by the units (Table 4). The results indicated that over 6 orders of magnitude of bacterial disinfection had occurred, which met the USEPA guide standard criteria for bacterial removal.

Table 4. Klebsiella terrigena Disinfection/Removal

Liters Pumped	Test Day	Water Type	Klebsiella terrigena Inoculum/Liter	Klebsiella terrigena Recoveries/Liter	Averaged % Removal
1	1	General	9.33×10^7	0	≥99.9999
15	1	"	9.33×10^7	0	≥99.9999
45	3	"	8.10×10^7	0	≥99.9999
75	5	"	1.20×10^8	0	≥99.9999
76.5	Stag.*	"	1.08×10^8	0	≥99.9999
91.5	6	Worst Case	1.40×10^8	0	≥99.9999
121.5	8	"	1.63×10^8	0	≥99.9999
151.5	10	"	1.70×10^8	0	≥99.9999
153	Stag.	"	1.13×10^8	0	≥99.9999

*48-Hour Stagnation Sample

3. Evaluation of Viral Disinfection/Removal--Enterovirus removal studies with the Pre-Mac FWP purifier utilized both poliovirus and echovirus challenge for comparative purposes to make sure that there were no differences in disinfectant sensitivity to the iodine resin from the purifiers. Sodium thiosulfate again was used to neutralize the iodine as soon as the samples were taken from the purifiers. The results indicated that, like the bacterial disinfection results, both enteroviruses were effectively eliminated (Tables 5 and 6). No virus of either type could be detected in product water samples over the test period for either regular samples or stagnation samples over the 150-plus liter throughput. The USEPA guide standard criteria were met with over 4 orders of magnitude (99.99 percent) removal.

Table 5. Echovirus 1 Disinfection/Removal

Liters Pumped	Test Day	Water Type	Echovirus 1 Inoculum/Liter	Recovery/Liter	Averaged % Removal
1	1	General	1.33×10^7	*	≥99.99
15	1	"	1.33×10^7	*	≥99.99
45	3	"	1.79×10^7	*	≥99.99
75	5	"	8.40×10^7	*	≥99.99
76.5	Stag.*	"	8.54×10^7	*	≥99.99
91.5	6	Worst Case	5.27×10^7	*	≥99.99
121.5	8	"	4.27×10^7	*	≥99.99
151.5	10	"	9.20×10^7	*	≥99.99
153	Stag.	"	5.13×10^7	*	≥99.99

* 48-Hour Stagnation Sample

Table 6. Poliovirus 1 Disinfection/Removal

Liters Pumped	Test Day	Water Type	Poliovirus 1 Inoculum/Liter	Recovery/Liter	Averaged % Removal
1	1	General	2.00×10^7	*	≥99.99
15	1	"	2.00×10^7	*	≥99.99
45	3	"	3.33×10^7	*	≥99.99
75	5	"	3.52×10^7	*	≥99.99
76.5	Stag.*	"	8.54×10^7	*	≥99.99
91.5	6	Worst Case	2.86×10^7	*	≥99.99
121.5	8	"	4.53×10^7	*	≥99.99
151.5	10	"	3.80×10^7	*	≥99.99
153	Stag.	"	4.43×10^7	*	≥99.99

* 48-Hour Stagnation Sample

4. Oocyst and Latex Bead Removals--The evaluation of the physical removal and possible disinfection/destruction of Cryptosporidium parvum oocysts was evaluated, and physical removal was compared to the removal of the latex beads. Tables 7 and 8 show that the removals of both oocysts and beads were significant but did not continuously meet the USEPA guide standard criteria of continuous 3 orders of magnitude (99.9 percent). In these experiments Cryptosporidium oocysts were not used in the initial tests up through day 3. When oocysts were provided, their removals appeared to be very similar to the latex bead removals. Pre-Mac purifier unit 1 appeared to have the best capability to remove both oocysts and beads over the experimental procedure and, in every case, met the 99.9 percent removal criteria. However, units 2 and 3 did not consistently meet the

criteria, and sporadic breakthroughs were noted in which slightly less than 99 percent of the latex beads were removed. There did not appear to be any difference in particle removal using either the 1.0 or 3.0 μm rated final filter on the units.

Table 7. Cryptosporidium parvum Oocyst Removals

Liters Pumped	Test Day	Challenge Water	Cryptosporidium parvum Oocysts Inoculum/Liter	Recovery/Liter	% Removal
			Pre-Mac Filter Unit 1		
1	1	General	None		
15	1	"	None		
45	3	"	None		
75	5	"	2.39×10^6	*	≥99.90
76.5	Stag.**	"	2.02×10^6	*	≥99.90
91.5	6	Worst Case	3.10×10^6	*	≥99.90
121.5	8	"	2.24×10^6	*	≥99.90
151.5	10	"	2.48×10^6	*	≥99.90
153	Stag.	"	2.00×10^6	*	≥99.90
			Pre-Mac Filter Unit 2		
1	1	General	None		
15	1	"	None		
45	3	"	None		
75	5	"	2.39×10^6	3.10×10^3	99.87
76.5	Stag.	"	2.02×10^6	6.60×10^3	99.67
91.5	6	Worst Case	3.10×10^6	*	≥99.90
121.5	8	"	2.24×10^6	*	≥99.90
151.5	10	"	2.48×10^6	*	≥99.90
153	Stag.	"	2.00×10^6	6.00×10^3	99.70
			Pre-Mac Filter Unit 3		
1	1	General	None		
15	1	"	None		
45	3	"	None		
75	5	"	2.39×10^6	1.17×10^3	99.95
76.5	Stag.	"	2.02×10^6	2.06×10^3	99.89
91.5	6	Worst Case	3.10×10^6	*	≥99.90
121.5	8	"	2.24×10^6	*	≥99.90
151.5	10	"	2.48×10^6	*	≥99.90
153	Stag.	"	2.00×10^6	1.26×10^4	99.37

* Below Detection Limits
** 48-Hour Stagnation Sample

Table 8. Latex Bead (Cyst Simulant) Removals

LIters Pumped	Test Day	Challenge Water	Latex Beads (Cyst Simulant) Inoculum/Liter	Recovery/Liter	% Removal
			Pre-Mac Filter Unit 1		
1	1	General	1.40×10^7	*	≥99.99
15	1	"	1.40×10^7	*	≥99.99
45	3	"	9.20×10^6	*	≥99.99
75	5	"	2.00×10^7	*	≥99.99
76.5	Stag.**	"	1.75×10^7	*	≥99.99
91.5	6	Worst Case	8.06×10^6	*	≥99.99
121.5	8	"	2.08×10^7	1.20×10^4	99.94
151.5	10	"	1.94×10^7	*	≥99.99
153	Stag.	"	2.44×10^7	*	≥99.99
			Pre-Mac Filter Unit 2		
1	1	General	1.40×10^7	7.08×10^3	99.93
15	1	"	1.40×10^7	1.28×10^4	99.91
45	3	"	9.20×10^6	1.25×10^4	99.86
75	5	"	2.00×10^7	1.80×10^4	99.91
76.5	Stag.	"	1.75×10^7	2.31×10^4	99.87
91.5	6	Worst Case	8.06×10^6	*	≥99.99
121.5	8	"	2.08×10^7	1.20×10^4	99.94
151.5	10	"	1.94×10^7	4.80×10^4	99.75
153	Stag.	"	2.44×10^7	6.00×10^4	99.75
			Pre-Mac Filter Unit 3		
1	1	General	1.40×10^7	2.70×10^4	99.81
15	1	"	1.40×10^7	1.50×10^4	99.89
45	3	"	9.20×10^6	3.03×10^4	99.67
75	5	"	2.00×10^7	2.46×10^4	99.88
76.5	Stag.	"	2.44×10^7	3.46×10^5	98.58
91.5	6	Worst Case	1.94×10^7	6.60×10^3	99.99
121.5	8	"	2.08×10^7	1.20×10^4	99.94
151.5	10	"	1.94×10^7	2.70×10^5	98.61
153	Stag.	"	2.44×10^7	3.46×10^5	98.58

* Below Detection Limits
** 48-Hour Stagnation Sample

5. Evaluation of New Improved Final Filters for Cyst and Bead Removals--Since cysts and beads were able to pass through the nominally rated 3.0 and 1.0 μm final filters, tests were conducted to evaluate the new experimental final filters on the three Pre-Mac units. The units were fitted with new improved 1.0 μm final filters for the first test and 1.0 μm filters sandwiched between 3.0 μm mesh filters for the second test. Two additional tests were set up without final filters on the Pre-Mac

units to evaluate filter efficiencies for removal of latex beads only.

For each test 16 liters of modified worst case challenge water were prepared and seeded with Cryptosporidium parvum oocysts and latex beads. (The oocyst challenge level was increased to that of the latex beads for more direct comparison.) Five liters were manually pumped through each Pre-Mac unit, and the final liter was collected. During the last test Pre-Mac unit 3 appeared to become clogged, and only 1350 ml could readily be pumped over a 26-minute interval. After iodine neutralization, the water constituents were concentrated as previously described to a final volume of 2.0 ml and then examined for cysts and beads. Table 9 shows the results of these experiments.

Table 9. Evaluation of Pre-Mac Filters

Challenge Organism	Challenge Inoculum/Liter	Pre-Mac Unit #	Oocyst Recovery/Liter	Simulant Recovery/Liter	Percent Removal
		TEST #1 (Pre-Mac with new 1.0 μm final filters)			
C. parvum	2.52×10^6	1	6.00×10^3		99.76
		2	6.00×10^3		99.76
		3	1.80×10^4		99.29
Latex Beads	2.19×10^7	1		4.20×10^4	99.81
		2		2.70×10^5	99.20
		3		4.02×10^5	98.16
		TEST #2 (Pre-Mac with 1.0 μm Between 3.0 μm Mesh)			
C. parvum	1.82×10^7*	1	1.60×10^4		99.91
		2	9.60×10^4		99.47
		3	1.85×10^5		98.99
Latex Beads	1.99×10^7	1		2.40×10^4	99.88
		2		1.60×10^5	99.20
		3		2.32×10^5	98.83
		TESTS #3 & #4 (Pre-Mac minus Final Filters)			
Latex Beads	2.05×10^7	1		2.40×10^5	98.80
		2		8.13×10^5	96.03
		3		1.38×10^6	93.27
Latex Beads	2.17×10^7	1		7.80×10^4	99.64
		2		1.02×10^6	95.30
		3**		3.48×10^5	98.40

* Oocyst inoculum increased to increase detection limits.
** Only 1350 ml pumped through before unit clogged.

The results above indicate that neither of the new types of final filter combinations provided any improvement to the total removal of either oocysts or latex beads. In fact, it appears that the Pre-Mac FWP purifier prefilters and other internal removal mechanisms, such as the carbon cloth, provided removals almost as well as the addition of the final filters, and differences appeared to be within 1 order of magnitude.

COLLABORATIVE STUDIES

1. Phase I

a. Oocyst Recovery Efficiency and Infectivity for Positive Controls--The two 5 °C test waters (distilled deionized and worst case) were seeded with 1.19×10^8 oocysts per liter. After 20 minutes, the preparations were stirred and 1.0 ml withdrawn for the positive infectivity control challenge doses. The challenge waters were filtered through 1.0 µm polycarbonate filters to recover the oocysts.

distilled deionized water mean recovery = 71.1%
worst case water mean recovery = 45.0%

These values were used to determine the volume of concentrate needed to prepare the challenge doses for each experiment.

Two groups of neonatal BALB/c mice were challenged with oocysts exposed to each of the test waters. One group was given the ID_{50} of 115 oocysts (low test dose), while the other received 1150 oocysts (high test dose). Complete data are shown below in Table 10. Mice challenged with the high dose in distilled deionized water showed a high level of infection (80 percent). Mice challenged with worst case water showed the 50 percent and 100 percent infection expected for the low and high dose respectively. The outcome of the positive control experiment supported the expected ID_{50} and established the infectivity of the oocysts used in the tests.

Table 10. Positive Control Mouse Infectivity Data

Water	Dose # of oocysts	Treatment	Percent Mice Infected
DD* 5 °C	15	Control	50 (2/4)
DD 5 °C	1150	Control	80 (4/5)
Worst Case 5 °C	115	Control pH 5	50 (2/4)
Worst Case 5 °C	1150	Control pH 5	100 (2/2)

* DD = distilled deionized

b. Iodine Resin Infectivity Test Results--Distilled deionized and worst case 5 °C waters containing equal parts of iodine resin were seeded with the Cryptosporidium oocysts and sampled after 1 and 5 minutes of contact. After sample processing and quantification, the concentrated sample materials were given to the neonatal mice at challenge levels of 115 and

1150 oocysts. Table 11 shows the mouse infectivity data. Generally, the low-dose infectivity ranged from 50 to 60 percent while the high dose ranged from 64 to 100 percent infectivity. These data suggest that the iodine resin may have had some modest effectiveness for disinfection. It was uncertain whether the increased effectiveness was due to the iodine or to mechanical abrasion of the oocysts by the resin during the stirring process. Measurement of residual iodine in the liquid phase of control samples after a 5-minute contact time yielded 4.8 to 6.2 mg/l of available disinfectant.

Table 11. Mouse Infectivity

Water	Dose # of Oocysts	Treatment	Percent Mice Infected
DD* 5 °C	115	Iodine 1 min	64 (7/11)
DD 5 °C	1150	Iodine 1 min	92 (12/13)
DD 5 °C	115	Iodine 5 min	50 (5/10)
DD 5 °C	1150	Iodine 5 min	85 (11/13)
Worst Case 5 °C	115	Iodine, pH 5 1 min	58 (7/12)
Worst Case 5 °C	1150	Iodine, pH 5 1 min	100 (11/11)
Worst Case 5 °C	115	Iodine, pH 5 5 min	50 (7/14)
Worst Case 5 °C	1150	Iodine, pH 5 5 min	64 (7/11)

* DD = distilled deionized

Recovered oocysts were also evaluated for potential viability using MAb OW64 monoclonal antibody. After treatment, many oocysts had clearly been damaged, including some which had excysted. The percentage of OW64-positive oocysts increased from no binding in untreated oocysts to 4 to 5.5 percent binding in each of the treatment groups.

2. Phase II

The phase II studies were performed to determine if the second generation, improved final 1.0 μm filters on the Pre-Mac water purifiers would physically eliminate oocysts from product water. The Pre-Mac units used in these evaluations had already been used for the USABRDL in-house efforts, and thus had several hundred liters of challenge water passed through them. Only the final 1.0 μm filters were new. The basic protocol for the tests at the UA followed those of USABRDL except that the source and preparation of the Cryptosporidium oocysts was that of the UA investigative team. Samples taken from the two Pre-Mac purifier product waters at 2, 12, and 25 liters (for both distilled deionized and worst case waters) were immediately neutralized with sodium thiosulfate; and oocysts and latex beads were counted using phase contrast and epifluorescent microscopy.

Table 12 shows that the oocysts could be found at low numbers in all of the samples collected from the Pre-Mac product water samples. Only the sample from the second liter of distilled deionized water from filter unit #1 showed a high percent of passage of oocysts. This may have been due to a leak detected in the filter housing which allowed oocysts to bypass directly into the collection vessel. Oocyst recovery in the rest of the samples ranged from 0.2 to 7.2 percent, thus indicating a general oocyst removal capability of around 1.5 to 2 orders of magnitude. All of the product water samples were very turbid because the iodine resin bled into the filtrate during the pumping process. This high turbidity prevented the enumeration of latex beads in any of the samples. The data would suggest that the filtration efficiency improved with increased volumes of water passage through the filters. Partial filter blockage may have accounted for this increased efficiency. Many damaged oocysts and empty shells were recovered from the filtrate. However, it is difficult to assess the meaning of this finding; and since the filters had been used in previous experiments containing a Cryptosporidium oocyst challenge, the oocysts could have been contained within the main body of the purification unit for long periods. Considering the condition of the oocysts recovered during the iodine resin study, however, it is entirely possible that the damage noted for the residual oocysts may have been caused by the combined mechanical and chemical stresses of filtration and iodine resin.

Table 12. Pre-Mac Water Purifier Treatment Effectiveness for Oocysts

	Distilled Water		Worst Case Water	
Sample	Filter #1 % Oocysts Removed	Filter #2 % Oocysts Removed	Filter #1 % Oocysts Removed	Filter #2 % Oocysts Removed
Liter 2	64.7	92.7	97.5	98.8
Liter 12	97.9	99.0	99.8	97.5
Liter 25	99.0	97.4	99.7	99.3

NOTE - The actual oocyst challenge levels achieved ranged from 5.8 - 6.8 X 10^5 cysts per liter of distilled deionized water and 3.1 - 3.6 X 10^5 cysts per liter of worst case water.

DISCUSSION

IN-HOUSE STUDIES

The results of these studies indicated that both Klebsiella terrigena bacteria and Poliovirus 1 and Echovirus 1 challenges were readily removed by the Pre-Mac FWP hand-held water purifiers. No bacteria or viruses were detected in product water at any sampling point. The purifiers met the USEPA guide standard and protocol criteria for removal of both types of challenge. The results indicated that a significant iodine residual was provided to the product water which, in a military setting, would be provided to the canteen to treat post-purification contamination. The units all provided at least 5 mg/l of disinfectant when operated for a period of time before sampling. On the other hand, it was noted that there was a diminished iodine residual when the first liter of water was produced after a 48-hour stagnation period. Although reduced after the stagnation period, the residual would probably be adequate to remove most typical bacterial contamination. If there were a concern, users could be instructed to void the first liter or two of water before taking water for consumption. With regard to protozoan cysts and their simulant, the latex beads, it was observed that the Pre-Mac FWP purifiers did not continuously meet the USEPA removal criteria. Modification of the final filter configuration from a single 3.0 or 1.0 μm pore size to the new 1.0 μm filter and 1.0 to 3.0 μm sandwich filters did not increase the effectiveness of the purifier; in fact, studies suggested that the purifiers without the final filters were nearly as effective in removing cyst-size particles as when final filters were present. It is probable that the prefilters and carbon material in the units were effective in physically removing the cyst-sized particulates. Because there was no significant difference between oocyst and latex bead removals, it is suggested that there was little activity of the iodine resin in terms of oocyst disinfection.

COLLABORATIVE STUDIES

The results of the collaborative USABRDL-UA studies indicated that the iodine resin may have a modest capability to disinfect Cryptosporidium oocysts in both distilled deionized and worst case waters. Further studies are necessary to determine if the damage detected for cysts was due mainly to the iodine or possibly to mechanical destruction from agitating the beads during the reaction period. The results of testing with the complete Pre-Mac purifier units including the second-generation 1.0 μm final filters indicated that they did not perform as anticipated. The final filters theoretically should have eliminated oocysts or other particles with sizes of 3.0 to 5.0 μm diameters from entering the product water. Either the filters

were flawed, or were not fitted onto the units properly, or the cysts had a capability to partially bypass the final filter. The physical removal of around two orders of magnitude would not satisfy the USEPA Guide Standard and Protocol for Testing Microbiological Water Purifiers. The modest capabilities of the iodine resin to further reduce infectious cyst levels could not guarantee removal of 3 orders of magnitude. Improved final filtration is still considered essential to provide the microbiological removals needed in a military field setting.

Appendix 1

EXCYSTATION OF CRYPTOSPORIDIUM OOCYSTS

Materials: Freshly washed oocysts (Pelleted in a 15 ml conical tube)
Tissue Culture PBS (TPBS)
2X Excystation Medium (Frozen medium must be allowed to reach room temperature before use.)

Procedure: (Note: This procedure may be adapted for use with very small volumes. In this case, use 1.5 ml Eppendorf tubes, wash with 1.0 ml TPBS, centrifuge at setting #7, aspirate down to 100 µl of 2X excystation medium.

1. Carefully add 5 ml of TPBS to the freshly washed oocysts without disturbing the pellet. Centrifuge at 3000 rpm for 3-5 minutes. Repeat this step once. (Note: Centrifuge at 3000 rpm for 20 minutes if the pellet was disturbed.)

2. Aspirate down to 0.5 ml, trichurate with a pasteur pipette to break up the pellet, and bring the volume to 2.0 ml with 2X excystation medium. Place in 37 °C water bath for 60 minutes. Remove from the water bath and let the sample sit at room temperature for 30 minutes before counting. Place on ice if counting cannot proceed immediately.

3. Count and record at least 200 intact cysts + shells and the sporozoites in the same area of the counting chamber. Repeat this once using another sample from the excystation mixture.

4. To calculate the percent excystation, divide the number of shells by the sum of intact cysts and shells and multiply by 200. Calculate the number of sporozoites produced per shell by dividing the number of sporozoites by the the number of shells.

$$\text{Percent Excystation} = \frac{\text{Shells}}{\text{Intact Cysts + Shells}} \times 100$$

$$\text{Sporozoites per Shell} = \frac{\text{Sporozoites}}{\text{Shells}}$$

5. Calculate the percent theoretical sporozoite yield by dividing the number of sporozoites observed by 4X the sum of the number of shells and the number of intact cysts.

$$\text{Percent Theoretical Sporozoite Yield} = \frac{\text{Sporozoites}}{4(\text{Intact Cysts + Shells})} \times 100$$

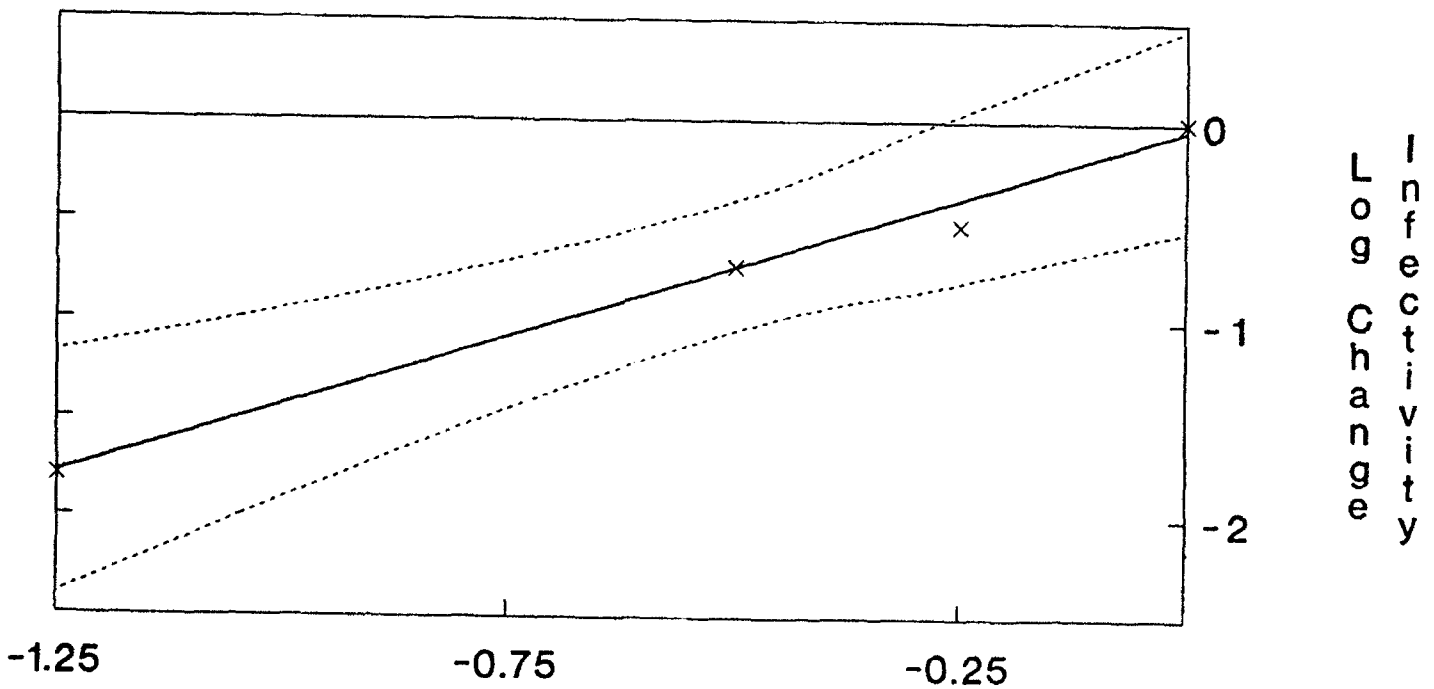
Ref: Woodmansee. 1987. J. Protozool. 34(4):398-402

Appendix 2

Infectivity v Sporozoite Yield

Linear Regression Analysis

80% Probability to Predict Viability with One Trial



Regression line: y = 1.39(x) - 0.066
r = 0.995, P<0.0048

GLOSSARY OF TERMS

BGMK	Buffalo Green Monkey Kidney
CFU	colony forming units
CPE	cytopathic effect
EBSS	Earle's Balanced Salt Solution
FITC	fluorescein isothiocyanate
HBSS	Hanks' Balanced Salt Solution
MEM	Minimum Essential Medium
MOI	multiplicity of infection
NCS	newborn calf serum
PFU	plaque forming units
PBS	phosphate buffered saline
ROWPU	reverse osmosis water purification unit
TDS	total dissolved solids
TOC	total organic carbon
UA	University of Arizona
USABRDL	US Army Biomedical Research and Development Laboratory
USEPA	US Environmental Protection Agency

LITERATURE CITED

1. Report of Task Force. 1987. Guide standard and protocol for testing microbiological water purifiers. U.S. Environmental Protection Agency, Office of Drinking Water and Office of Pesticide Programs, Wash, DC.

2. Sharp, D.G. and J.D. Johnson. 1977. Inactivation of viruses in water by bromine and its compounds: Influence of virion aggregation. U.S. Army Medical Research and Development Command Contract No. DAMD-17-74-C-4013.

3. Waldman, E., S. Tzipori, and J.H.R. Forsyth. 1986. Separation of Cryptosporidium species oocysts from feces by using a percoll discontinuous density gradient. J. Clin. Microbiol. 23(1):199-200.

4. Arrowood, M.J., and C.R. Sterling. 1987. Isolation of Cryptosporidium oocysts and sporozoites during discontinuous sucrose and isopycnic percoll gradients. J. Parisitol. 73: 314-329.

5. Korich, D.G., J.R. Mead, M.S. Madore, N.A. Sinclair, and C.R. Sterling. 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on Cryptosporidium parvum oocyst viability. Appl. Environ. Microbiol. 56(5):1423-1428.

6. Novak, S.M. and C.A. Sterling. 1991. Susceptibility dynamics in neonatal BALB/c mice infected with Cryptosporidium parvum. J. Protozool. 38(6):102s-104s.

7. Woodmansee, D.B. 1987. Studies of in vitro excystation of Cryptosporidium parvum from calves. J. Protozool. 34(4):398-402.

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