The following reveals the results of months of stringent testing of all outflows of Tempico's ***Rotoclave***® System by Dr. Gary Braedt. Dr. Braedt, formerly Assistant Professor and Coordinator of the Microbiology Program at the University of New Orleans, has been conducting tests on the ***Rotoclave***® for many years. These tests were performed in July 1996 and the results were published in August of 1996.

We are very pleased to present these results to you in the form of an Environmental Impact Statement. It begins with a brief, two-page summary and is followed by an in-depth explanation of the empirical evidence, proving all ***Rotoclave***® outflows to be sterile, non-toxic, and non-carcinogenic.

ENVIRONMENTAL IMPACT STATEMENT

Tempico's ***Rotoclave***→ is a pre-vacuum-type autoclave with a patented internal rotating drum and the additional feature that the sterilizing steam is condensed and eliminated before the door can be opened. The ***Rotoclave***→ is a totally closed system with safety interlocks that ensure that the vessel's integrity cannot be breached until the conditions of time, temperature and pressure have been met, guaranteeing complete sterilization of the contents. Since the sterilizing steam must be evacuated and condensed before the door can be opened, the ***Rotoclave***→ emits no fumes or emissions of any kind, which would endanger the health and safety of the operator. The outflows from the ***Rotoclave***→, therefore, are as follows: solid waste discharged from the drum, water resulting from condensation of the sterilizing steam, which is routed to the sanitary sewer; and the air exhaust used to break the vacuum post-sterilization, which is vented outside of the building. The results of stringent tests of these outflows have shown them to be sterile, non-toxic and non-carcinogenic. Further tests have demonstrated the ***Rotoclave***→ to have a sterilization capacity that exceeds worldwide standards by several orders of magnitude. The following is a brief summary of the data we have provided in the accompanying document, "*Treatment of Regulated Medical Waste in Tempico's* ***Rotoclave***→ *produces an output that is sterile and non-carcinogenic*", which serves to demonstrate the environmental safety of the ***Rotoclave***→ Please consult this document for further details.

Bacterial contamination of *Rotoclave*→ outflows by source:

Aqueous:

Floor drain, during a cycle	None Detected
Floor drain, after a cycle	None Detected
Sight glass	None Detected
Air exhaust vent, during a cycle	None Detected
Air exhaust vent, after a cycle	None Detected

Assay limit: 10 bacteria/ml of sample.

Solid:

None detected

Assay limit: 1 bacterium/gram of sample

Results of a sterility Challenge:

Cell/Spore type	Log_{10}Kill		
	Trial 1	**Trial 2**	**Trial 3**
Bacillus stearothermophilus			
cells	-----	7.0	6.7
endospores	7.8	8.3	8.0
Bacillus subtilis			
cells	9.1	7.8	7.4
endospores	9.1	9.5	9.1
Staphylococcus aureus	8.2	9.5	9.5
Mycobacterium smegmatis	8.6	9.6	9.6
Mycobacterium phlei	8.5	-----	-----
Pseudomonas aeruginosa	10	9.5	9.5
Candida albicans	9.5	10	10

*Log_{10}Kill = Log_{10}Input – Log_{10}Recovered. Log_{10}Recovered was set to 0 since the assay limit is 1 cell/ml, and no cells were recovered from any of the samples tested.

Carcinogenic and toxic potential of *Rotoclave*→ outflows by source:

Aqueous extract from solid mass	None detected
Floor drain, during a cycle	None detected
Floor drain after a cycle	None detected
Sight glass	None detected
Air exhaust vent, during a cycle	None detected
Air exhaust vent, after a cycle	None detected
Assay: Ames test	

Treatment of Regulated Medical Waste in Tempico's *Rotoclave*® produces an output that is sterile and non-carcinogenic

INTRODUCTION

Processing of Regulated Medical Waste (RMW) is a problem of immense proportion. It has been estimated that 3.5 million tons of RMW waste requiring treatment are generated every year. Regardless of its source, all RMW must be treated as Potentially Infectious Medical Waste (PIMW) and rendered sterile prior to disposal. In addition, to be acceptable most states, as well as landfill operators, require that the product be rendered non-recognizable, e.g., sharps must be made harmless and unrecognizable. A number of treatments have been developed to address the disposal of RMW, each with its own strengths and weaknesses.

Incineration may be the most widespread treatment and has the historic advantage of common use to sterilize waste. However, ash from incineration contains high levels of heavy metals, and potentially carcinogenic heavy metal oxides are to be found in the emissions, as are dioxanes, furans and polycyclic aromatic hydrocarbons resulting from the incomplete combustion of plastics such as syringes and even the bags the waste is packaged in. Other stack emissions such as nitrogen and sulfur oxides contribute to acid rain. Thus, although incineration has the potential for rendering PIMW sterile, it creates another problem. Due to the toxic by-products, incineration creates a problem which is of the same or greater magnitude than the problem it was designed to eliminate. Additionally, the combustion process is destructive to the vessel, resulting in the need for frequent repairs. These and other considerations limit the usefulness of incineration as a practical means for the disposal of RMW, although incineration will no doubt continue to be necessary in the short run for the destruction of carcinogenic materials.

Chemical treatment of PIMW suffers from the inability to guarantee sterilization. The high lipid content of some bacteria, such as the Mycobacteria, make them notoriously resistant to chemical treatments. Given the current increase in the incidence of tuberculosis, an infection of *Mycobacterium tuberculosis*, this becomes a serious shortfall of chemical treatment. The chemicals pose safety problems as well because they are themselves toxic, corrosive and sometimes even highly explosive (for example gaseous ethylene oxide). These properties lead to equipment deterioration and potential health problems. There is, for example, an extensive literature which implicates ethylene oxide in the formation of chromosomal aberrations, hemoglobin adducts, and even an increase in the rate of spontaneous abortion (e.g., see Schulte, et al., 1992; Landrigan, et al., 1984; Laurent, et al., 1984; Garry, et al., 1979). Chemical treatment of Red Bag waste, therefore, is not an attractive technology because it requires the use and disposal of highly toxic compounds.

Microwave technology has recently entered the marketplace for the treatment of PIMW. There are two basic types of microwave processes. The first relies on the ability of microwaves to produce steam. These units are highly sensitive to the water content of the waste. For this type of process to be efficient, the Red Bags must be shredded prior to, or during processing to evenly distribute the waste permitting steam to contact all surfaces. However, shredding prior to processing

is illegal in most states as opening untreated Red Bags eliminates the protection afforded by containment of the waste and allows the release of pathogens into the atmosphere. Further, effective steam sterilization requires the steam be pressurized since many organisms survive steam treatment at atmospheric pressures and temperatures. Steam-generation microwaves are not generally capable of generating pressurized steam and are thus unlikely to achieve disinfection much less sterilization (i.e., no microorganisms). This type of microwave process is not suitable for very wet or dry wastes, body parts or metals which make up a large portion of the waste stream. The constant and persistent danger of microwave leakage requires the added burden of diligence on the part of the operator who must monitor the equipment, probably during each run. Additionally, the generation of microwaves consumes enormous amounts of electricity.

The second microwave technology is essentially a combustion technology where the microwaves are absorbed by a receiving device to produce a sufficient quantity of heat to initiate combustion. Some of these units have a conventional secondary natural gas flame combustion chamber. Such units suffer from the drawbacks of standard incinerators because it never is possible to achieve total combustion. Furthermore, the heart of a microwave, the magnetron, consumes a great deal of electricity and is vulnerable to damage by a high metal content. High electricity consumption is an unattractive prospect not only for the economic cost, but also because of the environmental cost of producing the electricity through the burning of fossil, or nuclear fuels. Hence microwaving is not an optimal technology for the treatment of RMW.

Steam sterilization, or autoclave technology, relies on high pressure and temperature steam and therefore, in principle, neither uses nor produces toxic substances. Autoclaving has been accepted for decades as the standard for sterilizing surgical instruments. However, because steam does not readily penetrate sealed Red Bags (Lueur, *et al*., 1982; Rutala, *et al*., 1982), stationary autoclaves are not a reliable means of RMW sterilization. In recognition of this fact, regulators have adopted standards, referred to as disinfection, that fall short of full sterilization of PIMW (e.g., a 6 $\log_{10}$ reduction in the number of a defined repertoire of vegetative cells and/or a 3 or 4 $\log_{10}$ reduction of *Bacillus stearothermophilus* endospores). PIMW that has been "disinfected" or "decontaminated" to meet these minimal standards is not sterile and may prove hazardous to transporters and to landfill operators.

Thus, incineration, chemical treatment, microwaving, and autoclaving all demonstrate significant drawbacks to their use, either by failure to render the product sterile, by producing toxic by-products, or both. Many of these same problems make the costs of a particular process economically prohibitive, e.g., the high energy demands of producing microwaves, the cost of frequent replacement of the containment vessel and the neutralization of caustic chemicals.

To address these problems, the ***Rotoclave***® (Tempico, Inc., Madisonville, LA) was developed, which is best described as a steam autoclave with an internal rotating drum. The combination of agitation and high temperature steam ruptures Red Bags and causes glass to break, allowing all surfaces to be exposed to the sterilizing steam. The results presented here demonstrate that Tempico's ***Rotoclave***® is capable of achieving a 10 $\log_{10}$ reduction of vegetative cells and an 8 $\log_{10}$ reduction of *Bacillus stearothermophilus* endospores. The results attained were probably not an upper limit, but rather were due to the difficulty of obtaining high density cell and spore suspensions. Furthermore, the solid and liquid outflows of the ***Rotoclave***® were determined to be

sterile and non-carcinogenic. This latter observation demonstrates that the ***Rotoclave***® process does not itself produce or use toxic or carcinogenic compounds, unlike the contemporary technologies. Because the ***Rotoclave***® can often be incorporated into an existing steam generation system, energy costs are low and operational costs are minimized. Once sterilized, the waste, which has been reduced to about 20% of its original volume, is passed through shredders and grinders to render it unrecognizable, satisfying requirements imposed by most states and landfill operators. An added benefit results from extending the life span of the landfill, an important consideration as land for these sites becomes more scarce. I therefore concluded that the ***Rotoclave***® is the most reliable and cost effective method for the treatment of RMW.

MATERIALS AND METHODS

Bacterial strains and growth media. The Ames tester strains *Salmonella typhimurium* TA98 and TA100 were obtained from the laboratory of Dr. Bruce Ames (U. Calif., Berkeley). Cells were propagated on Vogel-Bonner medium E plates (0.2 g/l $MgSO_4 \cdot 7\ H_2O$, 2 g/l citric acid monohydrate, 10 g/l K_2HPO_4, and 3.5 g/l $NaHNH_4PO_4 \cdot 4H_2O$) containing 3 μM biotin, 50 mg/l histidine, 1% glucose and 1.5% agar. Assay plates were of the same composition but lacking biotin and histidine. Assay top agar contains 0.05 mM of both biotin and histidine, and 0.6% of both agar and NaCl. Overnight cultures were grown in nutrient broth (Oxoid #2 or Difco). Petri dishes were from VWR or Falcon since these dishes are sterilized by γ-irradiation, not by ethylene oxide sterilization which has been reported to leave a residue that increases the spontaneous reversion rate of the tester strains (Maron and Ames, 1983; Vennit, *et al*. 1984).

The ATCC strains used for sterility testing are as follows: *Candida albicans*, ATCC 18804; *Mycobacterium phlei*, ATCC 11758; *Mycobacterium smegmatis*, ATCC 14468; *Bacillus subtilis*, ATCC 19659; *Bacillus stearothermophilus*, ATCC 7953; *Staphylococcus aureus*, ATCC 6538; and *Pseudomonas aeruginosa*, ATCC 15442. *B. subtilis, B. stearothermophilus, S. aureus, P. aeruginosa, M. smegmatis,* and *M. phlei* were grown in nutrient broth (Difco) or on nutrient agar plates. *Candida albicans* was grown using YPD media (1% yeast extract, 2% peptone, 2% dextrose).

Determination of sterility of cultures introduced into the *Rotoclave*®. Cultures of the spore-forming strains *B. subtilis* and *B. stearothermophilus* were incubated on nutrient agar plates for several days at 37^0 and 65^0, respectively, to increase the relative concentration of endospores in the culture. The cells were scraped from the plates and added to nutrient broth to achieve a high cell/spore density. A similar stratagem was employed for the other cell types except that a cell mass from a fresh overnight plate culture was added to a fresh overnight broth culture.

The initial assay involved allocating five ml of each cell type into individual 7.5 ml borosilicate tubes with Bakelite screw caps. It was found to be necessary to wrap the tubes with several layers of packaging tape to prevent the liquid from being lost during the vacuum phase of the cycle. Plastic vials of several types were also tried with the result that the content of the tube was lost in each case due to deformation either of the screw cap, or the body of the tube. For Trials 2 and

3, thick-walled chromatography 2.1 X 6.2 cm, 5 ml vials (VWR) with open screw-cap closures and a PTFE-faced silicone septum were inoculated with 1.5 ml of cell/spore suspension. For all of the assays, the vials were wrapped with 1 cm of paper toweling and inserted into a 3.25 X 20 cm, 60 ml syringe. The syringe was wrapped with an additional 1 cm of cotton wadding and inserted into a 5.25 cm inside diameter perforated stainless steel cylinder which had been fitted with a screw cap at one end. The paper toweling and cotton wadding served both to protect the vial from breakage, and to approximate the conditions likely to be found in a wad of hospital linens.

An untreated aliquot of each culture was diluted and plated in triplicate onto nutrient agar plates to determine cell/spore number. The cultures of the spore-forming strains *B. subtilis* and *B. stearothermophilus* were examined microscopically to determine the ratio of endospores to vegetative cells. The *B. subtilis* culture was estimated to contain 40% endospores for Trial 1 and 2% endospores for both Trial 2 and Trial 3. For *B. stearothermophilus*, no vegetative cells were observed in the broth for Trial 1, while the broth of Trials 2 and 3 each contained about 5% endospores.

One ml of the ***Rotoclave***® treated samples was added to ten ml of nutrient broth, additionally, 0.1 ml aliquots were plated in triplicate onto nutrient agar plates. The cells were then incubated for five days at 30^0 (*B. subtilis, C. albicans*), 37^0 (*M. phlei, M. smegmatis, P. aeruginosa* and *S. aureus*) or 55^0 (*B. stearothermophilus*). The plates were checked daily for colony formation, and the liquid cultures were monitored for an increase in turbidity at an OD_{600} with a Spec20 (Milton Roy) spectrophotometer. Small aliquots of the ten ml cultures were spotted onto nutrient agar plates to verify that the absence of an increase in turbidity was due to lack of viability rather than inhibition of cell growth. To insure that the broth containing the ***Rotoclave***® sample was not inhibitory to cell growth, at the end of the incubation period, cells were pelleted and the broth added to a fresh tube.

Plate incorporation assay. Cells (TA98 and TA100) were grown overnight at 37^0 C with shaking in nutrient broth. The following day the cultures were diluted five-fold into fresh nutrient broth and allowed to reach a cell density of about 1 X 10^9 cells/ml before use in the assay since the *Salmonella* strains TA98 and TA100 lose viability after extended incubation (Ames and Maron, 1983). The extract to be tested was mixed with 0.1 ml of cells, added to 2 ml of 45^0 C top agar and immediately poured onto fresh minimal assay plates lacking histidine and biotin. Duplicate plates were prepared for each extract volume. Extracts were also tested in the presence of rat liver S9 extract (Molecular Toxicology, Annapolis, MD), which was added last. The process of mixing and pouring took no more than 20 seconds in all cases. Revertant colonies were counted after incubation at 37^0 C for 2 days, and again each day for 5 days. The number of countable colonies by 5 days was never more than 10% greater than the number observed after a 2 day incubation. The presence and characteristics of the background bacterial growth (the so-called "lawn") were also noted since the quality of the background growth is a rough measure of extract toxicity. Negative controls consisted of plating cells alone, or in the presence of the S9 mix (where appropriate), to determine the number of spontaneous revertants. Positive controls were as follows. Daunomycin (Molecular Toxicology) was used for TA98, and sodium azide (Sigma) for TA100. 2-aminofluorene (Molecular Toxicology)

was used as a positive control for both strains. This compound does not give a positive result in the Ames assay in the absence of S9, since its carcinogenic ability is developed by oxidation carried out by P450 enzymes found in the extract. Therefore 2-aminofluorene -S9 mix serves as an additional negative control, while 2-aminofluorene + S9 mix serves as a control of both the activity of the S9 extract and the ability of the strains TA98 and TA100 to respond to the known mutagen 2-aminofluorene.

Sample collection. Samples were obtained from several ***Rotoclave***® sites: solid discharge, floor drain, sight glass and outside air exhaust vent. The floor drain receives standing water from the vessel as well as the steam condensate produced as the last step of a sterilization cycle. Water samples were taken from the floor drain during and after a cycle. The condensate produced from the sterilizing steam is routed to a reservoir tank before release via the floor drain. A sight glass connected to the reservoir allows one to inspect the reservoir volume. The water contained in the reservoir, and hence the sight glass, comes entirely from the steam condensate. Because the vessel is exhausted by vacuum, sight glass water can be sampled only after completion of a cycle. The air exhaust created by formation and dissipation of the pre- and post-vacuum is routed through a charcoal filter to outside of the building. Samples were taken of water dripping from the outside air exhaust vent during and after a cycle. In all cases, the outflow source was swabbed with 70% ethanol before sampling to prevent incidental contamination. Liquid samples were collected into sterile 50 ml Corning polypropylene tubes and kept at 4^0 C until use, and solid samples were collected into large plastic zip-lock bags which had been swabbed with 70% ethanol and allowed to dry. To generate a liquid sample from the solid sample for the Ames test, a one kilogram mass of solid waste was autoclaved in five liters of distilled water for 15 min at 121^0 C and 15 psi to extract water soluble components. A 50 ml aliquot was collected from the solution immediately following autoclaving and was stored at 4^0 until used, usually the next day. Aliquots of each sample were plated on nutrient agar plates to check for bacterial contamination that would interfere with the enumeration of revertants. Since these plates were always negative after a 24 hr incubation at 37^0 C, the Ames assay was performed on the ***Rotoclave***® samples within a day of collection.

Sterility Determination. Samples to be tested for microbial contamination were inoculated into nutrient broth or spread on nutrient agar plates. Colony formation was followed for 2 weeks to allow the development of colonies by slow growing organisms, such as some of the Mycobacteria. Incubation was usually at 25º C since the growth of some organisms is inhibited at higher temperatures. Growth in broth cultures was monitored for two weeks by an increase in A_{600} using a Milton-Roy Spec20 spectrophotometer. To ensure that the broth containing the ***Rotoclave***® sample was not inhibitory to cell growth, at the end of the incubation period, cells were pelleted, and the broth added to a fresh tube. The tube was then inoculated with about 10^3 organisms of the species originally held in the tube. Luxuriant overnight growth in the broth demonstrated the ability of the broth to support cell growth. Nutrient agar/broth was chosen since it will support the growth of most non-fastidious organisms. It was not deemed necessary to test for fastidious organisms since their presence or absence can be inferred from the presence or absence of the more hardy indicator organisms such as *Escherichia coli* and *Staphylococcus aureus*.

RESULTS

In this study I wished to address three questions concerning the operation of Tempico's ***Rotoclave***®, those being: 1.) the sterility of the material that leaves the vessel, both particulate and aqueous; 2.) the ability of the ***Rotoclave***® to sterilize cell/ spore cultures with a known high density; and 3.) whether the ***Rotoclave***® produces any products that show carcinogenic potential as judged by the Ames test.

Rotoclave® **discharges are sterile**. The ***Rotoclave***® (Fig. 1) is a pre-vacuum-type autoclave that is evacuated before pressurized steam is introduced into the vessel, replacing the air in the chamber with steam at a minimum temperature and pressure of 135^0 C (275^0 F) and 45 psi. (Standard autoclave values are 121° C at 15 psi.) The vessel is again evacuated following the sterilization cycle at which time the steam is condensed and shunted to a holding tank prior to discharge into the sanitary sewer system via a floor drain. A sight glass is attached to the condensate reservoir to monitor its fullness. A small amount of condensate is formed as the steam contacts the shell during the heat-up phase. This water, which does not come in contact with the waste, is conducted directly to the sanitary sewer by means of the same floor drain, bypassing the reservoir. Samples from the floor drain and the sight glass are, therefore, of essentially the same composition, except for the pre-cycle samples from the floor drain. The exhaust resulting from vacuum formation and dissipation is routed through a charcoal filter before release into the air outside of the building in which the ***Rotoclave***® is housed.

I wished to determine the quality of the air and steam condensate expelled from the ***Rotoclave***®. The former results from the vacuum formation prior to the introduction of high pressure steam and the latter is the steam condensate following sterilization of the chamber contents. The initial air exhaust has roughly the composition of the ambient air with the possible addition of any microorganisms aerosolized from the outside of the Red Bag waste in the chamber. To eliminate any possible microbial contamination, the air exhaust is passed through a steam jet for about a minute and then passed through an activated charcoal filter before it is released to the atmosphere. A small amount of air exhaust occurs following a cycle, after complete sterilization of the chamber contents, as the sterilizing steam is condensed to water for release into the sanitary sewer.

To determine if bacteria are found in ***Rotoclave***® outflows, samples were taken at various times and locations. Samples were aseptically collected from the floor drain, sight glass and outside air exhaust vent during (as the air was being exhausted to test ambient air) and after a sterilization cycle (except for the sight glass which can only be sampled post-cycle). For each of the samples, three nutrient agar plates received a 0.1 ml aliquot and were placed at 25^0 C. The plates were checked daily for an incubation period of two weeks. The data in Table 1 demonstrate that no bacteria were to be found in these samples (the limit of the assay is 10 bacteria/ml sample). To rule out the possibility that Rotoclave extracts inhibit bacterial growth, 10^3 *E. coli* K12 cells were added to a solution consisting of 1 ml of ***Rotoclave***® condensate and 9 ml of nutrient broth. These controls were turbid after an overnight incubation demonstrating that the ***Rotoclave***® extracts were not inhibitory to *E. coli*. I therefore concluded that neither the small numbers of airborne bacteria entering the system as the vacuum is relieved, nor those on the surfaces of the Red Bag Waste were sufficient to detectably contaminate the aqueous discharges from the ***Rotoclave***®.

To extend the above analysis to the solid discharge, solid samples of about 15 cm in diameter were aseptically collected from three different locations within the vessel. These samples were about as large as were to be found in this particular cycle. All of the samples were moist, roughly

spherical and loosely compacted, and contained a variety of substrates including paper pulp, cotton fibers and bits of plastic. Six one gram samples of the solid waste derived from each of the portions, and containing a random mix of materials, were inoculated into 25 ml of nutrient broth, placed at 25^0 C with gentle shaking, and checked daily for a period of two weeks. For each sample, three of the six inocula (#'s 4-6) were dissected from the core of the spherical mass to determine the efficiency of sterilization of the core of these spherical masses. None of the 18 inocula showed growth after extended incubation (Table 2) indicating that the materials subjected to a ***Rotoclave***® processing cycle were sterile, including those buried in a mass. Solid samples obtained from the conveyor belt at a distance of about one meter from the opening of the ***Rotoclave***® had a small number of gram positive bacilli (~100 bacteria/gram of sample) which were not further identified.

Sterilization of dense cell/spore populations in the *Rotoclave*®. Obtaining a quantitative measure of the ***Rotoclave***®*'s* sterilization capacity requires the introduction of cultures of known cell/spore density. For this purpose, high density cultures of various bacterial species were generated as described in Materials and Methods. This approach was chosen since the number of viable endospores on the commercially obtainable spore strips is not reliably known, nor is such a determination part of the assay, making the use of such strips qualitative rather than quantitative. The results of such an analysis are shown in Tables 3 and 4. The assay consisted of introducing samples into the ***Rotoclave***® in sealed glass vials which had been wrapped with cotton and placed in a perforated stainless steel cylinder fitted with a screw cap. The cylinder served both to protect the vials from breakage, and to approximate a "worst case scenario", i.e., to approximate a condition in which the samples are separated from the steam by the equivalent of wadded up hospital linens. In many cases, the cylinder was found in a tangle of linen sheets that frequently are found in the waste generated by the facility at which the testing took place. The strains were chosen as representing various classes of pathogenic and opportunistic species. Many of these strains have been chosen by state agencies for initial and periodic efficacy tests required of alternative technologies (e.g., the Illinois Environmental Protection Agency). Trial 1 differed slightly from Trials 2 and 3. In the first instance, 5 ml samples were introduced into standard 7.5 ml borosilicate screw cap test tubes. These tubes proved to be unsatisfactory since the seal effected by the screwcap was often times not sufficient to prevent the contents being lost during the vacuum stages of a ***Rotoclave***® cycle. In trials 2 and 3, 1.5 ml samples were sealed in 5.0 ml thick-walled borosilicate chromatography tubes with an open top screw cap closure and a PTFE-faced silicone septum.

The results in Tables 3 and 4 demonstrate that the ***Rotoclave***® achieves complete sterilization of dense populations of all of the strains tested, most notably *Pseudomonas aeruginosa* and *Candida albicans* at a cell densities of equal to or greater than 10^{10} cells/ml (10 $\log_{10}$) and *Bacillus stearothermophilus* and *B. subtilis* endospores (1.9×10^8, or 8.28 $\log_{10}$; and 1.23×10^9, or 9.09 $\log_{10}$, respectively). I thus concluded that Tempico's ***Rotoclave***® achieved full sterilization of its contents.

The *Rotoclave*® aqueous outflows are non-carcinogenic. I next wished to determine the mutagenic potential, if any, of ***Rotoclave***® extracts by subjecting them to the Ames test (Maron and Ames, 1983). It is not known if the ***Rotoclave*** process is sufficiently harsh to detoxify all carcinogenic compounds found in a medical setting. This determination was not the objective of this

study, however, since such compounds are pre-sorted from the waste that the ***Rotoclave*** receives. Instead, I wished to rule out the possibility that the high pressure and temperature steam liberates or creates hazardous compounds from the plastics or other components of the PIMW steam.

The Ames assay for carcinogenicity relies on the ability of a particular compound to cause mutations in the bacterial chromosome of *his*$^-$ biosynthetic genetic locus of strains of *Salmonella typhimurium,* which are unable to grow on media lacking the amino acid histidine. If the compound causes mutations at a sufficiently high rate, some of these mutations will by chance cause the *his*$^-$ cells to revert (mutate) back to *his*$^+$ and thus confer on the reverted bacteria the ability to grow on media lacking histidine. A high reversion rate indicates that the compound is highly mutagenic. The underlying assumption of the Ames assay is that a mutagenic substance is also carcinogenic. Reversion (mutagenicity) is measured as the number of colonies that appear on solid growth medium lacking histidine; and the number of revertants/plate is a quantitative measure of the mutagenic capacity of a substance. A negative control, i.e., cells not exposed to the compound, is included since there is a small strain-dependent reversion rate. A number of organic compounds are not carcinogenic until acted upon by liver-specific enzymes not possessed by bacteria, but which are found in humans. These compounds, while carcinogenic in the body, give negative results in an unmodified Ames assay. To provide a mechanism for detecting these compounds, a rat liver extract, called S9 mix, was added in some experiments along with the ***Rotoclave***® extracts. The S9 mix has been shown to activate a number of mutagens that would otherwise give negative results in the Ames test. A number of *Salmonella typhimurium* strains, each of which detects a different class of mutagens have been developed. I chose to use the standard strains TA98 and TA100 which between them are capable of detecting most mutagens (Maron and Ames, 1983, Vennit *et al*., 1984). The two strains differ in the precise nature of their *his*$^-$ mutation, thus making them responsive to a different spectrum of potential carcinogens.

The samples subjected to Ames tests were the same as those used to generate the data in Table 1. It was important that the samples be sterile, otherwise contaminant colonies would lead to an overestimation of the number of revertants induced by the samples since the contaminants will most likely form colonies in the absence of histidine. The results of the Ames tests are shown in Fig. 2. The negative control, i.e. TA98 cells alone, resulted in an average of 49 revertants/plate as can be seen from the zero extract control (Fig. 2A). This value was reproducible to within about 10% for a number of experiments (not shown) and is characteristic of this strain (Ames and Maron, 1983). The positive control daunomycin (100 μg/ml) elicited in TA98 a rapid increase in the number of revertants that was linear with the daunomycin dose over the range 1-10 μg. This extent of this increase is not obvious from Fig. 2 since I chose a Y-axis that would tend to exaggerate any potential mutagenic activity in the ***Rotoclave*** samples. As can be seen from the data in Fig. 2, ***Rotoclave***® samples did not stimulate an increase in the number of revertants of TA98.

A similar result was obtained when the strain TA98 is used in the presence of the S9 mix (Fig. 2B). The negative control, cells +S9 mix but without ***Rotoclave***® extract, resulted in the generation of 50 revertants/plate. A similar number of revertants resulted when a 100 μl volume of 2-aminofluorene (60 μg/ml) was added to the cells. A much different result was obtained when the same amount of 2-aminofluorene was incubated with cells in the presence of S9 mix. There was a

rapid and linear increase in revertants over the dose range of 0.6-6 μg. The large number of revertants produced by this latter treatment verified the activity of the S9 mix and established the positive control. Incubation of TA98 with ***Rotoclave***® samples in the presence of S9 gave a result virtually identical to that for the negative control, i.e., no evidence for mutagenic potential over that of the untreated control.

Strain TA100 was used in addition to TA98 since the two strains are reverted by different classes of mutagens (Maron and Ames, 1983). The zero dose negative control resulted in 148 revertants/plate establishing the background for this strain. Again, this number was reproducible within 10% for numerous experiments (not shown) and was similar to that reported for the stain. In the absence of S9 mix, sodium azide produced a large, linear increase in revertants over the dose range 0.65-6.5 μg (Fig. 2C). As for TA98, none of the ***Rotoclave***® samples produced an increase in the number of revertants of TA100 in the absence of S9 mix.

2-aminofluorene did not stimulate an increase in the number of TA100 revertants from the baseline of 150 over a dose range of 0.6-6 μg (Fig. 2D). A rapid rise in revertants was seen, however, when the incubation included the S9 mix. As before, none of the ***Rotoclave***® samples showed an increase in production of revertants for any of the volumes tested even in the presence of the S9 mix. The combined negative results given by both strain TA98 and TA100 allowed me to conclude that the ***Rotoclave***® process did not create carcinogens from PIMW.

The amount of background bacterial growth in the so-called "lawns" of TA98 and TA100 was identical in all cases, including those containing ***Rotoclave*** extracts, where a known carcinogen was lacking. The lawns produced in the presence of carcinogen were less extensive. This result is consistent with the postulate that ***Rotoclave***® extracts are not particularly toxic to *S. typhimurium*. Although the foregoing is not to be considered as conclusive evidence that ***Rotoclave*** extracts are entirely devoid of toxicity, it indicates that these extracts are suitable for disposal in the sanitary sewer. Similar results were obtained using *E. coli* and *Saccharomyces cerevisiae* as the test organisms, i.e., an identical number of colonies were formed in the presence or absence of ***Rotoclave***® extracts (not shown).

DISCUSSION

Treatment of Regulated Medical Waste (RMW, sometimes known as Red Bag Waste) presents a significant problem. Until recently, incineration has been the method of choice for treatment of Red Bag Waste. However, incineration produces toxic compounds from the incomplete combustion of plastics and other materials. Further, heavy metal contaminants are liberated from solid-phase confinement as the materials are combusted and are deposited in the ash. The bulk of the residue therefore ends up as a highly toxic residue that cannot safely be accepted at landfills. In addition, a significant amount of the ash and toxic by-products resulting from incomplete combustion ends up polluting our air. A possible alternative to incineration is steam sterilization. Two major devices for this purpose are the stationary autoclave and the ***Rotoclave***®. The conditions within these two devices are similar. However, the ***Rotoclave***® has a rotating drum with strategically placed paddles that serve to break open red bags, spilling their contents into the vessel. The contents are agitated and mixed so that every surface is exposed to the sterilizing steam resulting in the entire content of the vessel being sterilized. Two reports indicate that stationary autoclaves cannot make the same guarantee (Lauer, *et al*., 1982; Rutala, *et al*., 1982). These workers report that Red Bag contents are

not rendered sterile unless the bags are opened and placed, with additional water, in stainless steel vessels. If the waste to be treated contains pathogens, it is obviously not wise for personnel to open them before treatment. In this report, I wished to determine the sterility and carcinogenic potential of ***Rotoclave***® outflows.

Rotoclave discharges are sterile. The results in Tables 1 and 2 demonstrate a lack of detectable organisms in the solid, floor drain, sight glass and outside air exhaust vent output. This result was expected since the ***Rotoclave***® maintains a temperature of 135^0 C at 45 psi for 30 min, conditions much harsher than the standard autoclave conditions of 121° C at 15 psi. It was conceivable that the air exhaust emitted as the vessel was evacuated prior to steam injection contained a small number of organisms, particularly if there were a high concentration of organisms on the untreated waste. This possibility was made less likely, however, by the fact that the vacuum is created by a steam venturi, through which the exhausted air is passed as it is being expelled. Prior to atmospheric release, the exhaust is passed through a carbon filter which again should have the effect of eliminating bacterial release to the atmosphere. The results in Table 1 confirmed that the ***Rotoclave***® air exhaust did not contain microorganisms detectable by this assay, both prior to initiation of a cycle and after a cycle was completed.

The *Rotoclave*® exceeds regulatory standards for sterility. The results shown in Tables 3 and 4 demonstrate that the ***Rotoclave***® can achieve up to 10 $\log_{10}$ reduction in *Pseudomonas aeruginosa* and *Candida albicans* vegetative cells and greater than an 8 $\log_{10}$ reduction in *Bacillus stearothermophilus* endospores. These values are several orders of magnitude greater than those required by any regulatory agency in the United States (usually 6 $\log_{10}$ for vegetative cells and 3 or 4 $\log_{10}$ for endospores), or indeed the world community as a whole. The results reported here should be taken as lower, rather than upper limits. For practical reasons, cultures of densities greater than the range 10^8-10^{10} were not generated for this particular study. It must be stressed that the results of commercially available spore strips, unlike those reported here, are qualitative rather than quantitative. The number of viable endospores on a commercially available strip is not known with any accuracy, nor is it determined as part of the test. One assumes the value reported by the supplier to be accurate without proof and then notes the presence or absence of growth vs an untreated control. While the control demonstrates the presence of viable organisms, it in no way address their quantity. However, the density of the cultures here introduced into the ***Rotoclave***® was determined as part of the test.

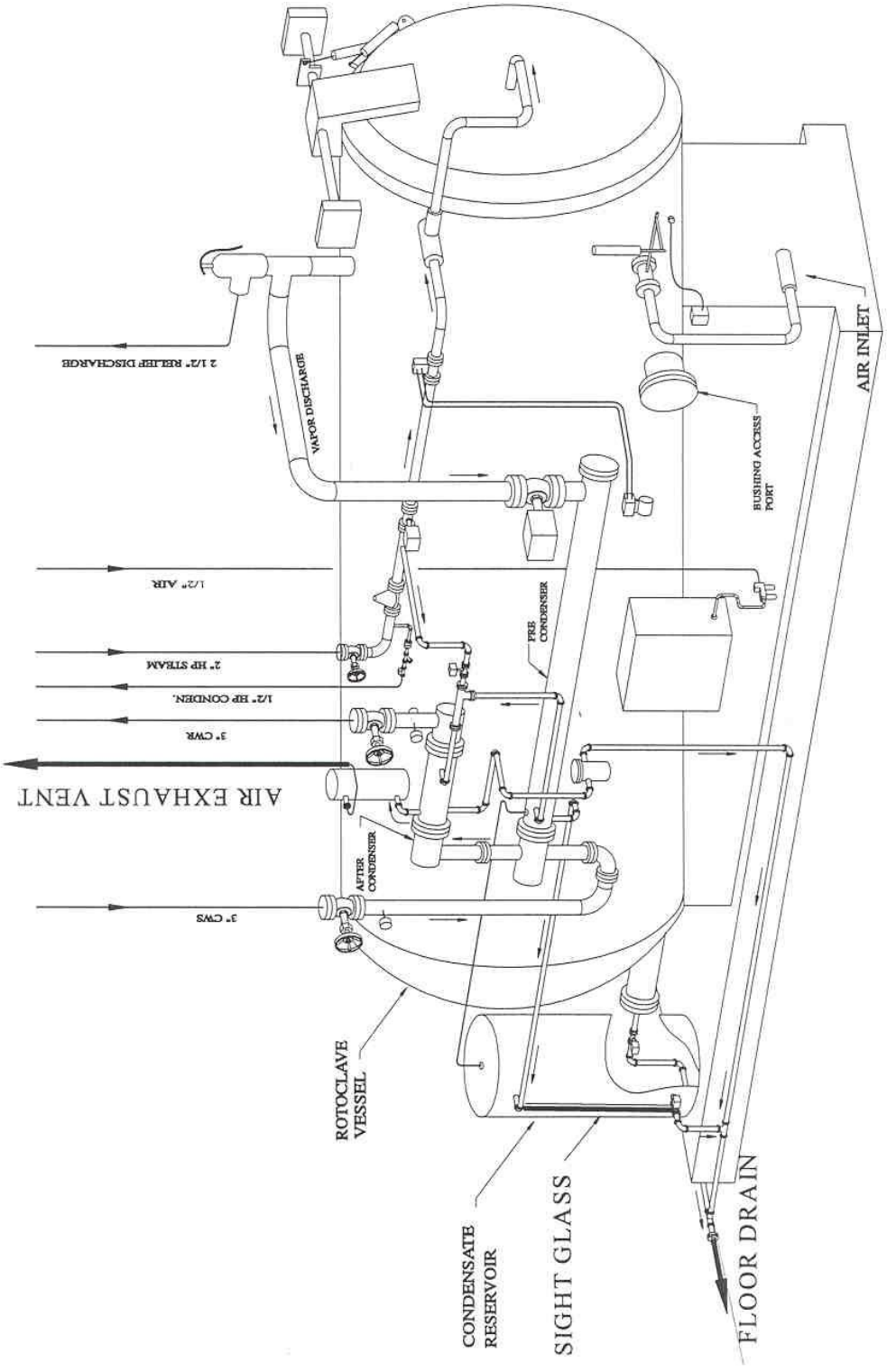
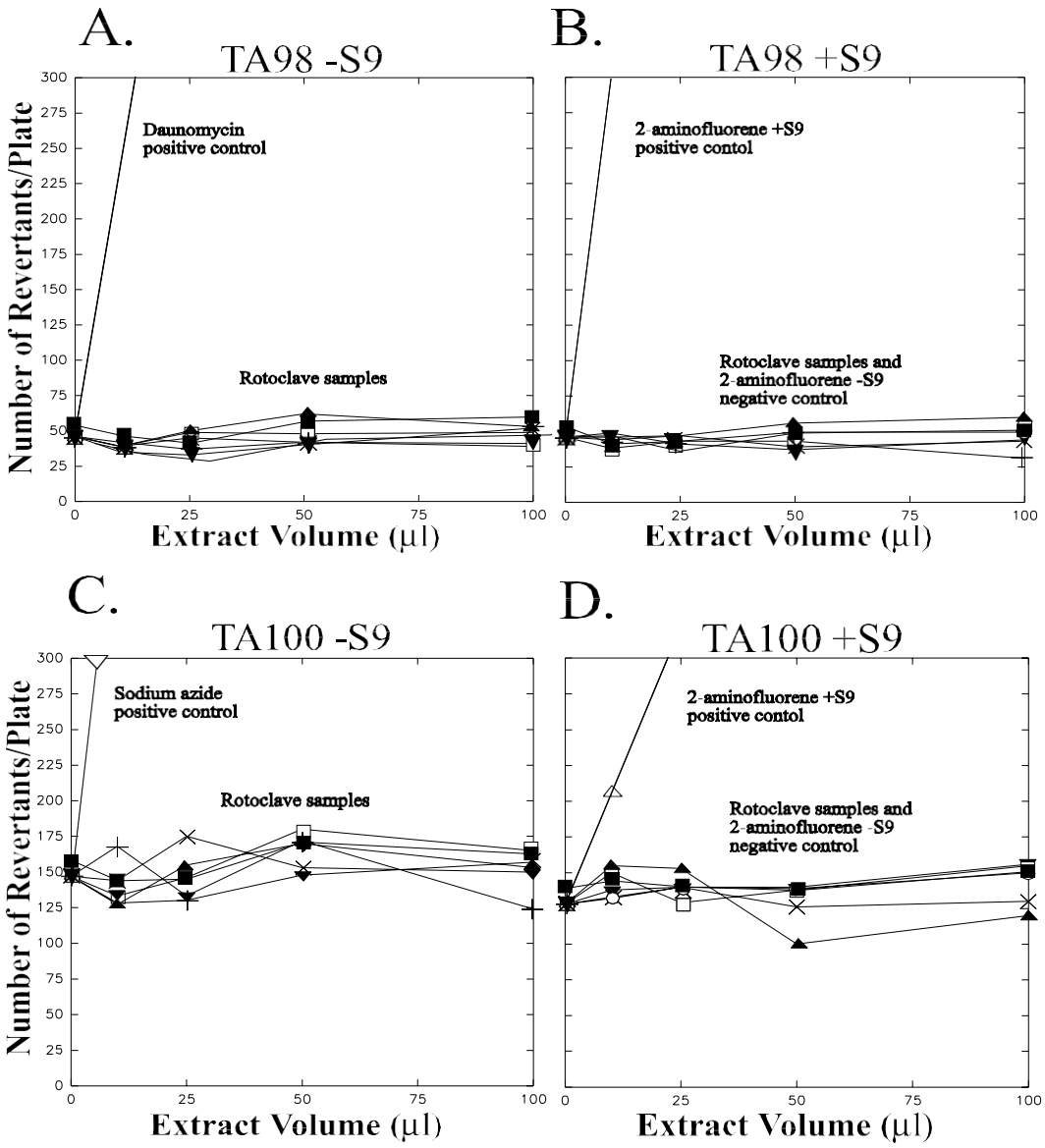


Figure 1. Sketch of a ***Rotoclave®*** showing sample sites.

Figure 2. Results of Ames tests without and with S9 using ***Rotoclave***® extracts.



It is generally assumed that conditions that kill thermostable endospores exceed, by at least a factor of 100, the conditions required to kill normally growing cells. However, to my knowledge, no quantitation of this commonly held assumption exists, and there are exceptions. For example, treatment of *Bacillus subtilis* endospores (a common test organism for chemical treatments of PIMW) with 1 M NaOH will most certainly result in a 4 $\log_{10}$ reduction in viability of the endospores, yet precisely these conditions are used to select *Mycobacterium tuberculosis* from a patient's sputum (although these same *Mycobacteria* are killed by exposure to 72^0 for 15 sec, i.e., pasteurization). Thus, given the chemical resistance of the tuberculosis-causing *M. tuberculosis*, one could arrive at the dangerous conclusion, by choosing inappropriate test organisms, that chemical processes rid PIMW of all pathogens. The fact, however, that the ***Rotoclave***® is capable of at least an 8.5 $\log_{10}$ reduction of *Mycobacterium spp.* vegetative cells, a 9 $\log_{10}$ reduction of *Bacillus subtilis* endospores and 8 $\log_{10}$ reduction of *B. stearothermophilus* endospores (as well as other organisms) led me to conclude that the ***Rotoclave***® vessel contents are rendered uniformly sterile.

The Rotoclave does not produce carcinogenic emissions. In addition to rendering RMW sterile, it's equally important that the technology itself be non-polluting. For this reason, I subjected the aqueous outflow of a ***Rotoclave***® to Ames tests, with and without S9 extract. The rationale of this experiment was not to demonstrate the ability of the ***Rotoclave***® to inactivate carcinogens, but to determine if the process in some manner created or liberated carcinogens from the waste. It is conceivable, for example, that some of the plastics in the waste stream contain dissolved solvents used in their manufacture that are carcinogenic, and that are released when the material is exposed to steam under pressure. Keep in mind that the combustion of plastics produces many toxic compounds. For the sake of thoroughness, I chose to test all of the ***Rotoclave***® portals of exit since they might be expected to have slightly different compositions. The floor drain accepts condensate produced both as the steam contacts the shell during the heat-up phase, as well as water produced from condensation of the sterilizing steam post cycle. The outside vent is the route by which air is exhausted from the vessel in creating a vacuum. This vent produces a condensate that drips out of the pipe at a rate of less than 100 ml/hr. None of the liquids, nor the water soluble extract from solid waste, showed detectable mutagenic potential (Fig. 2) demonstrating that The ***Rotoclave***® neither created nor liberated carcinogenic substances from components of the RMW stream. This is in stark contrast to the emissions emanating from the stack of an incinerator.

The validity of the Ames test has been demonstrated in several studies. Nearly 90% of the carcinogens tested in number of studies proved to be mutagenic in the Ames assay (for example see, McCann, *et al*., 1975; McCann and Ames, 1976; McCann and Ames, 1977; Purchase, *et al*., 1976). The correlation of mutagenicity to carcinogenicity has been estimated to be about 83% (Ames and McCann, 1981). The positive controls establish the sensitivity of the assay. Small amounts of these mutagens: 1.0 μg of daunomycin, 0.6 μg of 2-aminofluorene or 0.65 μg of sodium azide are all sufficient quantities to induce a dramatic rise in the number of revertants of either TA98 or TA100. the results of the Ames tests of ***Rotoclave***® extracts provides a strong evidence for the absence of carcinogenicity of ***Rotoclave***® outflows.

The top agar in which the bacteria are plated contains a growth-limiting amount of histidine, hence all of the cells grow for a short period following plating. This condition is necessary for a mutagenic compound to exert its genotoxic effect giving rise to revertants. A side consequence is that there is a background of growth which can be used as a rough measure of cellular toxicity. For all of the plates examined, there was no difference in the density of the lawn between cells exposed to water and those exposed to ***Rotoclave***® samples. This result corresponds to an earlier experiment that showed that the growth of *E. coli* cells was not inhibited by the addition of ***Rotoclave***® outflow. I am therefore confident that the material coming out of a ***Rotoclave***® is relatively non-toxic. More sophisticated analyses are planned to extend this admittedly limited result.

From the data I have obtained, I am confident that the ***Rotoclave***® achieves complete and reliable sterilization and does not release toxic or carcinogenic material into the environment, making it an ideal technology for the treatment of RMW.

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Rotoclave extracts were collected as described in Materials and Methods and volumes of 0, 10, 25, 50 and 100 µl subjected to Ames testing using the strains TA98 and TA100 without and with S9 mix. Positive and negative controls are included. Panel A: TA98 without S9, daunomycin (100 µg/ml) was used as the positive control for this experiment; Panel B: TA98 with S9 mix, 2-aminofluorene (60 µg/ml) was used as the positive control. This compound gives a positive Ames test only in the presence of the S9 mix and thus also serves as a control on S9 activity. Panel C: TA100 without S9, sodium azide (65 µg/ml) was used as the positive control. Panel D: as panel B except using strain TA100. The extracts are labeled as follows:

Aqueous extract from solid sample (■)
Floor drain, during processing (⊗)
Floor drain, after processing (∇)
Sight glass (+)
Outside air exhaust vent, after processing (X)
Outside air exhaust vent, during processing (☜)
sodium azide (∇)
2-aminofluorene without S9 (0)
2-aminofluorene with S9 (⊗)

The reported number of revertants/plate is the average of duplicate plates. The tests were repeated a minimum of three times on samples collected at different times and from different instruments.

Table 1. Result of sterility testing of ***Rotoclave***® aqueous samples.

Sample source	Bacteria/ml of extract
Floor drain, during a cycle	<10
Floor drain, after a cycle	<10
Sight glass	<10
Air exhaust vent, during a cycle	<10
Air exhaust vent, after a cycle	<10

Aqueous samples were collected as described in Materials and Methods and 0.1 ml aliquots were spread on nutrient agar plates. Colony formation was checked every day for 14 days.

Table 2. Result of sterility testing of a portion of the ***Rotoclave***® solid output.

Sample One Tube Number		Sample Two Tube Number		Sample Three Tube Number	
1	No growth	1	No growth	1	No growth
2	No growth	2	No growth	2	No growth
3	No growth	3	No growth	3	No growth
4	No growth	4	No growth	4	No growth
5	No growth	5	No growth	5	No growth
6	No growth	6	No growth	6	No growth

Solid samples were collected as described in Materials and Methods and 1 gram samples were inoculated into 25 ml of nutrient broth. Growth was monitored by checking for an increase in broth turbidity every day for 14 days. The samples consisted of a mixture of paper pulp, cotton fibers, small bits of plastic and unrecognizable material. A Kilit® vial (BBL) containing 3×10^4 *Bacillus stearothermophilus* endospores was included in this cycle, the contents of which were also rendered sterile.

Table 3. Quantitative Evaluation of the ***Rotoclave***® sterilization potential, Trial Number 1.

Culture	Cell/Spore Density (cells/ml)	Colony Formation	Growth in Broth	Log_{10} Kill
Bacillus subtilis				
cells	1.23×10^9	None Detected	None Detected	9.09
endospores	9.20×10^8	None Detected	None Detected	8.96
Bacillus stearothermophilus				
endospores	5.90×10^7	None Detected	None Detected	7.77
Candida albicans	3.02×10^9	None Detected	None Detected	9.48
Mycobacterium phlei	3.01×10^8	None Detected	None Detected	8.48
Mycobacterium smegmatis	4.16×10^8	None Detected	None Detected	8.62
Pseudomonas aeruginosa	1.01×10^{10}	None Detected	None Detected	10.0
Staphylococcus aureus	1.50×10^8	None Detected	None Detected	8.18

A 15 ml culture at the density listed for each microbial species was prepared as described in Materials and Methods and a five ml aliquot was introduced into the ***Rotoclave***®. After completion of the cycle, the cultures were retrieved, and a one ml aliquot was inoculated into 9 ml of nutrient broth (YPD broth for *Candida albicans*). Three 0.1 ml aliquots were also plated on individual nutrient agar plates (YPD agar plates for *Candida albicans*). The untreated aliquot was titred at the same time to determine cell density. Bacterial growth was monitored by an increase in turbidity of a broth culture, or the appearance of colonies on plates. Log_{10} Kill is defined as Log_{10} Introduced - Log_{10} Recovered where Log_{10} Recovered is set to 0 ($Log_{10}1=0$ in recognition of the assay limit of 1 cell/ml) when no cells are recovered.

Table 4. Quantitative Evaluation of the ***Rotoclave***® sterilization potential, Trial Numbers 2 and 3.

Culture	Cell/ Spore Density (cells/ml)		Colony Formation		Growth in Broth		Log^{10} Kill	
	Trial 2	Trial 3	Trial 2	Trial 3	Trial 2	Trial 3	Trial 2	Trial 3
B. stearothermophilus								
cells	1.0×10^7	5.0×10^6	ND*	ND	ND	ND	7.00	6.70
endospores	1.9×10^8	9.5×10^7	ND	ND	ND	ND	8.28	7.98
B. subtilis								
cells	6.2×10^7	2.4×10^7	ND	ND	ND	ND	7.79	7.38
endospores	3.0×10^9	1.2×10^9	ND	ND	ND	ND	9.49	9.07
P. aeuginosa	3.2×10^9	3.3×10^9	ND	ND	ND	ND	9.51	9.52
S. aureus	3.3×10^9	3.1×10^9	ND	ND	ND	ND	9.52	9.49
M. smegmatis	4.1×10^9	4.3×10^8	ND	ND	ND	ND	9.61	8.63
C. albicans	3.0×10^{10}	2.8×10^9	ND	ND	ND	ND	10.5	9.45

A 10 ml culture at the density listed for each microbial species was prepared as described in Materials and Methods and two 1.5 ml aliquots were introduced into the ***Rotoclave***®. After completion of the cycle, the cultures were retrieved, and a one ml aliquot was inoculated into 9 ml of nutrient broth (YPD broth for *Candida albicans*). Three 0.1 ml aliquots were also plated on individual nutrient agar plates (YPD agar plates for *Candida albicans*). The untreated aliquot was titred at the same time to determine cell density. Microbial growth was monitored by an increase in turbidity of a broth culture, or the appearance of colonies on plates. Log_{10}Kill is as defined in Table 2.

*ND = none detected.