Soil sterilization effects on in situ indigenous microbial cells in soil\(^1,2\)

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Soil was sterilized by various procedures, and then the resident microorganisms were physically separated and concentrated from the soil for viewing by transmission electron microscopy as thin sections and frozen-etched preparations. Remaining cell viability in the soil was tested by conventional plating before and after enrichment culture. The soil proved to be sterile after treatment with \(^{60}\)Co radiation, prolonged autoclaving, prolonged dry heat application at 200°C, or glutaraldehyde (if followed by subsequent mild heating), and could be considered sterile after OsO\(_4\) treatment. Treatment with glutaraldehyde alone, or 160°C dry heat for 3 h, did not sterilize the soil. Cellular fine structure was altered or destroyed by the heat treatments, but was not affected to any extent by any of the other treatments including glutaraldehyde followed by mild heating. These findings are considered in relation to the residual biological information observable by electron microscopy in soil samples which have been sterilized to eliminate possible pathogens before handling of the soil. These findings are also considered with the objective of obliterating the fine structure of the indigenous microorganisms during soil sterilization so that electron microscopy studies can be made of microorganisms inoculated into and grown in the presterilized soil.


Nous avons stérilisé le sol par diverses procédures et les microorganismes résidants furent physiquement séparés et concentrés à partir du sol pour leur examen microscopique par transmission électronique en couches minces et sur des préparations brisées au froid. La viabilité résiduelle des cellules dans le sol fut testée par des cultures conventionnelles sur plaques avant et après enrichissement. Le sol est stérile après un traitement de radiation au \(^{60}\)Co, un autoclavage prolongé, une application prolongée de chaleur sèche à 200°C, ou le glutaraldehyde (s'il est suit par un chauffage subseqüent moyen), et il peut être considéré stérile après un traitement au OsO\(_4\). Le traitement avec le glutaraldehyde seul ou une chaleur de 160°C pour 3 h, ne stérilise pas le sol. La structure cellulaire fine est altérée ou détruite par le traitement à la chaleur mais n'est pas affectée d'aucune façon par aucun des autres traitements incluant le glutaraldehyde suivi d'un chauffage moyen. Ces résultats furent étudiés en relation de l'information biologique résiduelle telle que vue par microscopie électronique dans des échantillons de sol qui ont été préalablement stérilisés pour éliminer les pathogènes possibles avant la manipulation du sol. Ces données sont aussi considérées en regard de l'objectif d'étude de la structure fine des microorganismes indigènes au cours de la stérilisation du sol de telle sorte que les études au microscope électronique peuvent être faites sur des microorganismes inoculés dans le sol ou qui se sont développés dans un sol pré-stérilisé.

[Traduit par le journal]

**Introduction**

Pure cultures of microorganisms can be inoculated into sterilized sand (21) or soil (13, 15) for maintenance of stock cultures, or one or more cultures can be added to sterile soil for microbial ecology studies (6, 12, 16, 19). In the latter instance, the soil, after inoculation and incubation, can be plated for total or differential counts (16) observed by various forms of light (8), ultraviolet fluorescence (5, 8–11, 20, 27), infrared (7), or electron (1–4, 14) microscopy, and can be evaluated for respiration capability (12, 17, 24–26), tested for \(^{14}\)CO\(_2\) uptake (23), and so forth. The various forms of light microscopy, however, and, in some cases, electron microscopy, are plagued by the fact that dead cells resulting from the soil sterilization are still present in the soil and, morphologically and structurally, they usually cannot easily be differentiated from the cells added after sterilization. Although acridine orange – ultraviolet fluorescence microscopy

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would seem to accomplish this by detecting living cells in the presence of dead cells, it has not proven to be reliable (8).

From a different viewpoint, at times it might be of interest to evaluate numbers, types, and cellular structures for microorganisms in a soil that, because of its possible content of virulent pathogenic microorganisms, might be more safely handled if it were sterilized before observations were initiated. An example of this would be a soil from an extraterrestrial source. Sterilization of the soil would virtually eliminate most methods of evaluating its microbial content, but evaluation of the above microbial parameters by electron microscopy might still be possible.

The present study was undertaken with both of these viewpoints in mind. Thus, soil sterilized in various ways was evaluated by transmission electron microscopy viewing of thin-sectioned preparations and of replicas of frozen-etched preparations (4) to determine the types of sterilization which would either destroy or, alternatively, leave unaltered the cellular structure of the indigenous microorganisms in the soil.

**Materials and Methods**

### Soil

The soil used for this study was a pH 6.0 Hagerstown silty clay loam. Its organic content was about 3.5% and moisture content 25%. It had been stored in bulk in a polyethylene bag for about 1 year.

### Cell Viability

Viable cells surviving the various soil-sterilization treatments were determined by preparing spread plates of soil dilutions on medium PYE: 0.1% peptone, 0.1% yeast extract, 1.5% agar, pH 7.0; and medium HIGYE: 0.2% heart infusion broth powder, 0.1% glucose, 0.1% yeast extract, 1.5% agar, pH 7.0. All media components were Difco products (Difco Laboratories, Detroit, Michigan). The lowest soil dilution plated was 1:20, and the plates were incubated 1 week at 30°C. To detect very low numbers of surviving cells, 10 g of treated soil were added to 100 ml of PYE medium (as a broth), and this was incubated stationary for 1 week at 30°C. In certain instances, however, the amount of treated soil available was not sufficient for this, and a proportional reduction was made in the volume of medium used. After incubation, the enrichment was plated on both media and incubated for 1 week at 30°C. For all trials, both with and without enrichment, the two plating media yielded similar numbers of colonies and, therefore, the respective counts from the two media were averaged together.

Bacteriophages naturally present in this soil for *Arthr. globiformis* American Type Culture Collection (ATCC) 8010 were detected by enrichment followed by plaquing. Four grams of treated or control soil was added to 100 ml of sterile broth consisting of 0.3% beef extract and 0.5% glucose. Cells (1 x 10^8) from a 1-day shake culture of *A. globiformis* in similar medium were also added, and the flasks were shaken 5 days at 27°C. Portion of these enrichments were then centrifuged and filtered (0.30-um membrane filters, Millipore Corporation, Bedford, Massachusetts). The filtrates were plated with strain 8010 on similar agar medium, and incubated 3 and 8 days, respectively, at 27°C for non-treated and treated soils. This procedure for non-treated soils yields about 10^8 to 10^9 plaque-forming units of mixed bacteriophage per milliliter.

### Soil Sterilization

Duplicate samples of soil were subjected to each sterilization treatment. One was used for testing of cell viability survival, and the other for analysis of ultrastructure survival of the cells residing in the soil. For dry-heat sterilization, 50-g portions of soil in 150-mm diameter glass Petri plates were placed, with lids partially offset, in a forced-air dry-heat oven (model 625, American Sterilizer Co., Erie, Pennsylvania). One pair of samples was subjected to 160°C for 3 h, and another set to 200°C for 24 h. For autoclave sterilization, two 50-g portions of soil were placed in 250-ml screw cap polycarbonate centrifuge bottles. Consecutive autoclavings at 121°C were applied to give 2 h of heating on days 1 and 2, and 17 h on day 4. The samples were incubated at room temperature between autoclavings.

Duplicate 25-g portions of soil were used for soil sterilization with OsO4. Four 50-g portions were required for the glutaraldehyde testing. The soil samples were placed in 230-ml screw cap polycarbonate centrifuge bottles, and the chemical sterilizing agents (fixatives) were added to provide one part soil (weight basis) to two parts fixative (volume basis). The fixatives were 1% OsO4 (Stevens Metallurgical Corp., New York, New York) in Kellenberger buffer (18), or 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pennsylvania) in 0.1 M phosphate buffer, pH 7.3. Incubations were stationary at room temperature for 17 h. One pair of the glutaraldehyde samples was subjected to an additional mild heat treatment at 87°C for 30 min in a water bath. After treatment, all of these samples were centrifuged 20 min in the cold at 23,100 x g, then washed four times by centrifugation with 50-ml portions of sterile Kellenberger buffer (for the OsO4-treated soil) or 100-ml portions of sterile phosphate buffer (for the glutaraldehyde-treated soil) to remove residual fixative.

Radiation sterilization was performed at the Pennsylvania State University Breazeale Nuclear Reactor Facility. Portions of soil (12.5 g) were placed in 16 x 125 mm glass screw cap tubes and subjected to 6 megarads from the 60Co source.

### Electron Microscopy

The procedures for release and concentration of the cells from the treated and control soils are from method B of Balkwill et al. (4). The technique for direct observation of the organisms in a "crude soil slurry" without release of the cells from the soil is also described in this paper, as are the procedures for transmission electron microscopy of thin-sectioned preparations and of replicas of frozen-etched preparations.
Results

Soil sterility was not achieved by dry heat at 160°C for 3 h (Table 1), and phase-contrast microscopic observation of the enrichment broth after 1 week of incubation revealed predominantly spore-forming rods. The heat, however, proved to be detrimental to the structural integrity of most of the in situ cells in the soil as observed in thin sections; compare Fig. 2 and non-heated control Fig. 1a. The cell walls were largely intact but, in general, there was considerable breaking of membranes and destruction of membrane structure. Dense blocks of cytoplasm (coagulated protein) were present, and the nucleoid region was poorly defined. These characteristics were previously reported (22) for autoclaved Bacillus cereus cultures. Replicas of frozen-etched preparations did not reveal entities which could with assurance be identified as microbial cells; a non-heated control is presented in Fig. 1b. Apparently, the heat had caused too much structural disorganization for cell detection by this method.

Sterility was attained by treatment of the soil with 200°C dry heat for 24 h (Table 1). The ultrastructure of the resident microorganisms was completely obliterated so that, except for an occasional partially destroyed endospore, they could not be detected by either means of electron microscopy viewing. Autoclaved soil proved to be sterile (Table 1). Thin sections of its resident microorganisms (Fig. 3) appeared similar to those resulting from 160°C dry heat for 3 h. However, the membrane damage did not appear as extensive. Again, microbial cells could not be detected with assurance in replicas of frozen-etched preparations.

The possibility was investigated that the destruction of cellular structure in these heated soils was not caused exclusively by heat, but that other contributing factors might have been operating during the cell release and concentration steps from the soil. This was done by preparing replicas of frozen-etched preparations of a crude soil slurry from untreated and heat-treated soils. Cells were easily distinguished in the untreated soil (4), but not in any of the heat-treated samples.

The incubation of soil with 1% OsO₄ was effective in obtaining sterility (Table 1), but there may have been a small amount of residual fixative in the soil after the washing steps. This was indicated by an observed blackening of the enrichment broth by the end of 1 week of incubation. Residual toxicity was also demonstrated by transferring 0.1-ml aliquots from a freshly prepared enrichment flask (for the OsO₄-treated soil) onto the surfaces of plates of PYE medium along with 0.1-ml aliquots of a 10⁻² dilution of untreated soil. After incubation, the colony counts were depressed about two and one-half orders of magnitude as compared with untreated controls.

As can be seen in Table 1, treatment of soil with 2.5% glutaraldehyde alone was not adequate to obtain sterility. The resident bacteria apparently had been killed, however, since the growth observed on all dilution plates was of fungal or streptomycete origin, and phase-contrast microscopic observations of the enrichment broth revealed only fungal mycelium and germinating streptomycete conidia. In contrast, application of a mild heat treatment (87°C for 30 min) after the incubation with glutaraldehyde was sufficient to render the glutaraldehyde-treated soil sterile (Table 1). The possibility that residual glutaraldehyde might be carried over from the washed soil onto the dilution plates or into the enrichment broth was tested by centrifuging out and discarding the cells and soil from the final soil suspension (after the washing had been completed). A portion of this supernatant fluid was then passed quickly through an ultrafine fritted-disc glass bacteriological filter, and 0.1-ml ali-

<table>
<thead>
<tr>
<th>Sterilization treatment</th>
<th>Plate counts per gram soil After sterilization</th>
<th>Plate counts per gram soil After sterilization plus enrichment</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>4.0 x 10⁶</td>
<td>4.3 x 10⁸</td>
</tr>
<tr>
<td>Dry heat 160°C</td>
<td>3.0 x 10⁶</td>
<td>2.2 x 10⁵</td>
</tr>
<tr>
<td>Dry heat 200°C</td>
<td>0</td>
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<tr>
<td>Autoclaving</td>
<td>0</td>
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<tr>
<td>OsO₄</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Glutaraldehyde</td>
<td>&gt; 2.0 x 10⁵</td>
<td>&gt; 2.0 x 10³</td>
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<tr>
<td>Glutaraldehyde followed by heat</td>
<td>0</td>
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<td>α⁴⁰Co radiation</td>
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*Average of combined counts for both media as described in Materials and Methods.

*Procedure and extent of treatment as described in Materials and Methods.

*Fungi and streptomycetes; bacteria were destroyed.
quot of the filtrate were added to the surfaces of plates of PYE agar medium along with dilutions of untreated soil. There was no decrease in numbers of colonies on these plates attributable to residual glutaraldehye, and the relative proportions of bacteria versus streptomycetes and fungi were unaltered.

Excellent preservation of microbial fine structure detail was observed during electron microscopy viewing of both thin sections and replicas of frozen-etched cells in soils treated with OsO_4, glutaraldehyde, and glutaraldehyde plus heat (Fig. 4). In fact the condition of these cells closely resembled that from untreated soil (Fig. 1), or soil which had been prefixed 1.5 h with OsO_4 (1, 2) followed by overnight OsO_4 post-fixation.

Gamma radiation at 6 megarads from a 60Co source sterilized the soil (Table 1). There was no apparent alteration of cellular structure for in situ microbes as determined from thin sections and replicas of frozen-etched preparations of the cells. In addition, enrichement for Arthrobacter globiformis bacteriophages naturally present in this soil showed that they had been destroyed.

Discussion

An additional medium, Difco heart infusion agar, was used for all of the platings in this study. The results were not reported, however, because the numbers of colonies on this medium were at least two orders of magnitude lower than with the other media.

For the soil used in this study, treatment with gamma radiation, or with glutaraldehyde followed by mild heat, sterilized the soil without a concomitant destruction of fine structure of the resident soil microorganisms. This probably also is true for OsO_4, but inability to remove the residual OsO_4 precludes a positive statement as to whether sterility has actually been achieved for the soil. Autoclave sterilization of soil, at least for most of its microorganisms, partially destroys cell structure, but enough remains that the cells usually can be detected in thin sections.

A lesser application of autoclaving heat, in our hands at least, has not been found to be reliable for achieving soil sterility, and the same can be said for the gamma radiation dosage level used. In contrast to the survival of some or all of the cellular structure with the above procedures of soil sterilization, the use of dry heat (200°C for 24 h) virtually obliterated cellular structure, and only a few "possible cells" could be detected in thin sections.

In these experiments, the use of thin sections for transmission electron microscopy provided a greater amount of fine-structure information on the cells which had been separated and concentrated from soil than did use of the replicas of the frozen-etched cells. In addition, freeze-etching could not detect cells which had suffered heat deterioration of their membranes and walls. This technique, however, might be advantageous for cell detection and study when autoclave-sterilized soils are to be inoculated. The residual dead cells will have suffered only a partial deterioration of fine structure as viewed in thin sections, in contrast to the total cellular destruction observed for replicas of frozen-etched preparations. Thus, in the latter procedure, there would be no residual cells to possibly confuse the cell detection.

Use of the freeze-etching procedure has two additional advantages. Obviously, it allows a determination of the overall shapes of the cells. But in addition, it can be used on crude slurries of soil in water in which no attempt has been made to separate and concentrate the cells from the gritty soil debris. This allows a determination of whether the separation and concentration procedures (required for thin-sectioning) in themselves are contributing to the apparent fine-structure deterioration with certain soil treatments, or whether the treatments might be interfering with release of the cells from the soil debris.

Our results have possible applications for two diametrically opposed types of experimental approaches. A major problem in the study of

Fig. 1. Examples of microbial cells from untreated (control) soil. Note cell walls (cw), cytoplasmic membrane (cm), and cytoplasm (cy). (a) Thin section. Bar marker represents 0.2 μm. (b) Replica of frozen-etched cell. Bar marker represents 0.3 μm; arrow indicates direction of shadowing. Fig. 2. Section of a microbial cell from soil subjected to dry heat at 160°C for 3 h. Note the condition of the wall (cw), lack of cytoplasmic membrane, and coagulated cytoplasm (cy). Bar marker represents 0.2 μm. Fig. 3. Section of a microbial cell from soil subjected to extensive autoclaving. Note the condition of the wall (cw), damage to membrane (cm), and coagulated cytoplasm (cy). Bar marker represents 0.3 μm.
soil microflora by microscopic methods is that cells added to soil cannot be easily distinguished from the cells which are already present. The acridine orange procedure of Strugger (27) cannot be relied upon (8), and many of the cells are too small for detection by this technique (1-3). A means of circumventing this problem would be to first sterilize the soil in a manner that would destroy the fine structure of the resident cells, or would alter their fine structure so that they could be recognized as having been resident before the sterilization. The sterile soil would be inoculated with one or more laboratory cultures or a small amount of unaltered soil. The growth and other activities of the added microorganisms could then be followed by electron microscopy, with the viewer being able to distinguish between the residual partially or totally degraded cells still in the habitat after sterilization and the added cells which would be establishing a new ecosystem. It would appear that sterilization of the soil by dry heat or autoclaving would accomplish this end.

From an opposing point of view, there may be instances in which known or presumed pathogenicity and virulence of microorganisms in the soil might raise a question as to safety in handling or examination of the soil. These microorganisms could be a normal component of the soil microflora which had assumed prominence because of the manner in which the soil had been incubated or handled, or they could be microorganisms which had been added to the soil. Another situation might be one in which it is not known whether the soil contains any microorganisms, or if present, whether they might be highly pathogenic and virulent. An example of this would be an extraterrestrial sample returned to earth from another planet. The presence of microorganisms and their cellular states in these soils could be determined with some degree of safety if the soil could be first sterilized without having a concomitant deterioration of cellular structure, then examined by electron microscopy. Sterilization by gamma radiation, and by glutaraldehyde followed by mild heat, would ap-
pear to meet these criteria. Autoclave sterilization might also be of value, although some degree of cellular deterioration would have occurred. Soil sterilization with 
OsO₄ would meet the criteria, because the soil is sterile in this context even though some residual 
OsO₄ might be present. The toxicity to man of this residual must be considered, however.

Cell detection in extraterrestrial soil samples could possibly be enhanced by combining the above two approaches. The microbes residing in such a soil might not be able to grow on laboratory media, or their numbers might be too low for detection by electron microscopy. It is still possible, however, that an extraterrestrial soil could be inoculated into terrestrial or extraterrestrial soil which had been sterilized so as to destroy the cellular structure of any resident microorganisms. This would then be incubated and observed with the electron microscope. The assumption is that even though the microorganisms may not be able to grow on laboratory media, they may be able to produce growth in sterile habitat material.

Acknowledgments

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