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Sterilize Methods Comparison for Soils: Cost, Time, and Efficiency

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ABSTRACT

Soil sterilization is generally used to eliminate or reduce microbial activity in studies involving microbial inoculations, soil enzymes, among others. Achieving an adequate sterility condition is not straightforward due to the variety of resistance structures that are generated in soil microbial ecosystems and the reservoirs that can form between soil aggregates. This is why finding an effective method to achieve good sterilization is important in methodological terms, so the present work aims to compare the effectiveness of three widely used methodologies to sterilize soil and to evaluate their cost/benefit in terms of time and inputs invested. Four treatments were tested: gamma irradiation, sterilization cycles at different times: three cycles of 1 h each and four cycles of 15 min each, and chloroform vapors. The evaluation and comparison of all samples sterilized by the different methodologies were based on the total aerobic heterotrophic bacterial count. The results of this study suggest that it is more efficient to use autoclaving methods because the process is more accessible in terms of equipment and methodologies, and the final results are the same. In the case of this work, sterilization with chloroform vapors had to be rejected. While the use of gamma radiation may be more efficient in terms of time, it can be a costly and inaccessible service for some laboratories that do not have the equipment. Therefore, the most viable options in terms of time, cost, and benefit are those using autoclaves. Among these, shorter treatment times mean a reduction in the cost of using the equipment, so the option of 15-minute cycles is desirable.

Keywords: Gamma irradiation, Autoclave, Soil bacteria.

1 Introduction

In research on the activity of microorganisms in soils and their influence on plant growth, it may be necessary to sterilize soils. Experiments with soil sterilization have been considered a suitable approach to understanding the recovery of microbial diversity [1].Sterilization is the process by which an object or substance is rendered free of all living organisms. It can be accomplished by exposing the material to physical or chemical lethal agents [2] The objective of sterilization is to destroy the microbial population without altering the physical and chemical properties of that soil. In all soil sterilization processes, effectiveness must be demonstrated in the absence of microbial growth [3]. Soil sterilization is generally used to eliminate or reduce microbial activity in studies involving microbial inoculations, soil enzymes, degradation/sorption/mobility of pesticides, and other xenobiotics [4]. There are numerous difficulties associated with soil sterilization, especially if large quantities need to be sterilized. Sterilization requires the destruction of resistant microbial cells and spores which may germinate days, weeks, or months after the sterilization process if conditions are conducive [5].

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In particular, spores, which can be produced by anaerobic, aerobic, or facultative bacteria, on the one hand, and fungi, on the other [6], are extremely resistant to high-stress levels; this resistance is origin in the structures that constitute them [7]. Spores exhibit great resistance to different agents such as drying, freezing, thawing, high temperatures wet or dry state, UV, and gamma radiation, high pressure, and a big number of toxic chemical products like oxidation agents, aldehydes, halogens, acids and bases. Spores are more resistant than growing cells of the same specie [8], [9]. Among the most commonly used methods are sterilization by autoclaving, gamma irradiation, and fumigation with volatile chemicals such as chloroform or methyl bromide [5], [10], [11].

Pressurized saturated wet steam as a sterilizing agent is one of the most widely used methods in laboratory practices [12]. Fumigation methods generate toxic residual effects, so autoclaving and irradiation methods are the safest and most effective. However, although irradiation greater than 20KGy generally eliminates actinomycetes, fungi, invertebrates and bacteria from many soils, the proportion of resistant bacteria may require doses greater than 70 kGy, raising costs [4]. Soil microorganisms are responsible for numerous and diverse biochemical activities. Elucidating indigenous microorganisms may be necessary to separate biological from chemical transformations in the case of some specific organic components; or to study the growth or metabolic activity of specific microorganisms inoculated in each soil [3]. Regarding autoclaved soil, it is known that there are influences on chemical properties, especially manganese, but there are also reports of influences on nitrogen, phosphorus, sulfur and organic matter [3], [11]. Autoclaving is recognized to increase or decrease pH and decrease cation exchange capacity and increase soluble organic carbon, increase electrical conductivity, increase available/exchangeable/extractable nutrients [4].

On the other hand, Gamma irradiation is effective and has been proposed as a selective sterilization method that can be administered in precise doses allowing the selective elimination of specific soil organisms [9]. Some authors tested that gamma irradiation produces effects on soil physical and chemical properties but is relatively less disruptive compared with other sterilization methods; effects on cation exchange capacity and pH are variable and do not show constant trends [9], [13], [14]. In this case, fungi are killed easier than bacteria, however, the treatment has shown an increase in manganese, NH_4-N and extractable organic N but in general does not influence other physical properties [3].

Fumigation with chemical compounds, in particular, chloroform, has been treated by an instantaneous bactericide for enterobacteria when is used in a liquid state and its vapor has been used for surface sterilization [15]. Chloroform, $CHCl_3$, is a colorless, clear, very volatile liquid with a characteristic odor; it boils at 61.2 ºC [16]. Despite chloroform vapors have been very used for sterilization, it is reported that it is not mutagen for bacteria such as *Salmonella typhimurium* or *Escherichia coli* but results are positive for mutagenicity in eukaryotic systems [17]. In all cases, is important to mention that partial soil sterilization accelerates the decomposition of organic material and increases the amount of carbon and nitrogen mineralized in the soil [18] so it is important to choose an effective protocol of sterilization according to the objective of each research.

The objective of the present work is to compare the efficiency of three widely used methodologies to sterilize soil and to evaluate their cost/benefit in terms of time and inputs invested to carry out the procedure.

2 Materials and Methods

A soil sample was taken from a field adjacent to an orchard in the district of Moreno, Province of Buenos Aires, Argentina. Some parameters such as pH and conductivity were measured on this sample, whose values were 5.72 and 0.080 mS/cm, respectively. In addition, from the ignition test, the percentage of organic matter in this soil was calculated to be 8.59 % and, on the other hand, the volume of water retained per gram of soil was estimated to be 0.88 mL. For the sterility tests, the samples were sieved on a 10- and 2000-micron sieve. The evaluation and comparison of all samples sterilized by the different

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methodologies were based on the total aerobic heterotrophic bacteria count. Once the samples were treated, 1 g of soil was weighed in a sterile test tube and suspended in 10 mL of physiological solution (g/L: NaCl 8.7). Serial 10-fold dilutions from 10-1 to 10-5 were made, 0.1 mL of each dilution was sown in duplicate on PCA-agar plates (g/L: Cassia peptone 5.0, Yeast extract 2.5, D(+)-glucose 1.0, Agar-agar 14.0) and incubated at 32 ºC for 48 Hs.

2.1 Experimental design

Twenty g of the sample were taken for each treatment and deposited in glass jars. Four flasks were selected and autoclaved at 200ºC and 1.5 atm of pressure for 15 minutes. This treatment was performed four times with a time between 24 and 36 hs of spacing. Each time the treatment was started, a sample was used for counting to estimate the minimum number of treatments to achieve the objective. In this way, samples were obtained with one, two, three and four treatments followed by 15 minutes. The same methodology was used with the counts for samples autoclaved for 1 hour up to 3 times. On the other hand, 1 g of soil was sterilized with ANEDRA chloroform vapors (AN 00654625) for 5 days. And, in parallel, 20 g of sieved soil was sent for irradiation with ^{60}Co source in glass containers at 2.5 Mrad (25 KGy) at a rate of 2Mrad/h.

3 Results

Table 1 shows a recount of colonies former units observed in each treatment for serial dilutions until 10^{-3}. In the case of Sterilization treatments with 15 minutes until four times, results show that the first and second cycle was not free of microorganisms. In the case of sterilization treatments with 1 hs until three times, results show good performance even for the first cycle, increasing the effectiveness of 15 min cyclins.

Table 1: *Recount of Colonies Former Units per gram of soil for each treatment CFU/g*

Treatment	Serial Dilutions- CFU/g					
	-1		-2		-3	
Irradiation	0	2	2	--	0	0
Irradiation	0	1	1	1	0	1
Irradiation	0	1	0	0	0	55
Sterilization 1 hs autoclave	0	2	0	2	0	1
Sterilization 2 hs autoclave	0	1	0	1	0	1
Sterilization 3 hs autoclave	0	0	0	0	0	0
First cycle-15 min autoclave	4	2	1	11	1	1
Second cycle-30 min autoclave	4	0	0	0	9	0
Third cycle-45 min autoclave	0	2	3	2	0	0
Fourth cycle-60 min autoclave	0	1	1	0	0	0
Chloroform vapors	>300	>300	82	69	>300	35

In the case of Chloroform vapors treatment, results reflect non-sterile soils (table 1), fungi hifes presence has been seen in the plates during the experiments. The counts in all cases have been above 30 CFU and, with the growth of hifes, it leads one to think that spores would survive and germinate in addition to the possibility of contamination during the process of manipulating samples. On the other hand, irradiated samples have presented low colonies former units including in plates with concentrated suspension. As can see in Fig 1, the third cycle of 1 hour in autoclave treatment presents better results even considering irradiation treatment. It is interesting to observe the lineal decrease between cycles of autoclave 1-hour treatment. On the other hand, figure 1 shows that in cycling treatments a lineal decrease in the counting of CFU beginning with a recount for the first cycle of 1 h below in comparison to the first cycle

of 15 min treatments. However, the cycle of 60 minutes (15 min. treatment) is equated with the 2-h accumulated cycle (2 cycles of 1 hour) so a possible explanation could be that the cumulative effect of 15 minutes is more efficient than 1h. It is observed clearly in figure 1 seeing that the accumulated 40` cycle equals the 1h cycle.

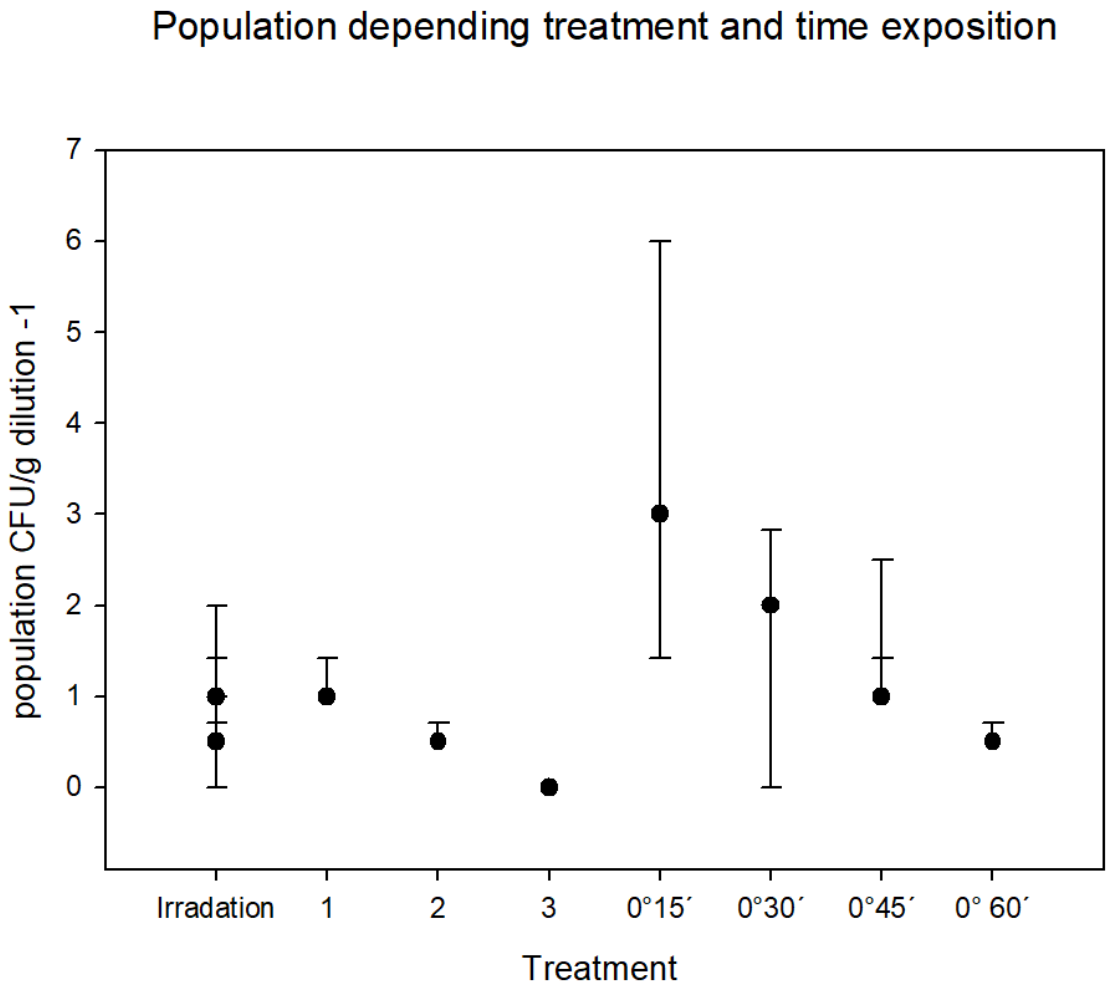

Figure 1: *Graphical representation of CFU/g for -1 dilution in case of irradiated and autoclaving samples*

The results presented in table 1 are not enough for quantifying CFU/g soil. The estimation of viable bacteria using colonies forming units has been applied when more than 30 CFU could be contabilized. In consequence, the majority of the results shown in table 1 could be undetectable. In the case of chloroform vapors treatment, values are quantifiable, so the method has been discharged because it did not result efficiently in finding adequate sterilization conditions.

4 Discussion

In soil bioremediation studies, it is necessary to develop abiotic controls that indicate decontamination by mechanisms other than biological ones; chemical processes and physical mechanisms such as evaporation, leaching and adsorption, among others [19]. Autoclave sterilization is a common and effective method that allows the production of absolutely pure cultures [20]–[23]. However, like any methodology, it can have disadvantages. On the one hand, it would increase production costs due to the consumption of electricity and gas, and, on the other hand, excessive heating of the support could lead to the formation of toxic compounds for some microorganisms [24]. On the other hand, soils subjected to gamma irradiation could have a taxonomically different biota than control soils, and the diversity of this biota will differ according to the radiation dose received [25].

In this work, since four 15-minute procedures did not show the expected sterility, a comparison was made by autoclaving the samples for one hour once, twice and three times. In the third treatment, almost no bacterial growth was detected so it could be presumed that the soil after three one-hour autoclaving processes would be sterile. If the treatments at three hours of autoclaving versus the samples that were irradiated are observed, for the same amount of soil, there are no significant differences between the results obtained from the count, that is, the appearance of colonies was observed below those detected at 15 minutes (Table 1). This is interesting given that the treatment for irradiating samples is more expensive and requires more sophisticated equipment when compared to autoclaving, which is a cheaper and more accessible methodology in any microbiology laboratory. However, fig. 1 shows clearly that fewer 15 min treatments are needed to reach an equivalent count compared to 1-h autoclave treatments. In terms of methodologic procedures, it is more accessible and practical to do fewer cycles, especially when no spore germination has been recorded so the comparison of the two methodologies of treatment in an autoclave could be an instrument to determine a cost-benefit rate.

In this study a dependence on the cycling of exposition has been more efficient in terms of the recount of colonies could be identified. It is reported that all microorganisms are susceptible to hot conditions and their effectivity depends directly on temperature and exposition time [26] but in this case could seem more dependent on cycles than the time of exposition to heat. Wet heat produces protein denaturalization and coagulation because at a cellular level, water excess would produce toxic levels of different compounds and water vapor heat transference coefficient is higher than air so leading to a fast increase in temperature which exceed up to lethal temperature [26]. However, the treated substrates using heat vapor are susceptible to recolonization because a biological vacuum is formed that can be filled by saprophytic organisms, pathogens, or organisms present in dust carried by wind and rain [12]. In line with this, each cycle would avoid the possibility of spore viability or other resistance structures, it could be a possible explanation for the low growth for advanced cycles [27]. Sterilization by autoclave has been reported in microcosm bioremediation studies [28]–[30] and, with irradiation, are de most used methods because do not produce chemicals residues, however, it is reported that could alter soil properties [31] and have taxonomic different microbiota in comparison of control soils and diversity which depends of the radiation doses [25].

The results of this study suggest that it is more efficient the use of autoclaving methods because of the process is more accessible in terms of equipment and methodologies, and the final results are the same. Some studies have used other mechanisms for soil sterilizing such as microwaves [5], [32]; and dry heat [14],and it is quite common sterilization by chemicals products such as sodium azide [33], [34], silver nitrate [35], mercury clorure [36], and chloroform [37] but in case of the present research, these techniques have not been effective. According to Mocali et al. [1], soil sterilization with fumigants leaves most of the soil's enzymes still active favoring recolonization.

For samples that were sterilized with gamma radiation, the results are in line with those reported by Hu, et al [38] and Luo et al [39] who tested with positive results of almost total sterility at around 25 KGy. In all cases, the efficacy of the proceeds must be checked by the absence of the microorganism's growth [40]**.** Also, special attention must be considered to opening and use of sterilized soil for avoiding contamination [37] It is important to note that several authors highlight the effect that both gamma radiation treatment and different autoclave sterilization protocols affect the physical and chemical properties [41], [42] This increases the variables to be taken into account when working with sterile soils.

5 Conclusion

The objective of the present work is to compare the efficiency of three widely used methodologies to sterilize soil and to evaluate their cost/benefit in terms of time and inputs invested to carry out the procedure. Here, the most efficient techniques were gamma irradiation and sterilization in the autoclave at

different times for which the results between the three procedures were comparable. While the use of gamma radiation may be more time efficient, it can be a costly and inaccessible service for some laboratories that do not have the equipment. Therefore, the most viable options in terms of time, cost and benefit are those that use autoclaves. Among them, those that translate into less treatment time mean a reduction in the cost of using the equipment, so the option of 15-minute cycles is convenient. In this sense, many papers reporting a soil sterilization process for different purposes perform the procedure in an autoclave, without referring to a specific protocol. In consequence, it is interesting to note that tests on this type of technique should be updated, as many times each author uses soil sterilization practices without a correct standardization. Consequently, a later comparison of results can be questioned, both for the quality of the sterilization itself, as well as for the possible alterations that each method can have on the soil.

6 Declarations

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6.2 Competing Interests

The author states that there is no conflict of interest.

6.3 Publisher's Note

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