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SOILS

Comparison of methods to quantify soil microbial biomass carbon

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ABSTRACT. Soil microorganism biomass is an important soil quality indicator. The microbial biomass of soil was determined by killing and lysing the soil microbes by fumigation with chloroform, irradiation with gamma rays, or irradiation with microwaves. Four soils with increasing carbon concentrations (5, 10, 15, and 30 g kg^{-1}) were analyzed using four methods: the direct application of chloroform, chloroform fumigation, microwave irradiation, and gamma ray irradiation with radiation doses of 15, 25, 35, 45, and 60 KGy. The fungi and bacteria in the soil were quantified by plate counting. Microwave irradiation and gamma irradiation with doses equal to or above 25 KGy killed all the soil microorganisms, but the chloroform methods did not. The carbon liberation increased with higher gamma doses, while the microbe mortality rates were the same, indicating that carbon was liberated from organic matter sources other than microorganisms. The biomasses determined by the microwave method correlated with those determined by the fumigation and 25 KGy gamma irradiation methods, but their values differed among all methods for at least one soil type. Despite this discrepancy, all methods were consistent in ranking microbial biomasses in increasing order of soil carbon concentrations, which corresponds with decreasing land use intensities.

Keywords: microwave; gamma irradiation; fumigation; incubation; microbial carbon.

Comparação de métodos para quantificação do carbono da biomassa microbiana de solos

RESUMO. A biomassa dos microrganismos do solo é um importante indicador da qualidade ambiental. Esta biomassa é determinada matando os microrganismos por fumigação com clorofórmio, irradiações gama ou micro-ondas. Quatro solos com concentrações crescentes de carbono (5, 10, 15 e 30 g kg^{-1}) foram submetidos a quatro métodos: clorofórmio fumigação ou aplicação direta; irradiação em micro-ondas; e irradiação gama de 15, 25, 35, 45 e 60 KGy. Fungos e bactérias foram quantificados por contagem em placa. Micro-ondas e irradiação gama ≥ 25 Gy mataram todos os microrganismos, mas não os métodos com clorofórmio. O C liberado aumentou com as doses de irradiação, apesar da mortalidade semelhante, indicando liberação de C da matéria orgânica além da microbiana sobrestimando a biomassa microbiana. As biomassas determinadas com micro-ondas correlacionaram-se com as da fumigação e irradiação com 25 KGy mas os valores diferiram entre os métodos em pelo menos um solo. Apesar da discrepância entre métodos, todos foram consistentes em ranquear as biomassas em ordem crescente das concentrações de C do solo, que corresponde a intensidades de uso decrescentes.

Palavras-chave: micro-ondas; irradiação gama; fumigação; incubação; carbono microbiano.

Introduction

The two main environmental problems affecting agricultural activities in the semiarid region of the Brazilian Northeast are low water availability and low soil fertility, which each limit the productivity of the main crops. Therefore, agricultural incomes are low, and crops are cultivated with minimum external inputs, i.e., usually without fertilizer application (Menezes, Sampaio, Giongo, & Pérez-Marin, 2012). Consequently, soil organic matter is a major source of nutrients, and its decomposition is vital to supporting plant production (Moura et al., 2016).

Soil microorganisms are a key component of organic matter nutrient cycling (Brookes et al., 2008). Therefore, knowledge of the soil microbiota functions is important to properly manage the processes that control nutrient availabilities to the plants (Arcand, Helgason, & Lemke, 2016). Several microbiological parameters have been used as indicators of these processes, but no single parameter is appropriate for all situations due to the dynamic and complex nature of agroecosystems (Brookes et al., 2008; Leite et al., 2010). A good indicator needs to respond to soil perturbations,

highlighting temporal changes in a rapid and low-cost manner.

Among soil quality indicators, microbial biomass is one of the best (Brookes et al., 2008; Jannoura, Bruns, & Joergensen, 2013; Makarov, Malyesheva, Maslov, Kuznetsovaa, & Menyailo, 2016). Soil microbial biomass (SMB), excluding plant roots and animals larger than 5 μm^3, corresponds with, on average, 2 to 5% of soil organic matter (Vance, Brooks, & Jenkinson, 1987). There is no consensus on the best method to quantify SMB under diverse edapho-ecological conditions, either because the results have not been very reliable, or the procedures are too labor intensive or too expensive (Hartmann, Frey, Mayer, Mäder, & Widmer, 2015; Makarov, 2016). Thus, it is advisable to use more than one method for SMB quantification.

Several methods are based on processes that cause the death and partial or total disintegration of the soil microorganisms (Powlson & Jenkinson, 1975; Araújo, 2010). After disintegration, some of the cell constituents can be extracted by even mild extractors. There are different methods to kill soil microorganisms, and the most common ones are chloroform fumigation (Brookes et al., 2008; King & Hofmockel, 2017) and microwave irradiation (Araújo, 2010; Souza et al., 2010); however, gamma irradiation has also been used (Powlson & Jenkinson, 1975). The most common method worldwide (Tang et al., 2016), which was developed by Jenkinson and co-workers (Powlson & Jenkinson, 1975) and later modified by Vance, Brookes, and Jenkinson (1987), consists of fumigation in a desiccator, followed by extraction with potassium sulfate and the determination of carbon by titration or colorimetry. Chloroform can also be applied directly to the soil (Witt, Gaunt, Galicia, Ottow, & Neue, 2000; Setia, Verma, & Marschner, 2012).

Comparisons of some of the different methods have been made, but they seldom verify the effectiveness of the methods in killing soil microbiota (Makarov et al., 2016). Chloroform fumigation does not kill all microorganisms, and the method is usually limited by the need for a large set of desiccators (Wolf, Dao, Scott, & Lavy, 1989). Microwave irradiation seems to be efficient in killing, and it is easily performed with commonly available equipment (Araújo, 2010). Gamma irradiation has seldom been used because the necessary equipment is only available at a few places; however, when it is used, the method seems to be efficient in killing and easily performed (Powlson & Jenkinson, 1975).

Considering the scarcity of data obtained on the gamma irradiation of tropical soils, the objectives of this study were: i) determine the proper gamma irradiation doses to kill soil microbiota and measure soil microbial biomass, and ii) compare the gamma irradiation method with the microwave radiation, chloroform fumigation, and direct chloroform application methods.

Material and method

Four soils samples with increasing carbon concentrations (4.8, 9.9, 15.8, and 29.2 g kg^{-1}) were collected in the Agroecological Center São Miguel (7°19'S and 33°51'W), the headquarters of the NGO ASPTA, in the municipality of Esperança, in the semiarid zone of Paraíba state, Brazil. All samples were collected within a radius of 500 m, and the soil was classified as Entisol, with a loamy sandy texture and 5% slope. The first three samples were collected in agricultural plots cultivated with increasing conservation practices: soil 1) continuous bean and potato cultivation without fertilization, soil 2) potato cultivation fertilized with manure, and soil 3) agroforestry system with maize and Gliricidia fertilized with manure. The last sample, soil 4, had the highest carbon concentration of the samples and was collected from Fluvic Entisol in the lower area of the landscape under apparently undisturbed dry forest vegetation. In each area, 20 individual samples were collected in the superficial soil layer (0 – 20 cm) and pooled into a single composite sample, which was analyzed for its chemical and physical characteristics (Table 1).

Table 1. Chemical and physical characteristics of soils with increasing carbon contents collected in the semiarid region of Paraíba, Brazil.

Soil	C	N	P	K	Ca	Mg	Na	pH (H_2O)	Density	Sand	Silt	Clay
	g kg^{-1}		mg kg^{-1}		$cmol_c$ kg^{-1}				g cm^{-3}		g kg^{-1}	
1	4.8	0.46	0.6	0.18	1.75	1.10	0.14	6.4	1.20	830	110	60
2	9.9	0.85	6.0	0.13	2.08	1.35	0.13	5.8	1.16	733	134	133
3	15.8	1.27	3.1	0.16	2.25	1.28	0.14	5.5	1.08	663	126	211
4	29.2	2.38	1.9	0.18	1.27	1.18	0.08	4.6	0.94	470	100	430

Twenty-gram subsamples were wetted to 40% of field capacity and incubated for 72 hours in polyethylene bags. These subsamples were then submitted to the four microbial killing procedures: fumigation with chloroform in desiccators, fumigation with chloroform applied directly to the soil, irradiation in a microwave oven and irradiation with gamma rays.

Part of the subsamples were initially submitted to gamma irradiation for 1.5, 2.5, 3.5, 4.5, and 6 hours in a ^{60}Co (GammaCell 220 Excel) irradiator to reach doses of 15, 25, 35, 45, and 60 KGy, respectively, to select the best dose for the second phase of the method comparison experiment. The soil microbial biomass was determined in these and in non-irradiated samples using the methodology recommended by Vance et al. (1987). The carbon concentrations were quantified by oxidation with potassium permanganate and colorimetry with a 495-nm absorbance wavelength (Bartlett & Ross, 1988).

To verify the efficiency of the different irradiation doses in killing the microbial population, the fungi and bacteria presences in the subsamples were determined. A 10 g portion of each irradiated subsample was placed in Erlenmeyer flasks with 90 mL of a 0.85% NaCl solution and shaken for 1 hour. Serial dilutions (10^{-1} to 10^{-5}) of the supernatant were made, and 100 μL aliquots were transferred, using a Drigalski spatula, to Petri dishes containing Sabouraud media with 100 mg mL^{-1} cycloheximide to isolate the fungi and TSA media with 100 mg mL^{-1} streptomycin to isolate the bacteria. All procedures were carried out in a laminar flow chamber. The Petri dishes were incubated at 30°C for 24 hours for the bacteria tests and for 48 to 72 hours for the fungi tests.

Next, subsamples were submitted to the four microbial killing methods. The subsamples that were gamma irradiated received a 25 KGy dose, as selected by the initial test. The subsamples submitted to the chloroform fumigation were placed in desiccators next to a beaker with 25 mL of chloroform and another beaker with water, which was introduced to maintain a high humidity level. Vacuum conditions were sustained for approximately 5 minutes, until the bubbling and volatilization of all the chloroform occurred, and the subsamples were maintained in the closed desiccator for 24 hours. The desiccator was then flushed, under vacuum, to remove excess fumigant (Vance et al., 1987). The direct application of chloroform was made by adding 1 mL chloroform to the soil subsamples and placing them under vacuum conditions for 24 hours, followed by the described flushing procedure. The microwave irradiation method was conducted with a commercial apparatus. The period of irradiation was calculated based on the tension, microwave frequency, and energy concentration values (Ferreira, Camargo, & Vidor, 1999; Araújo, 2010). In this method, microbes are killed by the increased temperature of their cytoplasm, which breaks their cell walls.

Data on the doses of gamma irradiation were submitted to an analysis of variance, according to a 5 x 4 factorial arrangement, which corresponded to the five doses and the four soils, with six replications in a completely randomized design. This was followed by a Tukey test at a 5% significance level. The interactions were split, and the doses underwent a regression analysis. Data on the microbial biomass determination methods were analyzed as a 4 x 4 factorial arrangement. The microbial counting data were transformed by the equation $\sqrt{x+1}$. A matrix of correlation was calculated for the microbial biomass data obtained with the two chloroform methods, the microwave method, and the 15 and 25 KGy irradiation methods. All analyses were performed using SISVAR version 4.8 (Ferreira, 2008).

Result and discussion

Irradiation with gamma rays at the lowest used dose (15 KGy) killed more than 99% of bacteria and fungi in all the soils (Table 2). This lethality may be adequate for the determination of soil microbial biomasses, but the radiation was not sufficient to sterilize the soils. The high dose (25 KGy), which was the dose level recommended by Powlson & Jenkinson (1975) to determine soil microbial carbon, sterilized all the soils, at least with respect to bacteria and fungi. Doses higher than 23 KGy had the same effect, as was found by Wolf et al. (1989), who irradiated soils with different total carbon contents using doses of 50 and 60 KGy. If sterilization is needed, 25 KGy is appropriate; 15 KGy killed only slightly less microorganisms than 25 KGy, so it should be sufficient for the determination of soil microbial biomasses if the sole requirement of the radiation is the death of the microorganisms.

If the effect of the radiation is restricted to the death and lyses of the soil microorganisms, the amounts of extracted carbon should be similar among all irradiation doses. However, the extracted carbon increased with the irradiation periods for all soils, although different patterns were observed for different soils (Figure 1). In the soil with the lowest organic carbon content, the increase was negligible, while in the other three soils, the extracted carbon increased more than four times from the shortest to

the longest irradiation period. For two of the soils (2 and 4), the increases had linear tendencies, indicating that higher doses of radiation extracted more carbon. Since the microbial biomass was similar in subsamples from the same soil sample, and all dose levels killed practically all of the microorganisms, the gamma radiation of the higher doses liberated soil carbon fractions from sources other than the killed microorganisms. Therefore, using higher doses of gamma radiation could cause the overestimation of microbial biomass if the biomass calculations assumed that all extracted carbon belonged to the killed microorganisms. However, these results open the possibility of using gamma radiation to determine soil carbon fractions that are relatively labile and include more than just microbial biomass. It is interesting to note that the soil with the most intense agricultural use (soil 1), and the lowest carbon concentrations, had almost no increase in the extracted carbon with increases in the irradiation period, indicating that its carbon is retained in more recalcitrant fractions than the other soil types (Singh & Nandita, 2014) and, probably, it has little more labile carbon than the carbon from its microbial biomass.

Table 2. Colonies of bacteria and fungi in growth media inoculated with extracts from soils with increasing carbon concentrations, which were collected in the semiarid area of the Brazilian Northeast and submitted to increasing doses of gamma radiation.

Soil	Organic carbon	Doses (KGy)					
		0	15	25	35	45	60
	g kg^{-1}	Number of colony units x 10^3 gss^{-1} -					
		Bacteria					
1	4.8	243	0.063	0	0	0	0
2	9.9	650	0.050	0	0	0	0
3	15.8	850	0.078	0	0	0	0
4	29.2	300	0.037	0	0	0	0
				Fungi			
1	4.8	3	0.013	0	0	0	0
2	9.9	28	0.017	0	0	0	0
3	15.8	28	0.015	0	0	0	0
4	29.2	8	0.010	0	0	0	0

Another observation is that in the control, non-irradiated samples, the highest numbers of bacteria and fungi colonies formed in the inoculation from soil 3, which had the highest extracted carbon content among all the soils after an irradiation of 25 KGy, although it did not have the highest carbon concentration (Table 2). This confirms that other factors besides the organic matter content affect the reproduction capacity of soil microorganisms, and it reinforces the current opinion that microbial biomass is a useful indicator of soil quality under different management regimes (Jannoura et al., 2013; Makarov et al., 2016). This peculiar behavior of soil 3 may reflect better conditions for microbial growth in its collection area, which are produced by the combination of high soil mobilization (maize cultivation) associated with the application of manure and litter inputs and the favorable microclimate due to the presence of the trees in the agroforestry system.

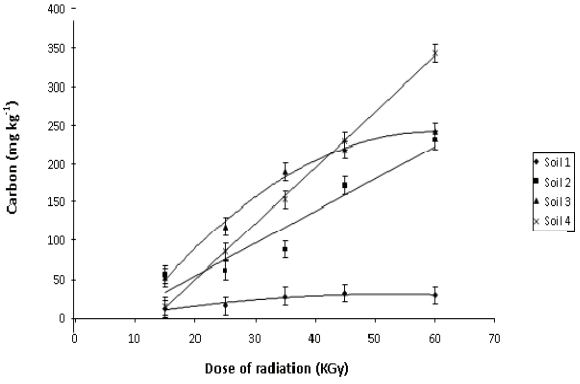

Figure 1. Carbon extracted from soils with increasing carbon concentrations collected in the semiarid area of the Brazilian Northeast and submitted to increasing doses of gamma radiation. Vertical bars represent minimum significant differences ($p < 0.05$).

The effect of the gamma irradiation can be further analyzed by comparing its results with those of other methods used to kill soil microorganisms and to determining their biomass data. Microwave radiation, like the 25 KGy gamma dose, killed all the bacteria and fungi, but the chloroform methods did not (Table 3). Since the original fumigation procedure was developed, it has been known that it does not kill all microorganisms. In fact, for the original method of fumigation, incubation, it was assumed that most of the microorganisms would be killed, and their biomass would be consumed by the new microorganism populations developing from the survivors of the fumigation, which would result in a flush of CO_2 evolution (Powlson & Jenkinson, 1975). Later, incubation was replaced by the immediate extraction with K_2SO_4 of the microbial biomass lysed by the fumigation (Brookes et al., 2008). By that period, Wolf et al. (1989) had already reported that chloroform fumigation within desiccators did not sterilize soil, while sterilization could be achieved with gamma radiation doses of 50 and 60 KGy.

Comparing fumigation with the direct application of chloroform, the consistent result for all soils was that higher numbers of bacteria colonies formed during the

incubation after the fumigation, while a higher number of fungi colonies occurred during the incubation after the direct application. For both methods, the numbers of colonies that formed after the chloroform treatments were not related to the numbers that formed from unfumigated soils; nor were they related to the total carbon concentrations of the four soils. The causes of the differences in the bacteria and fungi survival rates after the two treatments and of the different survival proportions are not known and warrant further research. The direct chloroform application might result in higher sorption interactions with the soil clay and/or organic matter constituents than fumigation, possibly making its complete evacuation more difficult (Rotbart et al., 2017). This effect on the determination of microbial biomass may be only slightly greater than the small effect found by Rotbart et al. (2017) with fumigation but its effect on the microbial population recovery is not known.

Table 3. Colonies of bacteria and fungi formed in growth media inoculated with extracts from soils with increasing carbon concentrations collected in the semiarid area of the Brazilian Northeast and submitted to different microbial killing procedures.

Soil	Organic carbon	Control	Chloroform in the soil	Chloroform fumigation	Microwave radiation
	g kg^{-1}	Number of colony units x 10^3 gss^{-1}-			
			Bacteria		
1	4.8	243	23 Ba[1]	42 Aa	0 Ca
2	9.9	650	11 Bb	37 Aa	0 Ca
3	15.8	850	13 Bab	33 Aa	0 Ca
4	29.2	300	11 Bb	32 Aa	0 Ca
			Fungi		
1	4.8	3	5 Ac	0.5 Bb	0 Ca
2	9.9	28	10 Aa	3.0 Ba	0 Ca
3	15.8	28	8 Ab	0.5 Bb	0 Ca
4	29.2	8	8 Ab	0.5 Bb	0 Ca

Averages followed by the same capital letter in the row and small letter in the column are not significantly different by the Tukey test at the 5% probability level.

The microbial biomasses calculated for the soil that was cultivated for the longest period (soil 1), which also had the lowest soil carbon content, were all low and not significantly different using the two chloroform methods, the microwave method, and the two gamma irradiation methods with doses of 15 and 25 KGy (Table 4). For the undisturbed soil (soil 4), which had with the highest carbon content, the 25 KGy gamma dose, microwave and chloroform fumigation methods were not significantly different, and the direct application of chloroform to the soil was inferior to the irradiation methods but not different from the chloroform fumigation method, while the 15 KGy gamma radiation dose resulted in the lowest, and most statistically inferior, biomass value. For the two soils with intermediate carbon contents (soils 2 and 3), the 25 KGy radiation dose also resulted in the highest carbon levels during extractions, although the values for soil 2 were not significantly different from the methods that used microwave radiation and the direct application of chloroform to the soil. In both soils 2 and 3, the microbial biomasses determined by the gamma irradiation method with the 15 KGy dose were included in the group of methods that produced the lowest values, which also comprised the biomasses determined by chloroform fumigation.

Correlating the five measurement techniques (the four primary methods, including the two gamma dose levels of 15 and 25 KGy) using the biomass data of the four soils (Table 4) only resulted in significant correlation coefficients (> 0.95; $p < 0.05$) for the microwave and 25 KGy gamma irradiation pair and the microwave and chloroform fumigation pair. However, four is a low number of compared pairs, which may overshadow real correlations with lower coefficients, similar to that of the paired microwave and soil applied chloroform fumigation methods ($R = 0.93$; significant at 0.07). If this pair is included, the microwave method correlates with all others, except for the gamma irradiation method using a 15 KGy dose. In fact, the 15 KGy dose correlated poorly with all other measurement techniques.

Table 4. Microbial biomass carbon in soils with increasing carbon concentrations collected in the semiarid area of the Brazilian Northeast and submitted to different microbial killing procedures.

Soil	Organic carbon	Chloroform in the soil	Chloroform fumigation	Micro wave radiation	Gamma radiation	
					15 KGy	25 KGy
	g kg^{-1}	---------- mg kg^{-1} ----------				
1	4.8	14.8 Ab[1]	16.1 Ac	22.5 Ac	19.2 Ab	22.6 Ac
2	9.9	69.4 Aa	46.0 Bb	64.7 ABb	52.1 Ba	74.0Ab
3	15.8	63.6 BCa	56.5 Cb	81.6 Bab	49.4 Ca	107.3 Aa
4	29.2	71.8 Ba	85.3 ABa	92.8 Aa	19.7 Cb	97.1 Aa

1 - Averages followed by the same capital letter in the row and small letter in the column are not significantly different by the Tukey test at the 5% probability level

With a more detailed analysis of the biomasses determined by the five ways of measuring biomasses in the four soils with increasing carbon contents, more information can be extracted. The low biomasses determined by the 15 KGy gamma irradiation method, particularly for soil 4, indicates this dose seems to be inefficient in the lysis of all soil microorganisms, despite of killing most of them (Table 2). On the other hand, the 25 KGy gamma irradiation level always resulted in the highest calculated biomasses, and this may have resulted from the extraction of carbon from sources other than the microbial biomass, which is more evident with doses higher than 25 KGy; therefore, this method may overestimate the soil microbial biomass compared to the other methods. Consequently, this dose level cannot be unquestioningly recommended for the microbial biomass determination of all soils (Powlson & Jenkinson, 1975), and it is possible that an intermediate dose between 15 and 25 KGy works better, at least for tropical sandy soils relatively poor in total organic matter.

A similar doubt could be raised for the microwave method if it is compared with the fumigation treatment; not only were the absolute averages always higher for the former, they were significantly higher in the case of soil 3 (Table 4). However, both methods have been used extensively, and in some cases, the microwave method resulted in consistently lower C extraction levels (Araújo, 2010). The direct application of chloroform also resulted in higher carbon extraction than fumigation in the case of soil 2, and the average extraction value was very similar to those obtained with the microwave and 25 KGy irradiation methods. A higher carbon extraction level with direct application than with other methods was also reported by Setia et al. (2012). Either all three methods are not adequate for all soils, or the fumigation method, which is considered to be the standard, is also sometimes inadequate.

Despite the discrepancies among the average extraction levels of the methods, in general, they were all consistent in ranking the microbial biomasses in increasing order from the lowest to the highest total carbon content. This is consistent with the results of several authors who have found that intensively cultivated soils have lower microbial biomasses (Araújo, Santos, & Monteiro, 2008) and microorganism diversities (Kong, Scow, Córdova-Kreylos, Holmes, & Six, 2011) than soils under native vegetation or rotated crops (Souza et al., 2010; Leite et al., 2011; McDaniels, Tiemann, & Grandy, 2015; Hartmann et al., 2015; King & Hofmockel, 2017). From this perspective, all the methods were valid for detecting changes in the land use intensity, but their absolute microbial biomass results should be carefully interpreted. Independent of variations, the microbial biomass carbon of the soils determined by all four methods was always less than 1% of the total soil carbon concentration. These proportions, which are low compared to those reported for soils of the same type in other regions (Matias, Salviano, Leite, & Araújo, 2009; Souza et al., 2010) but similar to the values reported for the same Brazilian region (Araújo et al., 2008), can be attributed to the semiarid climate conditions prevailing in the area where the soils were sampled. These low proportions also minimize any possible need to stress the importance of the absolute biomass values.

Conclusion

Microwave and gamma irradiation equal to or above 25 KGy killed all microorganisms, but the chloroform methods did not. Higher numbers of bacteria colonies formed during incubation after chloroform fumigation than after the direct application, while the opposite occurred with fungi colonies.

Biomasses determined by the microwave method correlated with those determined by the fumigation and 25 KGy irradiation methods, but the values differed among all methods for at least one soil type. Despite this discrepancy, all methods were all consistent in ranking the microbial biomasses in increasing order from the lowest to the highest total carbon content.

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