TITLE:

Simulating Temperature in a Soil Incubation Experiment

AUTHORS AND AFFILIATIONS:

Jianwei Li[1], Precious Areeveso[1], Xuehan Wang[1], Siyang Jian[1,2], Lahiru Gamage[1]

[1]Department of Agricultural and Environmental Sciences, Tennessee State University, Nashville, Tennessee, USA

[2]Department of Plant Biology and Microbiology, University of Oklahoma, Norman, Oklahoma, USA

Email addresses of co-authors:

Precious Areeveso (pareeves@tnstate.edu)

Xuehan Wang (xwang3@tnstate.edu)

Siyang Jian (sjian@ou.edu)

Lahiru Gamage (lgamage@tnstate.edu)

Corresponding author:

Jianwei Li (jli2@tnstate.edu)

SUMMARY:

Laboratory soil warming experiments usually employ two or more constant temperatures in multiple chambers. By presenting a sophisticated environmental chamber, we provide an accurate temperature control method to imitate the magnitude and amplitude of *in situ* soil temperature and improve the experimental design of soil incubation studies.

ABSTRACT:

The study of warming impact on soils requires a realistic and accurate representation of temperature. In laboratory incubation studies, a widely adopted method has been to render constant temperatures in multiple chambers, and *via* comparisons of soil responses between low- and high-temperature chambers, to derive the warming impact on soil changes. However, this commonly used method failed to imitate both the magnitude and amplitude of actual temperatures as observed in field conditions, thus potentially undermining the validity of such studies. With sophisticated environmental chambers becoming increasingly available, it is imperative to examine alternative methods of temperature control for soil incubation research. This protocol will introduce a state-of-the-art environmental chamber and demonstrate both conventional and new methods of temperature control to improve the experimental design of soil incubation. The protocol mainly comprises four steps: temperature monitoring and programming, soil collection, laboratory incubation, and warming effect comparison. The step-by-step procedure is modified according to a former publication. One example will be presented to demonstrate different methods of temperature control and the resultant contrasting warming scenarios; that is, a constant temperature design referred to as stepwise warming (SW) and simulated *in situ* temperature design as gradual warming (GW), as well as their effects on soil respiration, microbial biomass, and extracellular enzyme activities. In addition, we present a

strategy to diversify temperature change scenarios to meet specific climate change research needs (e.g., extreme heat). The temperature control protocol and the recommended well-tailored and diversified temperature change scenarios will assist researchers in establishing reliable and realistic soil incubation experiments in the laboratory.

INTRODUCTION:

Global surface temperature is expected to increase this century by 1.8–6.4 °C[1,2]. Global warming may increase CO_2 flux from soil to the atmosphere, resulting in positive feedback with warming[3–6]. Because microbial communities play a critical role in regulating soil respiratory responses to warming[7,8], the changes in microbial respiration and the underlying microbial mechanisms with warming have been a research focus. Though soil warming experiments deployed in the field condition, *via* a heating cable[9] and an open top chamber[10], were advantageous in capturing natural soil features such as temperature[11], their high cost for installation and maintenance have limited their application. Alternatively, soil incubation experiments subject to different temperatures are a favorable choice. The primary advantage of soil incubation in a laboratory is that the well-controlled environmental conditions (e.g., temperature) are able to disentangle the one-factor effect from other confounding factors in a field experimental setting[12,13]. Despite differences between growth chamber and field experiments for plant growth, translation from lab results to the field are readily available[14]. Incubating soil samples in a laboratory setting could help improve our mechanistic understanding of soil response to warming[15].

Our literature review identified several temperature control methods and, consequently, distinct temperature change modes in past soil incubation studies (**Table 1**). First, instruments used to control temperature are mostly through an incubator, growth chamber, water bath, and in a rare case, heating cable. Given these instruments, three typical temperature change patterns have been generated (**Figure 1**). These include the most implemented mode, constant temperature (CT), linear change (LC) with a non-zero constant temperature change rate, and nonlinear change (NC) featured with a diurnal type of temperature. For a case of CT pattern, the temperature may vary in magnitude over time, though constant temperature remains for a certain time period during the incubation (**Figure 1B**). For LC, the rate of temperature change could vary in different studies at more than two orders of magnitude (e.g., 0.1 °C/day vs. 3.3 °C/h; **Table 1**); For NC cases, most relied upon the intrinsic capacity of instruments used, thus leading to various modes. Despite a type of diurnal temperature, change was claimed through a heating cable or incubator[16,17]; however, the chamber temperatures in these experiments were not validated. Other major review results in **Table 1** include the range of incubation temperature of 0–40 °C, with most between 5–25 °C; the duration of experiments ranged from a few hours (<1 day) to nearly 2 years (~725 days). Also, soils subjected to incubations were collected from forest, grassland, and cropland ecosystems, with dominant mineral horizon, organic horizon, and even contaminated soil, located mostly in the US, China, and Europe (**Table 1**).

Given the three major temperature change modes, several distinct warming scenarios achieved in the past studies were summarized in **Table 2**. They include stepwise warming (SW), SW with varying magnitude (SW_v), gradual warming linearly (GW_l), gradual warming nonlinearly (GW_n), and gradual warming diurnally (GW_d).

In summary, past soil incubations usually captured the average air or soil temperature in a site. In many cases, as shown in **Table 1**, incubators or chambers were manually programmed at a fixed temperature but incapable of automatically adjusting temperature as desired, lacking the ability to control the mode and rate of temperature change with time (**Eq. 1**), and thus leading to difficulty to imitate diurnal temperature of the local soil. On the other hand, though attempted in two experiments[16,17], we identified no studies that explicitly imitated gradual warming diurnally (GW_d) in their incubation experiments (**Table 1**). Based on the literature review, the major obstacle lies in poor experimental design, particularly lacking a sophisticated instrument that enables implementation and validation of diurnal or other gradual warming scenarios.

$$\Delta T = f(m, r, t) \quad \textbf{(Eq. 1)}$$

Where ΔT is the quantity of temperature change, m is the mode of temperature change, r is the rate of temperature change, and t is the duration of change.

To improve the experimental rigor in soil incubation, an accurate and sophisticated temperature control method is presented in this study. Adopting a state-of-the-art environmental chamber, increasingly available and economically viable, the new design shall not only enable the accurate simulation of *in situ* soil temperature (e.g., diurnal pattern) but also, by accounting for possible temperature change extremes, provide a reliable way to minimize the artefacts of instrumental bias. The current soil incubation design should assist researchers to identify optimal strategies that meet their incubation and research needs. The overall goal of this method is to present soil biogeochemists with a highly operational approach to reform soil incubation design.

PROTOCOL:

1. Temperature monitoring and programming

1.1. Identify a sampling zone within a research plot. Install one or a few automatic temperature probes in soils at 10 cm depth. Connect the weather station to a computer *via* the data transmission cable and open the software on the computer.

1.2. Click on the **Launch**/**Properties** toolbar button to configure the logger for the external sensors being used.

1.3. On the **Properties** screen, set the logger/station name (i.e., Soil incubation exp.) and the data collection interval (i.e., 60 min). Then, on the **Properties** screen, click **Enabled** on the external sensor ports being used and select the sensor/unit from the dropdown button for each sensor port (i.e., Port A; "Enabled": Temperature ˚C). Finally, click on **OK** to save the settings.

1.4. Monitor the probes' reading weekly to avoid malfunction and download the dataset once a month. Obtain a complete record for several months covering the growing season (i.e., April to September).

1.5. Conduct data analysis of the temperature records. Obtain the mean hourly temperature of the growing season by averaging all observations.

1.5.1. Obtain the mean temperature of each hour on a daily basis by averaging temperatures of the same hour across all days during the growing season.

1.6. In the sophisticated chamber, launch the software and click on the **Profile** button on the main menu screen to create a new file. In the file name input line, enter “SW low”. By clicking on the **Instant Change** option, enter 15.9 °C as an initial temperature as obtained in step 1.5, and enter 2 on the **Minutes** row to maintain the temperature for 2 min and click on the **Done** button. Then, under the **Ramp Time** option, enter 15.9 °C as the target set point and on the **Hours** row enter 850 h to sustain the temperature. Fianlly, click on the **Done** button.

1.6.1. Repeat the above step in the second chamber by adding 5 °C to each temperature node and create a new file name “SW high".

1.6.2. Repeat step 1.4 in the third chamber by adding 23 additional steps corresponding to 23 observed hourly soil temperatures as obtained in step 1.5.1. At the last step, called **JUMP**, set 42 repeated loops (Jump Count 42). This leads to the scenario of gradual warming or GW low.

1.6.3. Repeat the above step in the fourth chamber with 5 °C added to each temperature node. This will allow a simulation of varying temperatures for 42 days at a higher temperature level (i.e., GW high).

1.7. Conduct a preliminary run for 24 h and output the temperatures recorded by the four chambers. Plot the temperatures recorded by the chambers against those as programmed (**Figure 2A–D**).

1.7.1. If the temperatures achieved in the chamber match the temperatures as programmed by a temperature difference <0.1 °C during the 24 h (**Figure 2A**,**B**,**E**,**F**), the chambers are suitable for the soil incubation experiment.

1.7.2. If the criteria were not satisfied in any of these chambers, repeat another 24 h test or seek a new chamber.

2. Soil collection and homogenizing

2.1. Near the temperature probe area, collect five soil samples at 0–20 cm depth and put them into one plastic bag after removing the surface litter layer.

2.2. Mix the sample thoroughly by twisting, pressing, and mingling the materials in the bag until no individual soil sample is visible.

2.3. Store the samples in a cooler filled with ice packs and transport the samples to the lab

immediately.

2.4. Remove the roots in each core, sieve it through a soil sieve of 2 mm, and thoroughly mix and homogenize the sample prior to the following analysis.

3. Laboratory incubation

3.1. Prior to incubation, weigh 10.0 g of fresh soil, oven-dry it for 24 h at 105 °C, and weigh the dry soil. Derive the difference between fresh and dry soil samples and calculate the ratio of difference over dry soil weight to determine the soil moisture content in a spreadsheet.

3.2. Use the derived moisture content to calculate the soil microbial biomass carbon (MBC), extracellular enzyme activity (EEA), and soil heterotrophic respiration as described in the following steps. These data will help understand the treatment effects on soil respiration and the underlying microbial mechanisms.

3.3. Prior to incubation, weigh the field moist soil subsample (10 g) and quantify the soil MBC by chloroform fumigation–K_2SO_4 extraction and potassium persulfate digestion methods[18].

3.4. Prior to incubation, weigh the subsample of field moist soil (1.0 g) and measure soil hydrolytic and oxidative EEA[19].

3.5. Weigh 16 field moist soil subsamples (15.0 g equivalent of dry weight) in 16 polyvinyl chloride (PVC) cores (5 cm diameter, 7.5 cm tall) sealed with glass fiber paper on the bottom.

3.6. Place the PVC cores in Mason jars (~1 L) lined with a bed of glass beads to ensure that the cores do not absorb moisture.

3.7. Place four jars in each of the four chambers as described in step 1.4. Turn on the chambers and launch the program simultaneously in four chambers.

3.8. During the incubation, at 2 h, days 1, 2, 7, 14, 21, 28, 35, and 42, take all jars in each of four chambers and use a portable CO_2 gas analyzer to measure soil respiration rate (R_s) by putting the analyzer's collar to the top of each jar.

3.9. Destructively collect all jars at the end of incubation (i.e., day 42) and quantify soil MBC as described in step 3.3.

3.10. Destructively collect all jars at the end of incubation (i.e., day 42) and quantify soil enzyme activity as described in step 3.4.

4. Warming effect comparison

4.1. By assuming a constant respiration rate (Rs) between two consecutive collections, use the

respiration rate times the duration to derive the cumulative respiration (R_c).

4.2. Conduct a three-way repeated measures analysis of variance (ANOVA) to test the main and interactive effects of time, temperature (warming), and temperature mode (warming scenario) on R_s and R_c. In addition, conduct a two-way ANOVA to test warming and warming scenario effects on MBC and EEA.

REPRESENTATIVE RESULTS:

The selected state-of-the-art chambers replicated the target temperature with high precision (**Figure 2A,B,E,F**) and met the technical requirement of the incubation experiment. Given the easy use and operation, this signified the technique to improve the temperature simulation in soil warming studies and in other applications such as plant studies. The procedure has been employed in our recent case study based on a switchgrass cropland in Middle-Tennessee.

Research results showed that warming led to significantly greater respiratory losses (R_s and R_c) in both warming scenarios (SW and GW), and GW doubled the warming-induced respiratory loss (R_c) relative to SW, 81% vs. 40% (**Figure 3)**. On day 42, MBC and EEA were also significantly different between SW and GW, such that MBC was higher in SW than in GW (69% vs. 38%; **Figure 4**) and glycosidases and peroxidase (e.g., *AG*, *BG*, *BX*, *CBH*, *NAG*, *AP*, *LAP*) were significantly higher in GW than in SW scenarios (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: The illustration of temperature change mode in a soil warming experiment as conceptualized from Table 1. (**A**) Constant temperature (CT) adopted by most studies. (**B**) Constant temperature with varying magnitude (CT_v). (**C**,**D**) Linear change (LC) with positive and negative rates. (**E**,**F**) Nonlinear change (NC) with irregular pattern and diurnal pattern.

Figure 2: Temperature targeted via programming and chamber temperature during a 24-h testing period. (**A**,**B**) Target temperature (grey line) and chamber temperature records (dashed line) under control and warming treatments of stepwise warming (SW); (**C**,**D**) Target temperature (grey line) and chamber temperature records (dashed line) under control and warming treatments of gradual warming (GW); (**E**, **F**) The temperature difference derived for records in panels **C** and **D**.

Figure 3: Mean (± SE) cumulative soil respiration rate (R_c, μg CO_2-C·g_{soil}^{-1}) under control (hollow) and warming (dark) treatments in SW and GW in a 42-day soil incubation experiment. The insets show soil respiration rates (R_s, μg CO_2-C·h^{-1}·g_{soil}^{-1}) applied to estimate cumulative respiration, assuming R_s was constant until the following measurement. (**A**) Stepwise warming (SW) and (**B**) gradual warming (GW). N = 4 in each collection.

Figure 4: Mean (± SE) MBC under control and warming treatments in SW and GW in a 42-day soil incubation experiment. MBC = microbial biomass carbon; N = 4 in each collection. S denotes significant effect of warming scenario (SW vs. GW), at $p < 0.05$, based on a three-way repeated measures ANOVA.

Figure 5: Mean (± SE) glycosidases and peroxidase (µmol activity $h^{-1} \cdot gsoil^{-1}$) under control and warming treatments in SW and GW in a 42-day incubation experiment. *BX =* β-1,4-xylosidase; *AP =* Acid Phosphatase; *LAP =* Leucine Aminopeptidase; *NAG =* β-1,4-N-acetyl-glucosaminidase; *OX =* Oxidative enzymes; *PHO =* Phenol oxidase; *PER =* Peroxidase. N = 4 in each collection. S denotes significant effect of warming scenario (SW vs. GW), at $p < 0.05$, based on a three-way repeated measures ANOVA.

Table 1: Literature review of temperature control methods and temperature change modes in soil incubation studies[12,13,16,17,20–62]. In total, 46 studies were included in the review.

Table 2: Major temperature change modes and the corresponding warming scenarios based on a literature review (Table 1). Five modes and scenarios were established to represent a wide range of possible temperature change and warming conditions.

DISCUSSION:

The constant temperature control method has been applied widely (**Table 1**). However, the magnitude and temporal pattern of temperature implemented in these procedures poorly simulate soil temperature observed in the field condition. Despite the emerging efforts imitating the diurnal pattern in the past, such studies were scarce and failed to clarify the equipment and procedure; neither did they validate the temperature simulation regarding accuracy and reliability[16,17]. As the community strived to improve its understanding of soil warming responses, optimizing the soil incubation procedure with realistic temperature and feasible control is imperative. Nevertheless, such new methods have not been developed, and thus, a standard method for future incubation experiments is still out of reach. In the face of the increasing complexity of global temperature change in magnitude, amplitude, seasonality, duration, and extremality, a comprehensive procedure is in high demand.

Here, a method for manipulating a diurnal temperature change procedure was presented, relying upon the sophisticated chamber, to offer the capacity to establish constant, linear, and nonlinear temperature change and subsequently various warming scenarios for meeting future research needs. There are four critical steps within the protocol. The first is to determine soil temperature in the field condition. Because the soil type and depth of interest—land use type of a specific research plot can vary from one study to another—the soil depth and number of temperature probes needed for the specific research site should be modified to best fit the soil characteristics and cover the plot landscape and conditions relevant to temperature as much as possible. In general, soil depth for temperature probes shall meet the most research needs at 0–20 cm, and the number of probes to represent the soil temperature should be limited to one to three. The key is to achieve a long-term continuous and consecutive temperature record in at least one typical soil location.

The second critical step is to set up the program to achieve the targeted temperature magnitude and pattern in the chamber. Because of the high sensitivity and accuracy of chamber (**Figure 4**), it is feasible to program for an accurate representation of temperature as observed in the field

condition. Although the current protocol only presented the observed hourly temperature as targeted in the chamber, a more frequent soil temperature monitoring, such as 30 min, 15 min, or even less, can be achieved through this procedure. Nevertheless, a test of the target and chamber temperatures must be conducted over 24 h, and prior to experiment, the test results must meet the criteria of less than 0.1 °C between the target and chamber temperatures at all time points. The more frequent the temperature observation is selected to simulate, the more steps are needed to set up the program in the chamber prior to the experiment.

The third critical step is to conduct the incubation itself. To reduce the influence of soil heterogeneities[63], homogenizing soil samples is key, and at least three replicates for each treatment are recommended. Prior to incubation, a pre-incubation treatment is required, and the current procedure can facilitate pre-treatment by programming the temperature and duration for this. This is advantageous for one to reduce the experimental disturbance and allow one to orchestrate the entire incubation seamlessly. The last critical step is to include both constant temperature and varying temperature treatments so that a comparison can be made as to the soil warming responses.

This protocol can be easily modified to allow one to manipulate the magnitude, amplitude, and duration of temperature change. For example, extreme temperatures during a heat wave in summer and sudden frost in early spring due to climate change, can be represented using this procedure, in addition to its capacity to account for their varying duration and intensity. Simulating the regular and irregular temperatures in combination also allow one to simulate long-term complex temperature change effects as projected in the future. As summarized in **Table 2**, those warming scenarios that have been studied in many distinct studies can be accomplished collectively in one study. This protocol is expected to provide a sophisticated method to simulate temperature in soil incubation studies. With hope for a wide application, the adoption of this protocol will help identify or validate a more accurate method for future soil warming studies based on laboratory incubation.

An important limitation of the procedure is that the chamber used in the current protocol has a relatively small volume, thus is only able to accommodate nine incubation jars in each chamber. Though a smaller jar will increase the capacity of the chamber, a big volume of chamber is recommended. A new model (e.g., TestEquity 1007) will offer eight times more capacity and is thus recommended for large scale experiments. Despite the improvement of temperature control procedure in soil incubations, the potential complications with moisture and soil homogenization will not be relieved by adopting the current protocol.

We demonstrate significant advantages of the sophisticated temperature control procedure. It provides a reliable and affordable temperature control strategy to obtain accurate temperature simulation and offers a feasible way to improve soil incubation experiment required for a better understanding of soil warming responses. Although the constant temperature control is widely accepted and logistically easy to operate, the artifacts of long-term constant temperature on soil microbial communities may divert efforts to capture the genuine soil responses. The other reported laboratory warming methods are largely less controllable and replicable. The current

protocol is superior due to its easy operation, high accuracy and replicability of temperature simulation, explicit programing, and capacity to combine various temperature change scenarios in a single experiment. The feasibility of temperature control with high accuracy will allow researchers to explore various temperature change scenarios.

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DISCLOSURES:

The author has nothing to disclose.

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