Novel Use of Chlorine Dioxide Granules as an Alternative to Methyl Bromide Soil Fumigation

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Abstract: Two greenhouse studies were conducted to evaluate the effectiveness of chlorine dioxide (ClO_2) granules as a soil fumigation alternative for methyl bromide. The objective of the first study was to determine the efficacy of chlorine dioxide granules to inactivate *Bacillus subtilis* spore samples placed in two soils and positioned at two soil depths in soil tubes. The objective of the second study was to measure ClO_2 gas concentrations released over multiple days in the two soils. The granules were evenly distributed in the soils with datalogger/sensors positioned at two different locations (headspace and soil matrix). The first study achieved a maximum spore log10 reduction of 4.12 and 5.82 for play sand and mixed soil, respectively, for inoculated samples placed 8 cm deep in the soil tubes with a chlorine dioxide rate of 240 g/tube. There was a 21-fold increase in percent organic matter for the mixed soil when compared to the play sand soil. The increase in organic matter in the mixed soil resulted in a 1.7 log10 reduction decrease due to the absorption of the gas onto the organic matter. In the second study, chlorine dioxide was collected at the bottom of the soil instead of volatilizing into the headspace of the soil tube because it is denser than air. The ClO_2 at the bottom of the soil was 3.95x and 3.8x higher than the headspace gas concentration for the mixed and play sand soil, respectively, as averaged over all test runs and periods. Both studies show that the chlorine dioxide granules are a promising alternative to soil fumigation with methyl bromide. Further research is needed to refine the granule formulation release rates and develop more economical application rates.

Keywords: Chlorine Dioxide, Z-Series Granules, Soil Fumigation, Methyl Bromide Alternatives, *Bacillus subtilis* spores.

1. INTRODUCTION

The 1992 Montreal Protocol was ratified as an international treaty that limited the production and use of ozone-depleting substances such as methyl bromide. Methyl bromide was widely used as a soil fumigant in specialty crops and commercial nurseries. The phase out of methyl bromide as a soil fumigant was accelerated due to a human toxicity review from the Center for Disease Control National Institute for Occupational Safety and Health (NIOSH) that states that methyl bromide is a potential, occupational carcinogen [1]. The directive of the Montreal Protocol led to a multi-decade search for alternative soil fumigation methods that would be effective across a broad spectrum of soil microbes and that was also environmentally friendly and cost-effective.

Alternative soil fumigation technologies are currently being evaluated for their effectiveness, and their green properties associated with low health risks and negative impacts on the environment. Ammonium bicarbonate, which was a forerunner to baking powder and baking soda, was recently evaluated as a soil sanitizer [2,3]. A soil tillage system [4] may soon be commercialized for sanitizing bio-contaminated soil. The tillage system generates ozone gas and ultra-violet light that converts ozone into reactive oxygen species and hydroxyl radicals that inactivate soil pathogens. Another environmentally friendly oxidant similar to ozone is chlorine dioxide, which has been evaluated by EPA for sanitizing soils bio-contaminated with *Bacillus* spores [5, 6]. These oxidants, such as ozone and chlorine dioxide, have potential as soil fumigants because they readily decompose into harmless metabolites when exposed to sunlight and air.

Chlorine dioxide is a stable free radical, green-yellowish gas (ClO_2) that acts as a broad-spectrum biocide due to its ability to "strip" up to five electrons from biomolecules. A series of granule biocide formulations developed by ICA TriNova (Newnan, GA) generates chlorine dioxide gas across a wide range of granule release rates. Their fumigant formulations (Z-Series) consist of one or two precursor chemistries impregnated onto zeolite carriers. The fast release formulation consists of two precursors, which are sodium chlorite (Part A - $NaClO_2$ on a zeolite carrier) and an acid activator (Part B - sodium bisulfate on a zeolite carrier). When the fast release formulation (Part A + Part B granules) is mixed in the soil, ClO_2 gas is generated over 60 to 80 hr. period, depending on the precursor chemistry, granule application rates, and ambient temperatures.

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A potential drawback of using chlorine dioxide as a soil fumigant is that it is partially inactivated by soil organic matter. Current research has focused on the ability of ClO2 to inactivate a variety of microbial surrogates. Several studies have evaluated the sporicidal efficacy of liquid and gas formulations of chlorine dioxide [5 - 8]. Future studies involving chlorine dioxide formulations should develop estimates for both soil absorption inactivation rates and microbial inactivation rates. Chlorine dioxide is photo-oxidized by sunlight and degrades very rapidly into chloride (Cl^-), chlorite (ClO^-), and chlorate (ClO_3^-) metabolites, which have a much lower toxicity ranking.

Bacillus subtilis was used as a surrogate microbe in this study to represent any hard-to-kill plant pathogen that may contaminate field crops or commercial nurseries. *B. subtilis* is an excellent surrogate because it is readily available, accessible to culture, and safe to use [9]. It is a gram-positive, rod shaped bacteria that is commonly found in the soil, air, and in plant compost. The survival structure of *B. subtilis* has a tough, dormant coat (endospore) to protect itself from harsh environmental conditions and toxic chemicals [9].

The goal of this soil fumigation study was to evaluate the ability of a novel chlorine dioxide granule formulation (Z-Series granules) to inactivate *B. subtilis* spore samples placed in soil tubes. The objectives of the first greenhouse study were to evaluate the sporicidal efficacy of the Z-Series granules when applied at three rates using two soil types, and with spore samples placed at two soil depths. The use of the two soil types provides necessary information on soil organic matter absorption rates for chlorine dioxide gas. The objectives of the second study were to determine ClO_2 gas concentrations in the same two soil types when collected by two datalogger/sensors with probes positioned in the headspace and in the soil matrix of a container of soil over a multi-day time.

2. MATERIALS AND METHODS

The two greenhouse studies were conducted in 2018 at the USDA-Agricultural Research Service greenhouse located in Fort Collins, CO. Greenhouse parameters were set at ambient light conditions, and a temperature range was set at 26 to 35 C. The experimental design for the first study was a completely randomized, factorial design with three factors. Study factors were two soil types, three application rates for Z-Series granules, and two *B. subtilis* spore sample locations in each soil tube.

A private microbiology laboratory (Microchem Laboratory, Round Rock, TX, USA) was contracted to prepare the *B. subtilis* inoculated, wool samples and to assay the samples for viable spores after the soil treatments. The lab inoculated the 2.5 x 5 cm wool strips with 300 µl of *B. subtilis* spore suspension. The initial spore count was 1.13×10^8 Colony Forming Units (CFU) per inoculated sample. Wool samples were stored at 4 C before and after the study was conducted to ensure the spores would not germinate during storage and shipping dates.

The chlorine dioxide fumigant granules (Z-Series) were supplied by ICA Trinova (Newnan, GA, USA). The Z-Series fast release formulation consists of Part A as the ClO_2 precursor (sodium chlorite) and Part B as the acid activator (sodium bisulfate). Granule rates for this study were: 0, 60, and 120 g/tube for both Part A and Part B granules, i.e., 60 g Part A + 60 g Part B per tube for granules applied at the low rate. Preliminary data from pilot tests were used to develop the application rates for the Z-Series fast release granules. The granules were evenly and individually hand mixed with the soil in a plastic tub for each treatment and soil tube and was then added back into a labeled soil tube. The average soil volume was 2,250 ml per tube. The total Z-Series application rates were 120 or 240 g/2250 ml soil, or 0.053 and 0.11 g/ml of soil for both soil types.

The two soil types were 100% play sand and a mixed soil (50% play sand + 50% potting soil). The potting soil was a Farfard – 4-MP (Sun Gro Horticulture, Agawan, MA, USA) mix, which is a Canadian sphagnum peat moss, processed pine bark, vermiculite, and perlite mix. Both soils were oven dried for five days to ensure initial low soil moisture (approx. 2% v/v) before starting the fumigant treatments.

The soil tubes were made of acrylonitrile butadiene styrene tubes (ABS), which are resistant to chlorine dioxide gas. The tubes prevented any ClO_2 gas release and allowed multiple fumigant treatments to be evaluated at minimal cost. Each tube was 30 cm by 10 cm (inside diameter). The tubes were capped on the bottom and covered with wax paper on the top.

Oven dried soil was added to each tube, and lightly packed down, until soil height reached 28 cm with a 2 cm head space. The evenly mixed Z-Series + soil mixture was added back into the tube while combining the two *B. subtilis* inoculated, wool strips at 8 and 18 cm from the soil surface. Tap water (250 ml) was added to the soil at 14 cm depth, and another 250 ml

was added to the soil surface. After adding the soil mixture, spore samples and water to each tube, two layers of wax paper were taped to the tube top to prevent the release of ClO_2 gas and to maintain the soil moisture levels. Soil moisture was determined for both soils using the gravimetric method (Table **5**). The *B. subtilis* wool samples were retrieved from the tubes 14 days after the soil fumigant treatment was initiated. The control and treated samples were stored at 4 C until they were returned to the private lab for assaying for viable spore counts.

The study design included seven control soil tubes that were not treated with the Z-Series granules. The untreated soil tubes served as field controls to determine whether the *B. subtilis* spores would germinate under the warm (30 C) greenhouse conditions over the 14-day study. In addition, nine storage and transit samples were stored in a refrigerator (4 C) to determine the effect of storage and transport on spore recovery rates from the wool samples. Viable *B. subtilis* spore counts from the storage and transport controls were used to calculate log10 reduction for each treatment.

A pilot study within the first study measured the ClO_2 gas concentration in the play sand soil using the fast release formulation in three test runs. A portable data logger/sensor (PortaSens II Model C16, Analytical Technology Inc., Collegeville, PA) was used to measure the ClO_2 gas concentration in the play sand soil over a 50 to 76 hr. period with data collection at one min. time intervals. The data logger sensor had a ClO_2 accuracy range from 200 to 1,000 mg/L and a sensitivity of 1 mg/L. The sensor probe was placed in a hole on the side of the soil tube located approx. 5 cm below the soil surface. The Z-Series granules were evenly mixed in the soil at the rate of at 60 g Part A and 60 g Part B granules per 2,250 ml of dry play sand per tube. The pilot study was used to develop a more detailed and accurate protocol for measuring ClO_2 soil concentrations that were used to initiate a second study.

A second greenhouse study was designed at a smaller scale, using cups to measure ClO_2 gas in the play sand and mixed soils at two sensor locations. The Z-Series fast release formulation, as previously described, was used in this study. The Z-Series granules were applied at a single application rate of 0.06 g Part A + 0.06 g Part B, or a total of 0.12 g/ml of soil. The granules were evenly and individually mixed by hand into 200 ml of air-dried soil, then added back to a 400 ml cup that is covered with a lid. The two soils used in the second study were described previously, with the exception that the soils were air-dried and had higher soil moisture (Table **5**).

Dynamic ClO_2 patterns in the soil were measured using two data loggers/sensors (PortaSens II, as previously described) that collected data at 10 min. time intervals over two to four test runs that ranged from 42 to 67 hr. One of the ClO_2 sensors had an accuracy range of 1 to 5 mg/L and a sensitivity of 0.01 mg/L. This sensor had a 20 cm flexible probe that measured ClO_2 in the 4 cm headspace in the cup. The second data logger used a sensor with a ClO_2 accuracy range from 200 to 1,000 mg/L and a sensitivity of 1 mg/L. This sensor had a 20 cm probe that measured ClO_2 gas concentrations in the soil with the probe inside a vertical perforated steel tube and placed at the bottom of the paper cup. The steel tube was covered with a permeable nylon fabric that was then inserted vertically into the paper cup with the Z-Series granules + dry soil added around the covered steel tube. Both sensor probes were sealed to the cup lid with tape to prevent ClO_2 gas from escaping the cup. In addition, the soil sensor probe was sealed with tape at the top of the steel tube to prevent ClO_2 gas from escaping the tube and confounding the ClO_2 measurements in the headspace. The density of ClO_2 gas is about 2.4-fold denser than air. Due to the density differences between ClO_2 and air, the second study was designed so that two ClO_2 sensor probes were placed in the bottom of the soil and in the headspace of the cup to measure whether ClO_2 tended to concentrate in either location.

The study design included 19 replications per treatment using the hidden replication statistical technique that limits data analyses to only two-way interactions. Treatments were significant at $\alpha = 0.05$. The storage and transit control and treated *B. subtilis* spore counts were transformed into log10 reduction data. The log10 reduction formula is as follows: A is the number of viable spores recovered from the control treatment surfaces, and B is the number of actual sores recovered from the treated surfaces:

Log10 Reduction of viable B. subtilis spores = Log (A/B)

The JMP Least Squares Fit program (SAS Institute Inc., Clary, NC, USA) was used to analyze the log10 reduction data.

3. RESULTS

Soil properties for both soils are listed in Table **1**. As anticipated, the play sand was low in percent organic

Table 1: Soil Properties for 100% Play Sand and 50% play Sand + 50% Potting Soil ((v/v) Mixed Soil). All Soils were Oven Dried before Initiating the Studies

Soil type	pH	Organic Matter	Sand	Silt	Clay	Soil moisture
		----------%----------				
Play sand	9.0	0.1	94	1.75	4.25	0.4
50/50 - mixed soil	7.6	2.2	89	1	10	1.0

matter, while the mixed soil had a high percentage of organic matter. The two soils covered a wide range in organic matter that should test the ClO_2 absorption rates and the ultimate efficacy of the fumigant. Percent soil moisture ranged from 12 to 14% and from 18 to 24% for the sand and mixed soil, respectively, and soil temperature ranged from 30 to 34 C for both soil types. The soil pH was 9.0 and 7.6 for the play sand and mixed soil, respectively. Soils with a lower pH are more acidic that combines with the acid released from the Part B granule and slightly increases the ClO_2 release rates from the granules (Table **1**).

The play sand trial reveals that only the Z-Series granules affected *B. subtilis* spore inactivation, and there was no interaction term in the final model (Table **2**). Analyses of the mixed soil (50% play sand and 50% potting soil) includes the Z-Series and the interaction between the Z-Series rate and sample burial depth as terms in the log10 reduction prediction model (Table **2**). The play sand soil treatment with the highest spore inactivation was Z-Series granules applied at 120 g/tube with a log 10 reduction of 5.81, averaged over both sample locations (Table **4**). The mixed soil treatment with the highest spore inactivation was the Z-Series applied at 120 g/tube for the 8 cm sample burial depth with a log10 reduction of 4.12 (Table **4**).

The storage and transit control tubes had 7.92 and 8.52 log10 CFU/sample, respectively, for the play sand and the mixed soils. These high spore counts per sample indicate that spore recovery was high despite the fabric samples being bio-contaminated from being in direct contact with the soil in each tube. Also, the high spore counts indicate that the spores did not germinate after 14 days in moist soil in a warm greenhouse.

The nine transit and storage controls were stored in a lab refrigerator (4C) for 186 days before the study could be conducted. The average *B. subtilis* spore count for these nine samples was 4.98×10^7. The private lab also prepared a control sample with an initial time of zero, which is the number of viable spores recovered from samples assayed a day after the spores were dried on the fabric samples. The spore count for time zero controls was 1.13×10^8. Therefore, the loss in viable *B. subtilis* spores for storing the samples for 186 days at 4 C was 0.36 log10 (8.05 – 7.69 log10). The decline in viable spore counts due to extended storage could be used to adjust the log10 reduction model predictions to account for the survival rates for spores in storage.

The first study revealed that the dynamic ClO_2 patterns in the play sand were closely correlated with air temperatures (Figure **1**). The ClO_2 release rates for all three test runs follow a cyclic curve pattern over 76 hr. study period. The cyclic pattern follows a diminishing three-peak release rate as the granules are virtually deleted over three test runs. The ClO_2 soil concentration ranged from 90 to 1,200 mg/l, with an overall average concentration of approximately 218 mg/L over at 5 cm below the soil surface of the three-day study period. The average soil temperature was 31 C due to higher greenhouse temperatures that occurred during the summer months when this study was conducted.

The dynamic ClO_2 patterns in the second study included both soils with the granules applied at 0.06 g Part A + 0.06 g Part B per 200 ml of soil. The ClO_2 release rates for both soils and both sensor locations were also correlated with diurnal air temperatures in the greenhouse (Figures **2-5**). The cyclic pattern also

Table 2: Least Squares Fit Model Terms and p-Value for Play sand Soil

Source	DF	Sum of Squares	F Ratio	Prob > F
Z-Series granule	2	468.44933	735.6519	<.0001*

*The LS test for the 100% sand soil shows that only the Z-Series granule wt. had a significant effect on log10 reduction of the B. subtilis spores. The other two study factors were not significant.

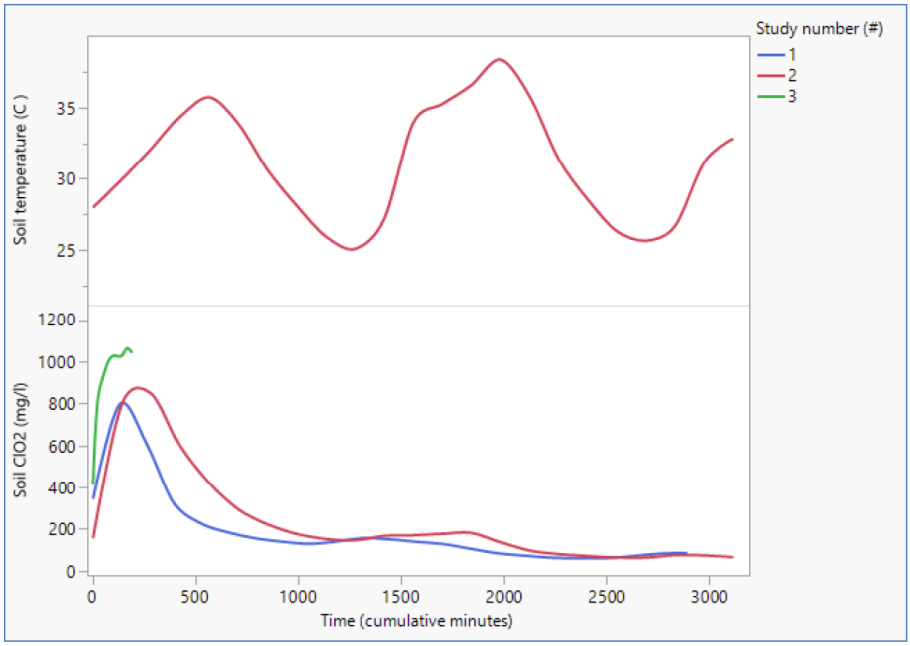

Figure 1: Concentration of chlorine dioxide gas in play sand soil when released from Z-Series granules in an acrylonitrile butadiene styrene (ABS) soil tube. Granules were evenly mixed in sand at 60 g Part A and 60 g Part B granules in 2,250 ml of soil. The ClO2 sensor probe was placed 5 cm below the soil surface. Data smoother lines were used to graph the temperature data (upper graph) and ClO2 concentration (lower figure) over the granule release time period (x axis) for four different test runs. The temperature data for run #1 and #3 were not recorded correctly so only the temperature data for run #2 was included in this graph.

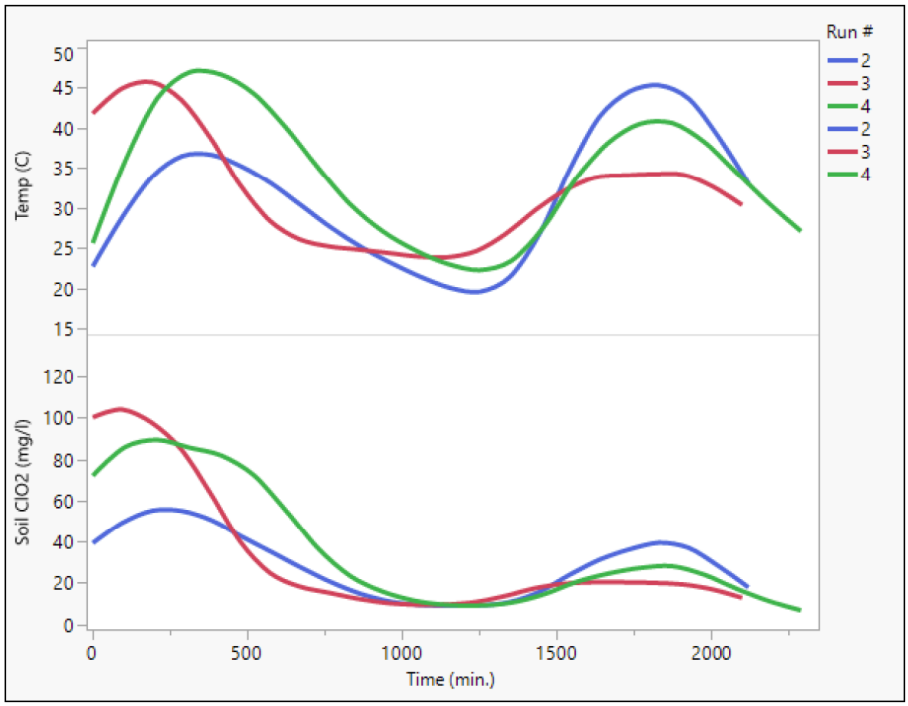

Figure 2: Concentration of chlorine dioxide gas in play sand soil when released from Z-Series granules in the paper cup study. Z-Series granules were added at the rate of 0.06 g Part A + 0.06 g Part B to 200 ml of soil. The ClO2 sensor probe was placed in a perforated steel tube and the probe nozzle is located near the bottom of the soil. Three data smoother lines were used to graph the temperature data (upper graph) and ClO2 concentration (lower figure) over the granule release time period (x axis) for three different test runs. ClO2 data for run #1 was deleted due to data collection errors.

displayed a diminishing, multi-peak pattern over the study time period. The daily maximum ClO_2 soil concentration for both studies is listed in Table **6**.

In the second study, the ClO_2 soil concentration for the play sand, when measured in the bottom of the soil, ranged from 10 to 130 mg/l with an overall, average

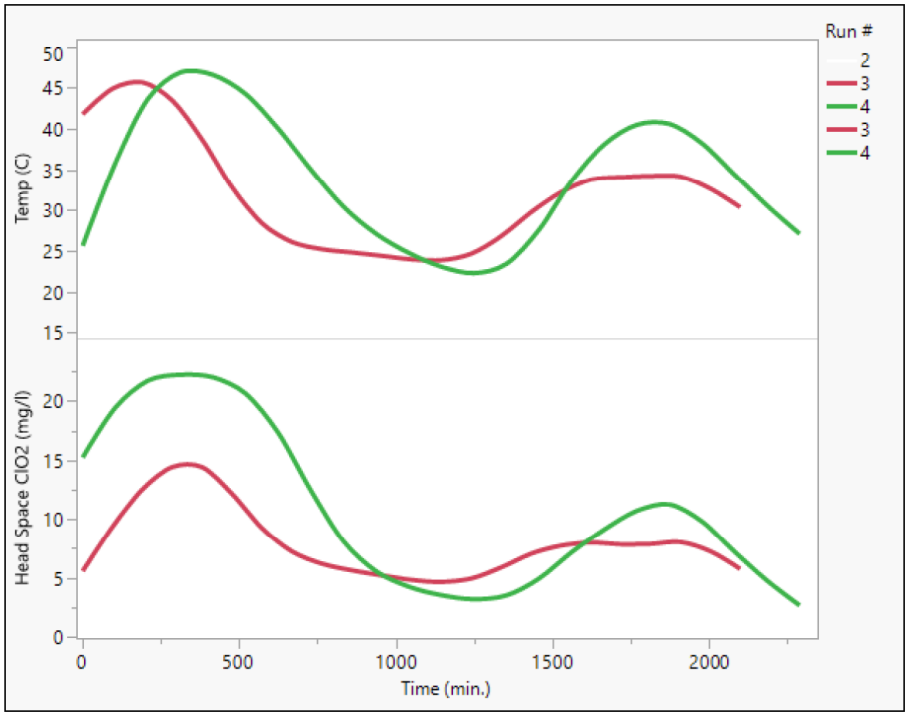

Figure 3: Concentration of chlorine dioxide gas in play sand soil when released from Z-Series granules in the paper cup study. Z-Series granules were added at the rate of 0.06 g Part A + 0.06 g Part B to 200 ml of soil. The ClO2 sensor probe was placed in the 4 cm head space above the soil surface. Two data smoother lines were used to graph the temperature data (upper graph) and ClO2 concentration (lower graph) over the granule release time period (x axis) for two different test runs. ClO2 data for run #1 and #2 were deleted due to data collection errors.

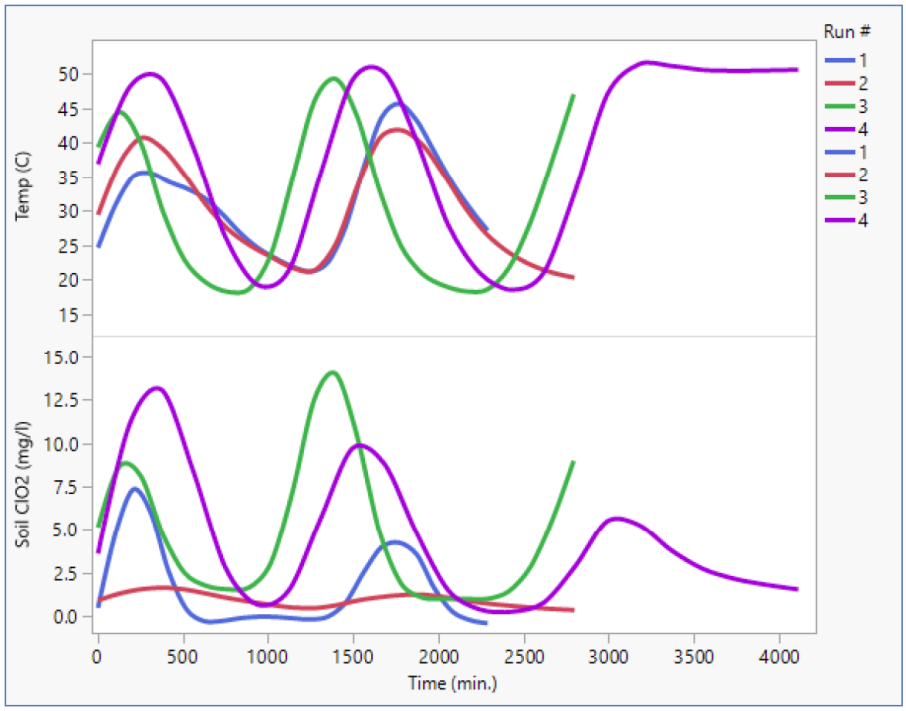

Figure 4: Concentration of chlorine dioxide gas in mixed soil (50/50 play sand and potting soil) when released from Z-Series granules in the paper cup study. Z-Series granules were added at the rate of 0.06 g Part A + 0.06 g Part B to 200 ml of soil. The ClO2 sensor probe was placed at the bottom of soil inside a steel tube. Four data smoother lines were used to graph the temperature data (upper graph) and ClO2 concentration (lower graph) over the granule release time period (x axis) for four different test runs.

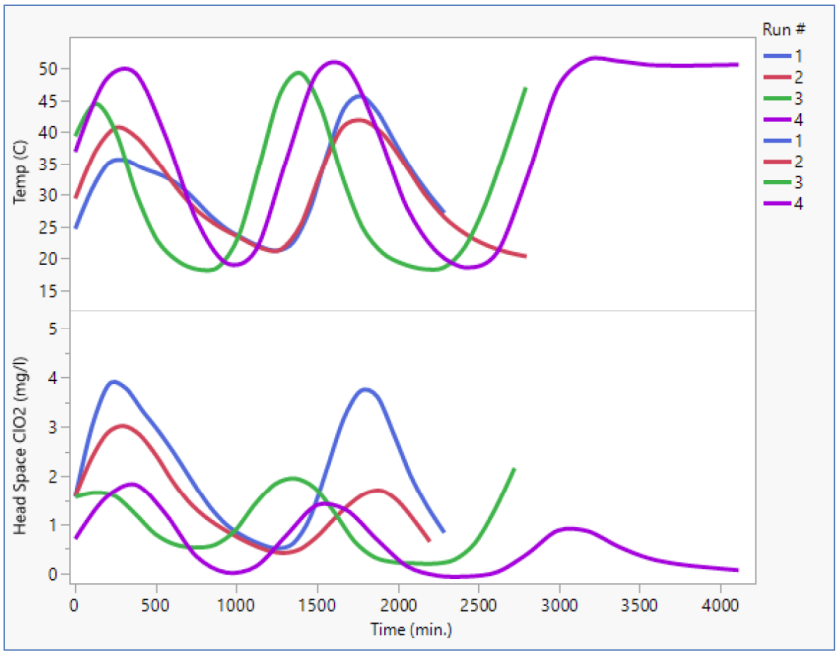

Figure 5: Concentration of chlorine dioxide gas in mixed soil (50/50 play sand and potting soil) when released from Z-Series granules in the paper cup study. Z-Series granules were added at the rate of 0.06 g Part A + 0.06 g Part B to 200 ml of soil. The ClO2 sensor probe was placed in the 4 cm head space above the soil surface. Four data smoother lines were used to graph the temperature data (upper graph) and ClO2 concentration (lower graph) over the granule release time period (x axis) for four different test runs.

Table 3: Least Squares Fit Model Terms and p-Value for 50% Play sand and 50% Potting Soil

Source	DF	Sum of Squares	F Ratio	Prob > F
Z-Series granule	2	58.399	111.741	<.0001*
Sample location	1	0.0	0.0002	0.9889
Z-Series granule *Sample location	2	3.908	7.478	0.0017*

*The LS test for the 50% sand and 50% potting soil shows that Z-Series granule wt., and sample location had a significant effect on log10 reduction of the B. subtilis spores. Also there was an interaction between these two study factors.

Table 4: Predicted log10 Reduction for *B. subtilis* Spores, along with 95% Confidence Intervals. The Least Squares Fit Model Predictions were Reported for both Soil Types, Z-Series Granule Application Rates, and the *B. subtilis* Sample Location in the Soil Tubes

Soil type	Z-Series granule (g/tube)	Sample location (8 or 18 cm from soil surface)	Log10 reduction of *B. subtilis* spores	Lower 95% CI Individual soil test	Upper 95% CI Individual soil test
Mixed soil	0	8	0.01	-1.08*	1.11
Mixed soil	0	18	0.01	-1.10*	1.11
Mixed soil	120	8	1.49	0.40	2.57
Mixed soil	120	18	1.35	0.27	2.43
Mixed soil	240	8	4.12	3.01	5.24
Mixed soil	240	18	2.72	1.61	3.84
Sand	0	8	0.16	-0.99*	1.31
Sand	0	18	0.16	-0.99*	1.31
Sand	120	8	5.32	4.17	6.47
Sand	120	18	5.32	4.17	6.47
Sand	240	8	5.82	4.67	6.97
Sand	240	18	5.82	4.67	6.97

*Any 95% confidence intervals (CI) with a negative lower value shows that the predicted log10 reduction is not different from zero, i.e. the log10 reduction values for all the Z-Series granule weights at zero are equivalent to zero. In other words, there was no log10 reduction of *B. subtilis* spores for all the control tubes for both soils.

Table 5: Average, Percent Soil Moisture, Based on Gravimetric Measurements, for Oven and Air-Dried Soils

Soil type	Average soil moisture (%)
Oven dry sand	2.0
Oven dry 50/50 mixed soil	1.1
Air dry 50/50 mixed soil	20.4
Air dry sand	12.6

Table 6: Maximum ClO_2 Gas Concentration in Soil at Peak of each Day for Soil Tubes and Cup Studies, for both Soils

Soil type	First day	Second day	Third day	Fourth day
	ClO2 concentration (mg/L)			
ABS tube sand	1190	1200	400	150
ABS tube sand	1200	170	90	.
ABS tube sand	1200	190	190	.
Cup sand	70	50	.	.
Cup sand	130	20	.	.
Cup sand	110	40	.	.
Cup 50/50	10	10	.	.
Cup 50/50	13	15	11	.
Cup 50/50	14	12	7.1	.

concentration of approximately 33 mg/L. The average soil temperature was 33 C for three test runs. The ClO_2 soil concentration in the 4 cm headspace ranged from 0.5 to 24 mg/L, with an overall average level of approximately 9 mg/L. The average soil temperature was 33 C for two test runs.

In the second study, the ClO_2 soil concentration for the mixed soil (50/50 play sand and potting soil), when measured in the bottom of the soil, ranged from 0.4 to 15 mg/L with an overall, average concentration of approximately 4 mg/L. The average soil temperature was 34 C for four test runs. The ClO_2 soil concentration in the 4 cm headspace ranged from 0.4 to 5 mg/L, with an overall average concentration of approximately 0.9 mg/L. The average soil temperature was 34 C for four test runs.

4. DISCUSSION

In the first study, the two soil trials were analyzed separately. The two experiments were conducted several months apart with more cooling diurnal air temperature patterns occurring during the second trial. The average, maximum daily soil temperature was 32, and 30.8 C for the mixed soil for Z-Series granules added at 120 and 240 g/tube, respectively. In contrast, the average, maximum daily soil temperature was 41.1, and 38.3 C for the play sand soil for Z-Series granules added at 120 and 240 g/tube, respectively. Analysis of air temperatures during both soil tests showed that both soil type and Z-Series granule rates were significant factors when tested for differences in maximum, daily air temperatures. Therefore, the two soil tests were analyzed separately due to the close correlation of ClO_2 gas release rates from Z-Series granules and air temperature patterns (Tables **2**, **3** and Figures **2-5**).

The lower pH in the mixed soil appears to have combined with the acid released from the Part B granule and affected the ClO_2 release rates into the soil. Visual comparison of the diurnal wave patterns for the play sand (Figures **2**, **3**) and the mixed soil (Figures **4**, **5**) shows a much steeper ClO_2 wave pattern for the mixed soil. The steeper release pattern in the mixed soil indicates that the lower soil pH releases the ClO_2 from the Part A granule at a faster rate when compared to the play sand.

Log10 reduction analyses for both soils resulted in different terms in the final models. The play sand model

only included the Z-Series granule rate per tube, and the mixed soil model included both the Z-Series granule rate and the spore sample location as well at the interaction term (Tables **2**, **3**). The final model terms for the play sand test indicates that play sand had a more uniform distribution of ClO_2 gas over time that caused equivalent log10 reduction values for both sample locations. The interaction term in the mixed soil indicates that the ClO_2 gas distribution was uniform when the Z-Series granule rate was 120 g/tube resulting in equivalent log10 reduction estimates. However, when the Z-Series granule rate was 240 g/tube, the log10 reduction was 4.12 and 2.72 for samples located at 8 and 18 cm from the soil surface. In other words, log10 reduction increased by approx. 1.5x for spore samples located at 8 cm from the soil surface.

The soil temperatures in this greenhouse study were probably much higher (35 to 45 C) than is expected under average field conditions, even at the height of summer. The ClO_2 soil concentrations rise and fall with the diurnal greenhouse temperatures (Figures **1**-**5**). The direct relationship between soil temperature and Z-Series granule release rates presuppose that soil treatments with the granules should be conducted during the hot summer months. If the Z-series granules were applied under cooler temperatures then the amplitude of the granule release rates would be lower but extended over a longer period of time.

The second study evaluated the putative effects of soil organic matter absorption rates of ClO_2 gas. The percent organic matter was 0.1 and 2.2% for play sand and mixed soil, respectively (Table **1**). The maximum log10 reduction for *B. subtilis* spores was 4.12, and 5.82 for play sand and mixed soil, respectively (Table **4**), for samples placed 8 cm from the soil surface at the highest Z-Series application rate. Log10 reduction decreased by 1.7 as the percent organic matter increased by 21x when comparing the play sand to the mixed soil properties (Tables **1**, **4**). The decrease in *B. subtilis* spore inactivation rates confirms the hypothesis that soil organic matter either absorbs and/or degrades chlorine dioxide. A study by Świetlik *et al*. [10] found the dissolved organic matter in water did absorb chlorine dioxide and ozone. These findings indicate that a soil buffering rate should be added into the calculations for granule application rates when soils contain a higher percentage of organic matter.

Preliminary tests were conducted on ClO_2 gas dynamics in play sand by adding 500 ml of water to a tube filled with air-dried soil. The extra soil moisture absorbed virtually all ClO_2 gas released from the granules so that ClO_2 gas never exceeded the lower range limit of the sensor during the first 24 hours of data collection. Therefore, only oven dried soil was used in the ClO_2 gas release study in order to avoid any water absorption of the ClO_2 gas and confounding measurements of Z-Series release rates into the soils.

In the first study, both soils were oven dried for five days to ensure they had equivalent soil moisture levels. After adding the same water volume (500 ml) to both soil types, the percent soil moisture was higher in the mixed soil, when measured the first day after the soil treatments (Figure **6**). Soil moisture was higher in the mixed soil due to the absorption rate of the peat moss. These findings suggest a tradeoff between soil moisture, gas diffusion rates, and increasing ClO_2 toxicity. Log10 reduction increased as soil moisture increased for the play sand test. However, log10 reduction was indirectly related to soil moisture for the mixed soil test (Figure **6**). Chlorine dioxide gas readily diffuses into water and soil moisture. As soil moisture absorbs ClO_2, the gas is transformed into a liquid biocide. This transformation stabilizes ClO_2, extends its exposure time, and increases its efficacy. Also, increasing soil moisture in the play sand appears to act as a reversible reservoir for the ClO_2 gas, where the gas is slowly released back into the soil matrix. The organic matter in the mixed soil also acts as a reversible reservoir that absorbs and releases excess soil moisture, but desorption of ClO_2 appears to be much slower if any desorption occurs at all. The combination of organic matter absorption of both ClO_2 gas and water appears to reduce the efficacy of the chlorine dioxide soil treatments in the mixed soil (Figure **6**).

A comparison of the greenhouse and transit/storage controls for viable spore counts shows that the warm greenhouse soils did not increase *B. subtilis* germination rates. The viable spore counts for the greenhouse samples that were exposed to 14 days of high temperatures were 8.5 and 7.9 log CFU/sample for the mixed and sandy soils, respectively. In contrast, the spore counts for the transit and storage samples that were stored at 4 C for the length of the study was 7.6 log CFU/sample. The greenhouse controls had spore counts that were 0.9 and 0.3 log CFU/sample higher than the transit/storage controls for the mixed and sandy soils, respectively. The spore counts for the samples buried in both soils were slightly higher than the non-buried, transit/storage spore counts because of

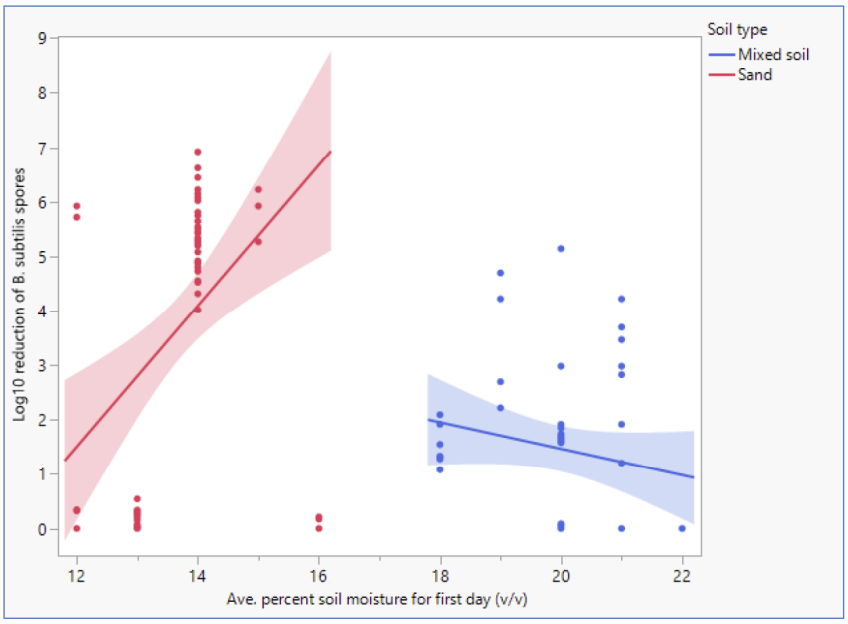

Figure 6: Relationship between log10 reduction of *B. subtilis* (y-axis) spores and average, percent soil moisture content (x-axis) for each soil type (legend), as averaged across both Z-Series application rates.

cross-contamination from the soil microbes in both soils.

In the first study, all the control and treated soil samples were cross contaminated with other microbial species. The soils were not autoclaved before initiating the soil fumigation treatments in order to preserve their natural properties, including their microbial communities. Cross contamination of the fabric samples made the identification and counting the *B. subtilis* spores problematic because the wool samples were not enclosed with an air permeable envelope. Semi-selective media was used to minimize the contamination issue. The lab assay report mentioned that cross contamination of the soil samples increased the spore counts due to the inclusion of misidentified microbes. The inclusion of microbial bio-contaminates in the final spore count may have increased the log counts by 0.9 and 0.3 for the mixed and sandy soils, respectively. This estimate of "spore plus bio-contaminant" was based on the average CFU differences between the greenhouse and transit/storage control samples. If the assumption that the spore counts were increased due to cross contamination of the samples is accepted, then the model predictions for log10 reduction for each soil treatment could be adjusted for the microbial contamination estimates (Table **4**). Future soil fumigation research should design inoculated soil samples that are enclosed with an air permeable (Tyvek) envelope to prevent cross contamination from native soil microbes.

The dynamic ClO_2 patterns in the second study reveal that the Z-Series formulation is more eco-friendly than the conventional, EPA registered fumigants due to its higher molecular density than air and its wide range of granule release rates. The ClO_2 concentrations in the soil and headspace show that the gas settled in the bottom of the soil instead of volatilizing into the headspace (Figures **2-5**). Chlorine dioxide measured at the bottom of the soil was 3.95-fold and 3.8-fold higher than the ClO_2 concentration in the headspace for the mixed and play sand soil, respectively, as averaged over all test runs and periods (Figures **2-5**). The average ClO_2 concentrations by sensor location verify that the density of ClO_2 (2.4-fold heavier than air) allows the gas to settle in the soil matrix. The density of ClO_2 minimizes any potential health risks that are inherent in EPA registered fumigants with high volatilization properties. Also, the ClO_2 dynamic patterns show that the granules were virtually depleted by the third or fourth day (Figures **2-5**). The Z-Series formulations can be readily manipulated to control release ClO_2 gas over a 2 to a 14-day time period. Future soil fumigation studies with the Z-Series granules should evaluate whether extending the release rates up to 7 or 14 days at sublethal concentrations would be more efficacious for controlling soil pathogens. Other studies could include a more complex set of treatments that combine fast and controlled release formulations that could provide both a front-end, lethal rate with an extended sublethal application rate.

The efficacy of chlorine dioxide gas on bacterial spores has been evaluated in several other studies. A study by Li *et al*. [11] tested the effectiveness of ClO_2 gas on six materials that were inoculated with *B. subtilis* spores and found a log reduction of 1.8 to 6.6 when testing the six materials. A sporicidal study by Nam *et al*. [12] evaluated the efficacy of ClO_2 for *Bacillus cereus* that achieved complete inactivation of the spores after 6 hr. of treatment. Another sporicidal study by Han *et al*. [13] evaluated ClO_2 gas on *Bacillus thuringiensis* spores that attained a 2.5 to 4.9 log reduction in spores inoculated onto four materials. EPA scientists also conducted a soil fumigant study that included a ClO_2 gas treatment using *B. anthracis* and *B subtilis* spores as the target microbial species. The EPA study [5] tested a gas formulation of ClO_2 (3,000 ppm) that was injected into Petri dishes with 1 and 2 cm soil depths. Despite the different study methods used between the EPA and this study, the log10 reduction results were comparable. They achieved a log10 reduction of 0.59 (75% RH) up to 3.72 (85% RH). In comparison, we achieved a log10 reduction ranging from 1.4 to 4.1 for the mixed soil tests.

Results from the first study offer evidence that the chlorine dioxide granules can effectively inactivate bacterial spores when applied to soils with a range of organic matter properties. The second study demonstrates the dynamic release patterns for ClO_2 gas from the granules over the multi-day test run in two soil types. This study reveals that the effects of soil organic matter on ClO_2 absorption rates and gas density effects on ClO_2 distribution patterns in a soil profile. Future studies should evaluate different Z-Series formulations on extended-release models for the inactivation of soil pathogens. Other research is needed to optimize the Z-Series application rates and develop more economical application rates. These studies verify that chlorine dioxide formulations could be an effective and green alternative to using methyl bromide as a soil fumigant.

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