SGM BIOTECH, INC.

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EFFECTS OF DELAYING POST-EXPOSURE INCUBATION TIMES ON D-VALUE DETERMINATIONS OF BIOLOGICAL INDICATORS

ABSTRACT

The effect of delaying incubation times after saturated steam or ethylene oxide exposure of EZTest®-Steam or EZTest®-Gas units and of SGMStrips for steam or gas was assessed under controlled laboratory conditions. The time of delay between exposure and culturing of three lots each of EZTest-Steam units, EZTest-Gas units, SGMStrip steam strips and SGMStrip gas strips was varied from immediate (within fifteen minutes of exposure) up to as much as 7 days. Units or strips were stored at controlled room temperature during the incubation delay. Average D-values (for the type of exposure, steam or EO) of each lot at each delay time were calculated and compared. There were no significant differences between the D-values calculated from immediate culturing (~fifteen minutes) and those obtained from delayed incubation times of four hours, twenty-four hours or seven days for any of the types of biological indicators tested. Such stringent culturing requirements for exposed biological indicators appear to be unnecessary.

INTRODUCTION

In some instances the guidance by ISO (1) and USP (2) that exposed biological indicators (BIs) be incubated within two or four hours of that exposure is difficult to accommodate. For instance, BIs may need to be exposed at one site and then shipped to another for subsequent culturing and D-value determination. Previous reports (3, 4) provided equivocal results on the effects of delaying incubation after exposure for longer than 2 hours for both EtO and steam. Conditions of delay were different among the earlier experiments with the one showing a 90% reduction in recovery (3) comparing storage under refrigeration with storage at 20 to 25C with a hold time of forty-eight hours. Graham (4) used hold times as long as five days and storage at 20 – 25 C or 32 to 35 C and found no effects. Shirtz et. al. (5) used only steam sterilization and found no effects for delays as long as 72 hours. To further assess the effects of such post-exposure delays incubation on different types of biological indicators a controlled laboratory investigation was undertaken.

Exposures of EZTest®-Gas and EZTest®-Steam self-contained units and of SGMStrip™-Steam and SGMStrip™-Gas paper carriers were done using the same exposure times used to establish the original D-values for each of the lots. Then set of BIs were cultured immediately and also allowed to sit at controlled room temperature for various delay times before culturing and subsequent D-value determination.

MATERIALS AND METHODS.

General Methods

All ethylene oxide exposures were done in a B.I.E.R. vessel (Joslyn Sterilizer Corp., USA) at 600 mg/l ethylene oxide (EO), 54ºC and 60% relative humidity (6). All steam exposures were done using saturated steam in a B.I.E.R. vessel (Joslyn Sterilizer Corp., USA) at 121°C (7). Exposed units and controls were incubated at the appropriate temperature and for the time required by the biological indicator type.

D_{121}-values and D_{EO}-values were determined using the Stumbo-Murphy-Cochran fraction negative procedure (SMCP) (8, 9). SMCP was chosen to reduce the effort and expense of this extensive testing by omitting the requirement of two successive total positives and two successive total negatives from each determination required by the Spearman-Karber procedure (2, 8). All values plotted are average SMCP D-values with standard deviations indicated by error bars.

The Prism 3.02 statistics software program, Graphpad, Inc., San Diego, CA, www.graphpad.com, was used to calculate descriptive statistics and perform linear regression analysis and graph the results.

Delayed Incubation

EZTest-Steam self-contained biological indicator units containing three different lots of *Geobacillus stearothermophilus* ATCC 7953 (S-246, S-255, S-257 – see Table I.) were exposed to the same time increments as used originally to determine initial D_{121}-values for the lots, using twenty samples per exposure. Incubation times were delayed by holding the units at controlled room temperature for less than two hours, for twenty-four hours or for seven days before subsequent incubation and D_{121}-value calculation. Each lot was tested in triplicate, a total of sixty units per exposure time.

EZTest-Gas self-contained biological indicator units containing three different lots of *Bacillus atrophaeus* ATCC 9372 (G-105, G-107 and G-108 – see Table I.) were exposed to the time increments used to determine initial D_{EO}-values for the lots, using 20 samples per exposure. A set was cultured immediately (within fifteen minutes), then incubation times were delayed for four hours, twenty four hours and seven days by holding at controlled room temperature before culturing. Each lot was tested in triplicate, a total of sixty units per exposure time.

SGMStrip biological indicators containing three different lots of *Geobacillus stearothermophilus* ATCC 7953 (Bst-252, Bst-258, Bst-262 – see Table I.) were exposed to the same dosages of steam used to determine the initial D_{121}-value for the lots, using 20 carriers per exposure. One set was cultured immediately and then incubation times were delayed by holding the carriers at controlled room temperature for four hours, twenty-four hours or seven days before culturing/incubation.

SGMStrip biological indicators containing three different lots of *Bacillus atrophaeus* ATCC 9372 (Bsub-255, Bsub-259 and RS000121 –see Table I.) were exposed to the same dosages of EO used to determine the initial D_{EO}-value for the lots, using 20 carriers per exposure. One set was cultured immediately and then incubations times were delayed by holding at controlled room temperature for four hours, twenty-four hours or seven days before culturing/incubation.

Table I. Description and Characteristics of Biological Indicator Lots

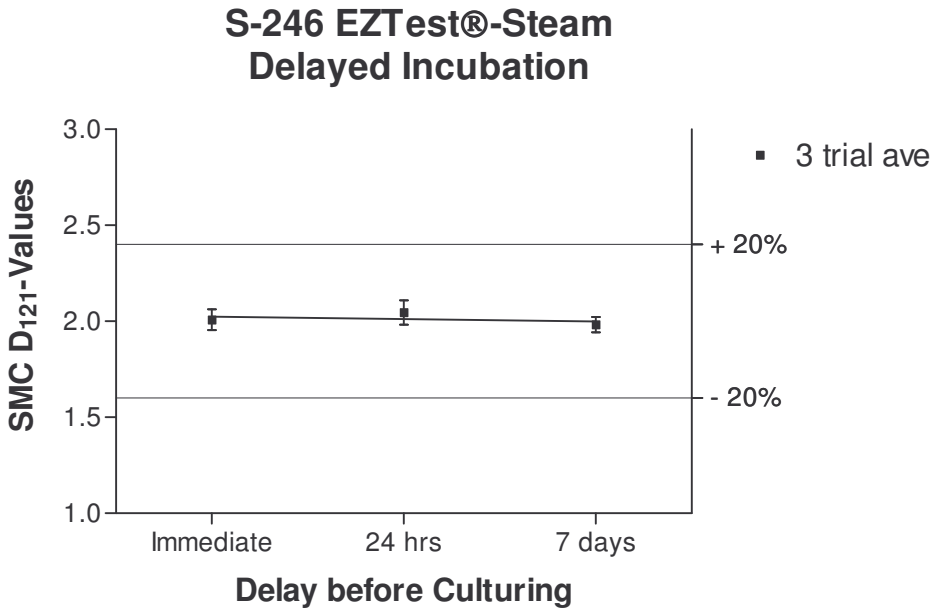
Lot #	Species	Crop #	Population	SMCP D-value*
S-246	G. st	Bst-081898/E5-4	1.7 x 10^5/unit	2.0
S-255	G. st	Bst-081898/E5-4	1.7 x 10^5/unit	2.1
S-257	G. st	Bst-081898/E5-7	1.9 x 10^5/unit	2.2
G-105	B. atr	Bsub-100200/E2-1	1.8 x 10^6/unit	4.0
G-107	B. atr	Bsub-042801/E2-1	2.2 x 10^6/unit	3.5
G-108	B. atr	Bsub-042801/E2-3	1.8 x 10^6/unit	4.0
Bst-252	G. st	Bst-110700/S7-2	1.9 x 10^5/carrier	1.7
Bst-258	G. st	Bst-052499/S6-1	1.5 x10^5/carrier	2.9
Bst-262	G. st	Bst-051601/S3-1	2.1 x 10^6/carrier	1.9
Bsub-255	B. atr	Bsub-042801/S3-2	2.2 x 10^6/carrier	3.9
Bsub-259	B. atr	Bsub-060501/S2-2	1.8 x 10^6/carrier	3.6
RS000121	B. atr	Bsub-071100/S5-1	1.7 x 10^6/carrier	3.4

* = D-value at < 2 hrs incubation delay

RESULTSAND DISCUSSION

EZTest-Steam biological indicators exposed in 20 unit groups, in triplicate, were allowed to sit for less than two hours, twenty-four hours or seven days before incubation and subsequent D_{121}-value determination by SMCP. The results are presented in Figures 1, 2 and 3. All error bars represent standard deviations.

There are no significant differences among the various delay times as determined by linear regression analysis for S-246, S-257 or for S-255. Slopes of regression lines calculated on data from each replicate for each lot were not significantly different from zero.



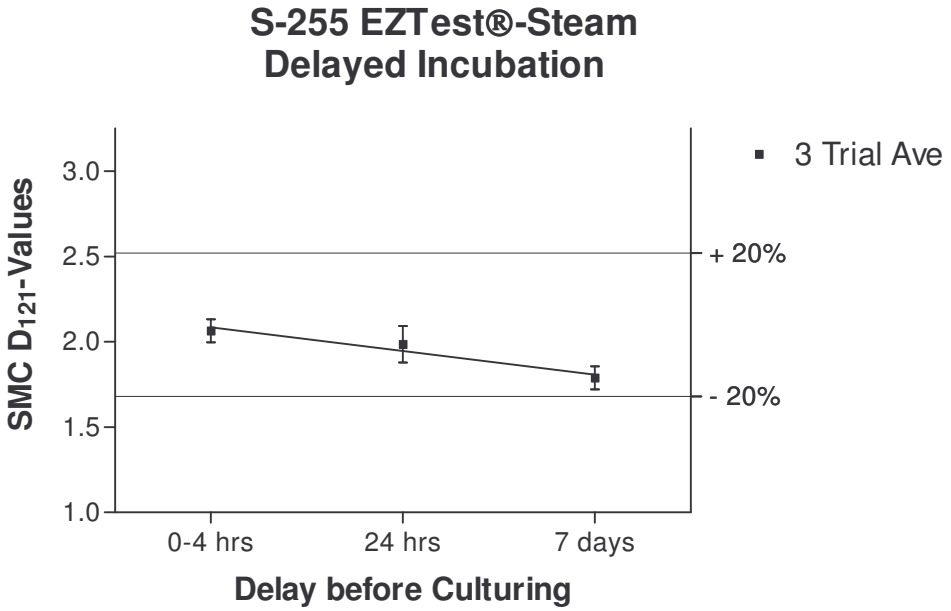
BIs Positive/20 Exposed

Exposure	Immediate			24 hr delay			7 day delay		
Time (min)	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
11	19	17	20	19	17	20	20	15	15
12	19	18	16	20	18	18	13	10	11
13	16	11	14	16	14	9	10	6	13
14	3	1	6	2	8	8	3	1	3
15	2	1	1	0	2	1	2	12	3
16	6	2	1	6	0	0	6	3	1
17	2	0	1	1	2	0	0	0	1
D_{121}-Values	2.08	1.95	2.04	2.11	2.04	1.99	2.03	1.97	1.99

D_{121}-value Stats	Immediate	24 hrs	7 days
Mean	2.0	2.0	2.0
Std. Deviation	0.067	0.063	0.030

Linear Regression	Average
P value	0.7367
Deviation from zero?	Not Significant

Figure 1. SMCP D_{121}-Values and Delayed Culturing Results for Lot # S-246 including raw data, descriptive statistics and regression analysis.



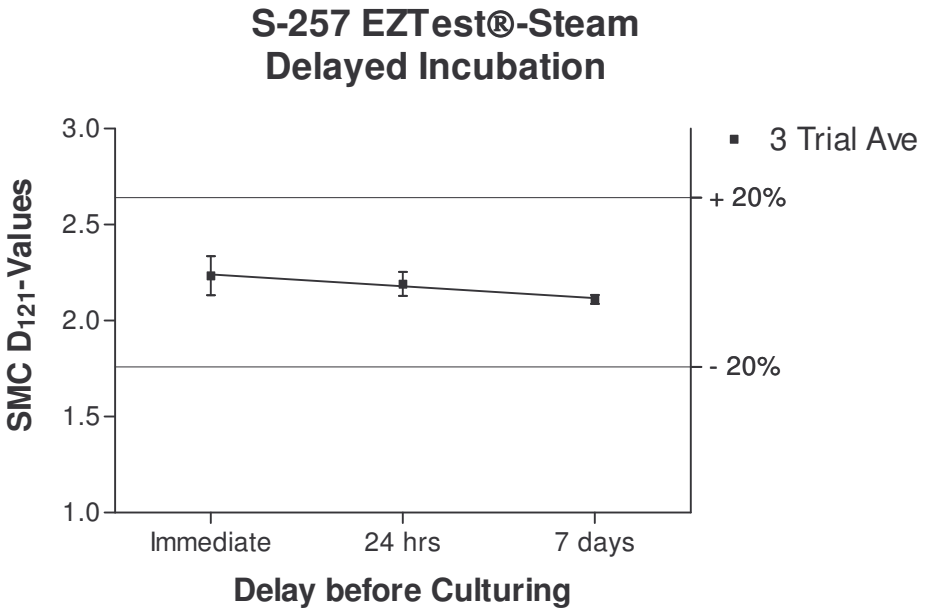
BIs Positive/20 Exposed

Exposure	Immediate			24 hr delay			7 day delay		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
[8]Time (min)	20	20	20	14	20	16	13	11	13
9.5	15	18	17	17	16	16	6	4	9
11	6	4	17	14	19	9	4	7	2
12.5	5	4	6	1	0	3	1	2	2
14	0	4	1	2	0	0	0	0	0
D_{121}-Values	1.99	2.08	2.12	1.96	2.10	1.89	1.74	1.87	1.76

D_{121}-Value Stats	Immediate	24 hrs	7 days
Mean	2.1	2.0	1.8
Std. Deviation	0.068	0.11	0.068

Linear Regression	Average
P value	0.1544
Deviation from zero?	Not Significant

Figure 2. SMCP D_{121}-Values and Delayed Culturing Results for Lot # S-255 including raw data, descriptive statistics and regression analysis.



BIs Positive/20 Exposed

Exposure	Immediate			24 hr delay			7 day delay		
Time (min)	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
8	20	20	20	20	20	20	20	20	20
9.5	20	20	18	20	16	19	19	16	18
11	11	16	20	16	14	16	13	11	13
12.5	6	9	17	7	6	5	3	10	4
14	1	4	8	3	3	1	3	1	5
D_{121}-Values	2.12	2.27	2.32	2.23	2.12	2.22	2.11	2.09	2.13

D_{121}-value Stats	Immediate	24 hrs	7 days
Mean	2.2	2.2	2.1
Std. Deviation	0.10	0.062	0.024

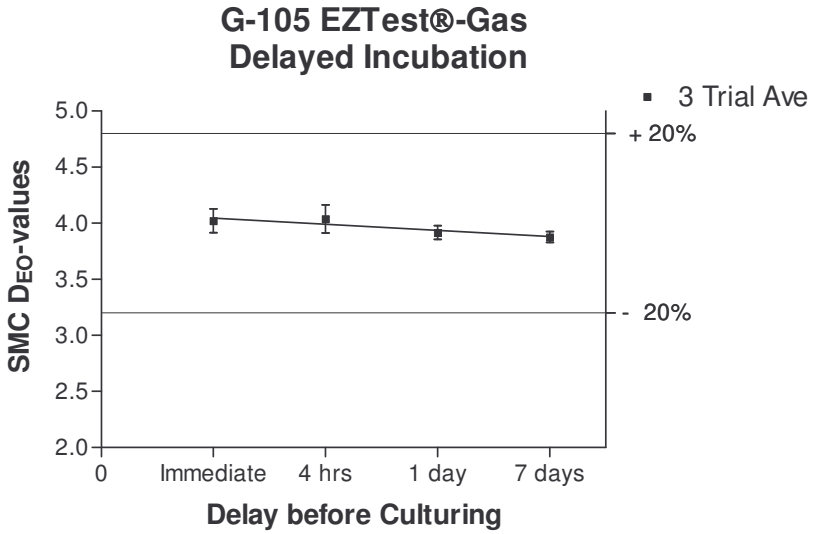
Linear Regression	Average
P value	0.1136
Deviation from zero?	Not Significant

Figure 3. SMCP D_{121}-Values and Delayed Culturing Results for Lot # S-257 including raw data, descriptive statistics and regression analysis.

Since the difference between the means at each delay time also all remain well within +/- 20% as stated by USP as depicted graphically, delaying incubation time for as long as seven days has no adverse effects on calculated D_{121}-values.

Results of three separate lots of EZTest-Gas biological indicators exposed in triplicate and then cultured after delayed holding times are present in Figures 4, 5 and 6. Linear regression analysis of replicates for each of the lots showed no differences between

slopes of the regression lines and pooled slopes could be calculated for each lot number. The slopes were also not significantly different from 0. Descriptive statistics depicted graphically and by table show that all D_{EO}-values calculated again remain within the limits of +/- 20%.



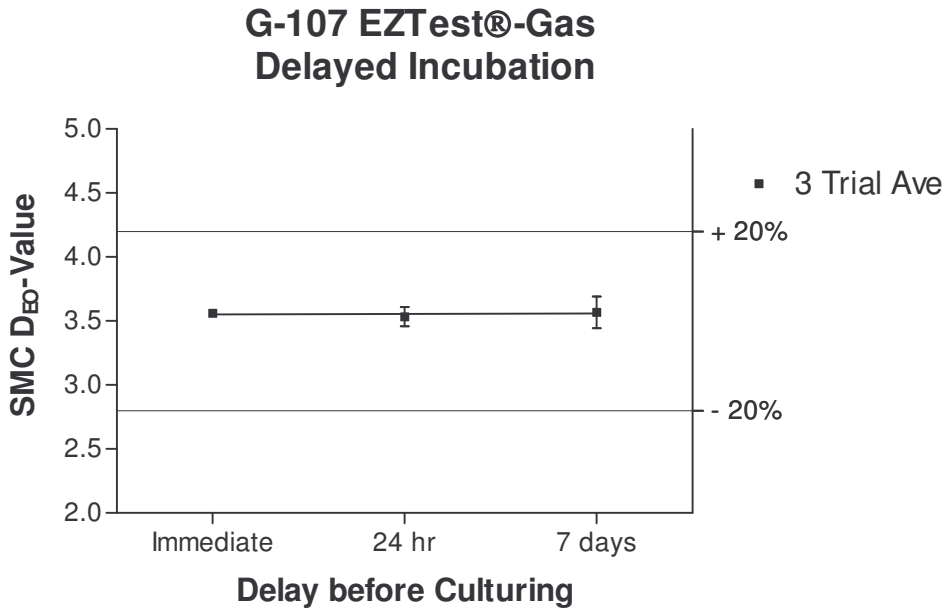
Bls Positive/20 Exposed

Exposure	Immediate			4 hr delay			24 hr delay			7 day delay		
time (min)	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
20	20	20	20	20	20	20	20	20	20	20	20	20
22	20	17	20	20	20	20	20	18	20	18	18	19
24	18	14	17	18	18	15	17	19	13	16	15	14
26	11	5	8	9	15	8	9	5	3	5	11	4
28	5	3	4	1	7	3	2	2	2	1	3	3
30	3	3	1	0	2	2	1	2	1	1	0	0
32	-	-	2	-	-	0	-	-	0	-	-	1
D_{-EO}-Values	4.14	3.93	4.00	3.93	4.18	4.00	3.96	3.95	3.85	3.84	3.93	3.86

D_{EO}-value Stats	Immediate	4 hrs	24 hrs	7 days
Mean	4.0	4.0	3.9	3.9
Std. Deviation	0.11	0.12	0.061	0.048

Regression Analysis	Average
P value	0.0885
Deviation from zero?	Not Significant

Figure 4. SMCP D_{EO}-Values and Delayed Culturing Results for Lot#G-105 including raw data, descriptive statistics and regression analysis.



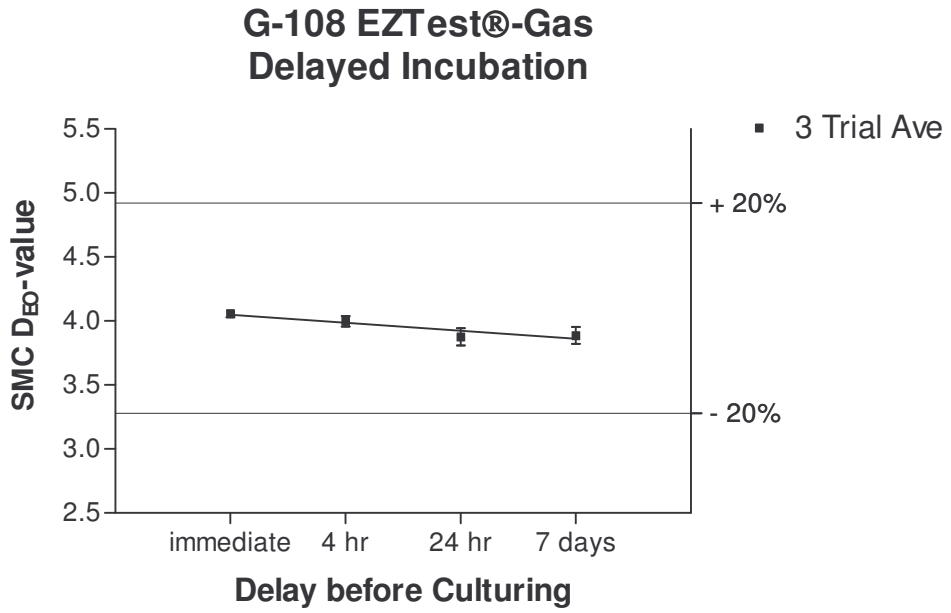
BIs Positive/20 Exposed

Exposure	Immediate			24 hr delay			7 day delay		
time (min)	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
20	20	19	20	20	20	20	20	20	20
22	20	8	17	20	16	17	10	12	18
24	18	9	7	17	11	3	3	12	8
26	9	2	2	9	2	0	2	0	1
28	1	1	0	2	0	0	0	2	1
D_{EO}-Values	3.55	3.48	3.43	3.55	3.62	3.68	3.59	3.50	3.59

D_{EO}-value Stats	0-2 hrs	24 hrs	7 days
Mean	3.6	3.5	3.6
Std. Deviation	0.022	0.076	0.12

Linear Regression	Average
P value	0.8712
Deviation from zero?	Not Significant

Figure 5. SMCP D_{EO}-Values and Delayed Culturing Results for Lot # G-107 including raw data, descriptive statistics and regression analysis.



BIs Positive/20 Exposed

Exposure	Immediate			4 hours			24 hr delay			7 day delay		
time (min)	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
20	20	20	20	20	20	20	20	20	20	20	20	19
22	19	20	20	18	20	19	19	19	20	19	18	19
24	14	15	17	11	13	11	5	8	12	7	8	14
26	10	10	11	8	10	6	4	5	9	6	4	9
28	6	3	2	5	2	4	4	0	3	3	3	4
30	1	3	0	5	3	0	0	2	1	1	1	0
32	1	0	0	0	0	0	1	0	0	0	0	0
D_{EO}-Values	4.08	4.07	4.02	4.03	4.02	3.95	3.84	3.83	3.95	3.88	3.82	3.96

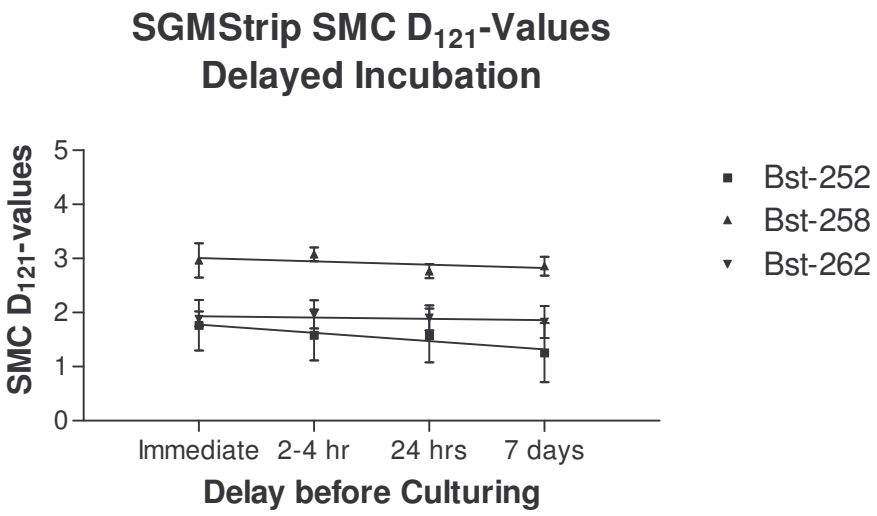
D_{EO}-value Stats	immediate	4 hr	24 hr	7 days
Mean	4.1	4.0	3.9	3.9
Std. Deviation	0.030	0.041	0.067	0.067

Linear Regression	Average
P value	0.0705
Deviation from zero?	Not Significant

Figure 6. SMCP D_{EO}-Values and Delayed Culturing Results for Lot #G-108 including raw data, descriptive statistics and regression analysis.

Results of delaying incubation times on three separate lots of *Geobacillus stearothermophilus* ATCC7953 on SGMStrips for steam are presented in Figure 7. below. Regression analysis of the SMC D_{121}-values showed no significant deviation from zero for the calculated slopes. Results for Bst-252 were not significant although the P-value of 0.064 is the lowest in these results. This lot is also the oldest (closest to expiration date) lot to be tested.

Results of delaying incubation times on ethylene oxide exposed SGMStrip biological indicators carrying *Bacillus atrophaeus* ATCC 9372 are presented in Figure 8 below. Again regression analysis substantiates the lack of differences from zero for the slopes of the lines and therefore an absence of affect. Descriptive statistics were not presented for SGMStrip data since replicate values (parallels) were not performed.



Regression Analysis	Bst-252	Bst-258	Bst-262
P value	0.0636	0.3964	0.4879
Deviation from zero?	Not Significant	Not Significant	Not Significant

Bst-252 # positive/ 20 exposed

Exposure	Immediate	4 hr delay	24 hr delay	7 day delay
4	20	20	20	16
6	20	18	18	19
8	18	12	12	6
10	4	4	2	2
12	3	2	3	0
`14	0	0	0	0
D_{121}-Values	1.8	1.6	1.6	1.4

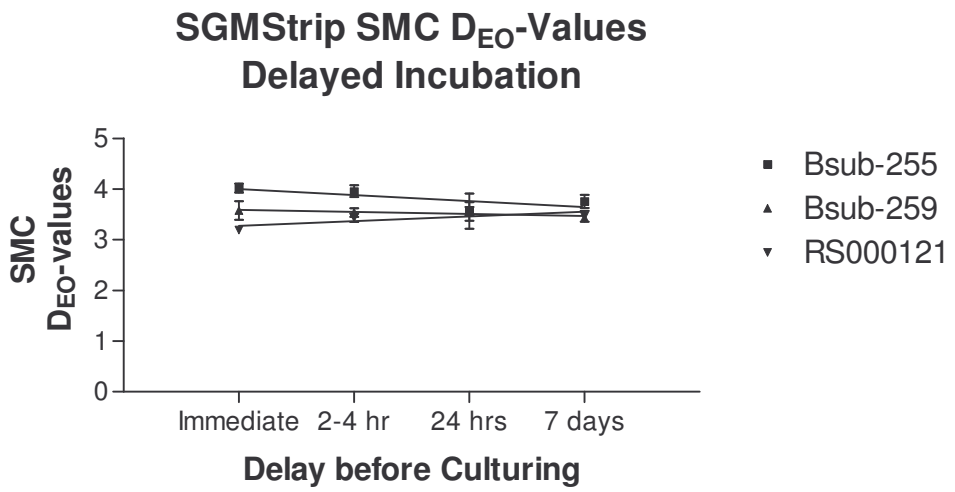
Bst-258 # positive/ 20 exposed

Exposure	Immediate	4 hr delay	24 hr delay	7 day delay
11.5 min	20	20	20	20
13	18	20	17	19
14.5	16	19	11	18
16	13	9	5	5
17.5	10	7	2	2
19	2	2	0	0
D_{121}-Values	3.0	3.1	2.8	2.9

Bst-262 # positive/ 20 exposed

Exposure	Immediate	4 hr delay	24 hr delay	7 day delay
9	20	20	20	20
10.5	19	20	20	15
12	8	10	7	9
13.5	1	6	4	4
15	0	1	1	0
17.5	0	2	0	0
D_{121}-Values	1.9	2.0	1.9	1.8

Figure 7. SGMStrip Saturated Steam Exposure and Delayed Culturing Results including raw data and regression analysis.



Regression Analysis	Bsub-255	Bsub-259	RS000121
P value	0.2709	0.2011	0.2259
Deviation from zero?	Not Significant	Not Significant	Not Significant

Bsub-255 # positive / 20 exposed

Exposure	Immediate	4 hr delay	24 hr delay	7 day delay
20	20	20	17	20
22	20	20	18	19
24	19	18	10	7
26	9	11	2	7
28	3	2	1	2
30	2	0	0	1
D_{EO}-Values	4.0	4.0	3.6	3.8

Bsub-259 # positive / 20 exposed

Exposure	Immediate	4 hr delay	24 hr delay	7 day delay
20	20	20	20	18
22	20	20	19	10
24	9	4	2	4
26	1	2	0	0
28	0	0	0	1
D_{EO}-Values	3.6	3.5	3.6	3.4

RS000121 # positive / 20 exposed

Exposure	Immediate	4 hr delay	24 hr delay	7 day delay
16	20	20	20	20
18	20	20	20	20
20	20	20	19	17
22	3	8	14	15
24	0	4	7	3
26	0	0	0	1
D_{EO}-Values	3.2	3.4	3.6	3.5

Figure 8. SGMStrip Ethylene Oxide Exposure and Delayed Culturing Results including raw data and regression analysis

CONCLUSIONS

Delaying the culturing of exposed biological indicators, whether they have been exposed to saturated steam at 121°C or to ethylene oxide at 600 mg, 54°C and 50% RH or contain *Geobacillus stearothermophilus* or *Bacillus atrophaeus* respectively, beyond the two hours specified by ISO (1) or the four hours recommended by USP (2) does not affect resistance performance. There was no significant affect on SMCP calculated D-values of either self-contained or strip biological indicators. Delays of up to seven days at controlled room temperature still allowed the determination of all D-values within ±20% of those calculated when cultured immediately (less than fifteen minutes of delay before incubation). Such stringent time limitations therefore appear to be unnecessary.

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