Running title: *S.* Mississippi inactivation on hazelnuts

Thermal Inactivation of *Salmonella* Mississippi on Hazelnuts

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Abstract

Salmonellosis has been linked to consumption of tree nuts and nut products, including almonds, pecans and hazelnuts. In Tasmania, Australia, where hazelnut production is a growing industry, validated process controls are needed to reduce risk posed by endemic strains of *Salmonella* Mississippi. Thermal inactivation is commonly used to control *Salmonella* on nuts, as documented in published studies. However, no reports describe thermal inactivation of *Salmonella* on hazelnuts, a product increasingly popular worldwide. Inactivation kinetics of *Salmonella* Mississippi strains M1 and M14 were measured on hazelnuts from 50—70 ˚C, demonstrating an initial linear inactivation phase followed by a lower rate of tailing. Linear models were fitted separately to both inactivation phases, as well as to the full curve, demonstrating Z-values ranging from 16.2 to 27.8 °C. The time to achieve a 5-log reduction at 70 and 50 °C ranged from 49 - 125 min and 668 - 2020 min, respectively. A Weibull model was also evaluated, however a weak correlation was observed between temperature and parameters *p* and δ over the temperature range.

1. Introduction

The demand for hazelnuts has increased, driven in part by nutritional properties including bioactive compounds, antioxidants, and dietary fiber, some of which have been reported to reduce cardiovascular risk (Wani et al., 2020). This and global demand have strengthened the hazelnut industry in the Australian state of Tasmania where climate and soil conditions are favorable for cultivation (Baldwin et al., 2007). However, such positive economic prospects reinforce the need for validated preventive controls to ensure food safety, such as achieving a 5-log reduction in *Salmonella* species (FDA, 2009; FDA, 2011).

Salmonella can potentially contaminate many types of nuts, including hazelnuts, originating on incoming products, from within facilities, on equipment contaminated with rodent droppings or by infected workers (Podolak et al., 2010). For example, in a 3-year-survey of Australia hazelnuts, 34% of pre-

roasted hazelnuts were contaminated with *Salmonella*, at an average level of 2.5 log CFU/g (Eglezos et al., 2008). Similar surveys have been conducted in the USA and England (Little et al., 2010; Zhang et al., 2020). Furthermore, hazelnuts have been recalled due to contamination with *Salmonella* (Yada and Harris, 2020).

For undefined reasons, the infection rate of *Salmonella* Mississippi in Tasmania is relatively high for a single *Salmonella* serotype, when compared to other Australian states and many countries of the world (Ashbolt and Kirk, 2006). In a case-controlled study, *Salmonella* Mississippi infections were found to be associated with exposure to native animals and untreated drinking water, suggesting that wildlife species serve as a reservoir (Ashbolt and Kirk, 2006). As such, the potential for *Salmonella* Mississippi to contaminate hazelnuts in the environment necessitates effective prevention controls.

Thermal treatment is a common method used to process edible nuts, by which drying and roasting inactivates pathogens while improving flavor (Ban and Kang, 2016; Brandl et al., 2008; Izurieta and Komitopoulou, 2012; Jeong et al., 2011; Venkitasamy et al., 2017; Villa-Rojas et al., 2013). For almonds, Villa-Rojas et al. (2013) measured *Salmonella* Enteritidis PT30 inactivation in kernels at 56-80˚C, with an associated product water activity of 0.601-0.946. These studies were extended by Ban and Kang (2016) who compared survival of *Salmonella* Typhimurium and *Salmonella* Enteritidis PT30 in almonds, as well as on in-shell pistachios under saturated steam at 100˚C, and for superheated steam at 125-200˚C.

Among the limited reports for hazelnuts, Farakos et al. (2017) measured survival kinetics of *Salmonella* Anatum, *Salmonella* Enteritidis, *Salmonella* Oranienburg, *Salmonella* Sundsvall and *Salmonella* Tennessee at a low temperature range of 4—25 ˚C. Also, Izurieta and Komitopoulou (2012) assessed the effect of moisture on *Salmonella* Oranienburg and *Salmonella* Enteritidis PT30 survival on hazelnut shells at 75 and 80 ˚C. However, there is no information about inactivation of *S*. Mississippi, or other *Salmonella* serovars, on hazelnut kernels at relevant processing temperatures.

2. Materials and methods

2.1. Hazelnut source and processing

Fresh raw in-shell hazelnuts were collected directly from a hazelnut orchard in Kettering, Tasmania on two separate days (i.e. trials 1 and 2). Using standard commercial procedures, in-shell hazelnuts were spread in a single layer on a wooden shelf in a ventilated room and dried for two weeks at 20—25 ˚C. Three to four days prior to each trial, in-shell hazelnuts were manually opened using a nut cracker, and kernels stored in a sealed glass jar at 20—25 ˚C.

2.2. Sample preparation

On the day of experimentation, 100—110 g shelled hazelnuts were transferred from the container to a 1-L stainless steel blender jar (Waring, USA), and autoclaved at 121 ˚C for 25 min. Sterilized hazelnuts were then homogenized and water activity measured (Aqualab CX-2, Aqualab, WA, USA) for two separate 2-g samples. Water activity was again measured at the end of each experiment; initial and final water activity ranged from 0.500—0.652 and 0.540—0.676, respectively.

2.3. Bacterial strains and inoculum

Salmonella Mississippi strain M1 and M14 were obtained from the University of Tasmania, Centre of Food Safety & Innovation culture collection. Strain M1 was originally isolated from a sewage treatment plant at Macquarie Point in Hobart, Tasmania; M14 was originally isolated from lizard droppings, collected by the University of Tasmania, Department of Zoology. Bacterial cultures were stored at -80 ˚C prior to experimentation. A frozen bead of each strain was streaked on tryptic soya agar (TSA; tryptic soya broth [Oxoid CM0219, Thermo Fisher Scientific Inc., Australia] and agar [Grade J3, Gelita, Australia]), incubated at 37 ˚C for 24 h, and then the agar culture stored at 4 ˚C. Before each experiment, TSA cultures were sub-cultured in 10 mL tryptic soy broth (TSB) at 37 ˚C for 24 h without agitation.

86 One milliliter of each culture was added to duplicate 2-mL microcentrifuge tubes (3810X, Eppendorf
87 South Pacific Pty. Ltd., New South Wales, Australia) and centrifuged at 5,000 rpm for 10 min at 25 ˚C.
88 Bacterial pellets were washed twice with peptone water (1% Bacterial Peptone, Oxoid LP0037, Thermo
89 Fisher Scientific Inc.) by centrifugation, and then re-suspended in 0.5 mL peptone water. Finally, the
90 content of both microcentrifuge tubes was combined into a single tube and vortexed to suspend cells.

91 *2.4. Experimental studies*

92 Aliquots of blended hazelnuts (2 ± 0.05 g) were transferred to separate sterile 50 mL polypropylene
93 centrifuge tubes (Cellstar Polypropylene tube, Greiner Bio-One, USA), with a single tube used to record
94 temperature at 1-min intervals by a data logger (i-button, DS1922L-F5# Thermochron, Maxim
95 Integrated, USA). Experimental tubes containing blended hazelnuts were placed in a temperature-
96 controlled water bath (SWB20, Ratek, Australia) pre-adjusted to 50, 55, 60, 65, and 70˚C. Next, 20 µL of
97 strain M1 or stain M14 (10^9—10^{10} CFU/ml) was added to each tube. The same volume of sterile distilled
98 water was added to two separate tubes used to measure water activity at the end of the experiment. At
99 each sampling time, two tubes containing inoculated hazelnuts were removed, immediately immersed
100 in 4 ˚C ice water, diluted 10-fold in 18 mL peptone water, and then stomached for 1 min (BagPage Plus
101 400, Interscience, France). One millilitre of stomached sample was diluted in 10-fold serial increments of
102 peptone water, and then 0.1 mL plated on TSA, in duplicate. Plates were incubated at 37 ˚C for 24-28 h,
103 and CFU values transformed to $\log_{10}$ CFU/g sample.

104 *2.5. Data analysis*

105 The linear regression function in Excel® was used to estimate inactivation rate. D- and Z-values were
106 calculated as described by Willey et al. (2008). Data were also fitted by the modified Weibull model of
107 Albert and Marfart (2005) using Glna FiT (version 1.6) (Geeraerd et al., 2005).

108 **3. Results and discussion**

Salmonella Mississippi strains M1 and M14 were inactivated on blended hazelnuts from 50—70 ˚C. In general, inactivation curves displayed bi-phasic patterns (Fig. 1), with the highest inactivation rate observed in phase-1, followed by a lower inactivation rate in phase-2. Similar patterns have been reported for other *Salmonella* strains, which can be influenced by the food matrix and/or incubation temperature, as well as distributions of inactivation sensitivity (rates) among a bacterial population (Izurieta and Komitopoulou, 2012; Farakos et a., 2013; Villa-Rojas et al., 2013). Examples of intrinsic factors reported to influence inactivation rate include lower water activity and lipids that can produce two-and three- phase inactivation curves with 'shoulders' and 'tails' (Farakos et al., 2013, 2016; Juneja et al., 2001; Podolak et al., 2010; Shachar and Yaron, 2006; Villa-Rojas et al., 2013).

Linear models have been used to measure rate for individual inactivation phases, such as *Salmonella* in wheat flour and other low moisture foods (Farakos et al., 2013; Smith et al., 2016). Average inactivation rates for phase-1 increased with temperature (Table 1); plots of temperature versus $\log_{10}$ D-value for M1 and M14 (Fig. 2) are described by the following equations:

M1 $y = -0.0566x + 4.956$ $r^2 = 0.876$ (1)

M14 $y = -0.0553x + 5.141$ $r^2 = 0.943$ (2)

where $y = \log_{10}$ D-value and x = temperature (°C)

Z-values for M1 and M14 were 17.7 and 18.1 °C, respectively (Table 2). The time to achieve a 5-log reduction at 70 and 50 °C ranged from 49 - 1188 min, respectively.

Inactivation rates were generally lower for phase-2 curves, along with markedly higher variability reflected in r^2 values (Table 1). Specifically, the average r^2 for M1 and M14 phase-1 curves was 0.79 and 0.88, compared to 0.41 and 0.56 for phase-2, respectively. Secondary plots of temperature versus log D-values for M1 and M14 (Fig. 3) are described by the equations:

M1 $y = -0.036x + 4.518$ $r^2 = 0.364$ (3)

M14 $y = -0.0515x + 5.546$ $r^2 = 0.453$ (4)

Z-values for M1 and M14 were 27.8 and 19.4 °C, respectively (Table 2).

A second modelling approach was done by fitting a primary linear model across the entire inactivation curve (Table 1). This could be a more practical and conservative approach, considering thermal inactivation kinetics would likely be influenced by variations in the hazelnut matrix (e.g. oil content and water activity) and among strains of *S*. Mississippi. Following this approach, a secondary plot of temperature versus log D-value for M1 and M14 (Fig. 4) is described by:

M1 $y = -0.0532x + 5.123$ $r^2 = 0.834$ (5)

M14 $y = -0.0616x + 5.686$ $r^2 = 0.942$ (6)

Z-values for M1 and M14 were 18.8 and 16.2 °C, respectively (Table 2). The time to achieve a 5-log reduction at 70 and 50 °C ranged from 118 - 2020 min, respectively.

Relatively high Z-values similar to those observed in this study have been previously reported for other foods contaminated with *Salmonella* spp., including cocoa beans (Z-value = 102.6 °C), cocoa nibs (Z-value = 50.3 °C), cocoa liquor (Z-value = 20 °C), dark chocolate (Z-value = 14 °C), and hazelnut shells (Z-value = 11 - 15 °C) (Izurieta and Komitopoulou, 2012; Krapf and Gantenbein-Demarchi, 2010; Nascimento et al., 2012). Also, thermal inactivation of *Salmonella* Oranienburg on crushed hazelnut shells and cocoa shells had Z-values of 11.85 and 15.36 °C, respectively, as well as 15.38 and 17.36 °C, for *Salmonella* Enteritidis PT30 (Izurieta and Komitopoulou, 2012).

A third modelling approach used the modified Weibull model as described by Villa-Rojas et al. (2013) using GInaFit v1.6 software (Geeraerd et al., 2005; Villa-Rojas et al., 2013).

$\log S(t) = -(t/\delta)^p$

and

M1 $\log\delta = -0.07T + 3.773 = -0.06 \times (T - 62.83)$ $r^2 = 0.833$

$p = 0.0222T - 0.371$ $r^2 = 0.0294$

$\delta = -0.4311\ p + 1.4431$ $r^2 = 0.0862$

M14 $\log\delta = -0.06T + 3.708 = -0.06 \times (T - 61.83)$ $r^2 = 0.951$

$p = 0.0078T + 0.3789$ $r^2 = 0.0677$

$\delta = -2.6275\ p + 3.7191$ $r^2 = 0.1515$

where $S(t) = N/N_0$, and N and N_0 is the bacterial population at the initial time (t=0), T is the time of heat treatment, *p* the parameter that describes the curve shape, δ the time for the first decimal reduction, and T the temperature (°C).

Unlike the linear models described earlier, a weak correlation was observed between temperature and parameters *p* and δ (r^2 for M1 = 0.0294, M14 = 0.0677; r^2 for M1 = 0.0862, M14 = 0.1515; respectively), as similarly described by van Boekel (2002). In the studies of Farakos et al. (2013, 2017), best-fits to inactivation curves varied by temperature, and the Weibull model produced better fits than linear models at temperatures >21 ˚C, whereas at 4 ˚C log-linear models resulted in improved fits. Villa-Rojas et al. (2013) observed that *Salmonella* inactivation curves for almond kernels were upward concaved (T=56—80 ˚C, aw=0.6—0.95) and were fitted best with a modified Weibull model with $p<1$ (0.29—0.76), depending on different a_w-temperature combinations. In the present study, there was not a strong correlation between parameters *p* and δ, or between these parameters and temperature, indicating that linear models may be preferred when designing thermal process controls over the lower temperature range used in the present study.

In conclusion, this study provides a quantitative description of *S.* Mississippi inactivation on roasted hazelnuts. The resulting models can be used by hazelnut processors, especially those located in *S.* Mississippi-endemic areas, to aid in the design of process preventive controls. However, all models must be validated before being implemented as process preventive controls.

Declaration of competing interest

None.

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253

Figure legends

Figure 1. Inactivation kinetics of *Salmonella* Mississippi M1 at 50 (upper left), 55 (upper right), 60 (middle left), 65 (middle right) and 70 ˚C (lower left).

Figure 2. Secondary plot of temperature versus $\log_{10}$ D-value for phase-1 inactivation rates of M1 and M14.

Figure 3. Secondary plot of temperature versus $\log_{10}$ D-value for phase-2 inactivation rates of M1 and M14.

Figure 4. Secondary plot of temperature versus $\log_{10}$ D-value for inactivation rates over the full inactivation curves of M1 and M14 based on Equation 5 and 6..

263 Table 1. Inactivation rates for *S.* Mississippi M1 (top) and M14 (bottom) based on linear fits to phase-1,

264 phase-2, and the full kinetic curve.

Temp	**Trial**	**Phase-1** (log CFU/min)		**Phase-2** (log CFU/min)		**Full curve** (log CFU/min)	
50	1	-0.011 (0.65)[a]	-0.008[b]	-0.001 (0.35)	-0.001	-0.002 (0.59)	-0.003
	2	-0.006 (0.89)		-0.002 (0.20)		-0.004 (0.83)	
55	1	-0.007 (0.78)	-0.012	-0.003 (0.77)	-0.003	-0.004 (0.85)	-0.005
	2	-0.016 (0.83)		-0.002 (0.60)		-0.006 (0.70)	
60	1	-0.038 (0.48)	-0.035	-0.013 (0.24)	-0.013	-0.017 (0.45)	-0.024
	2	-0.032 (0.84)		-0.014 (0.78)		-0.027 (0.92)	

65	1	-0.054 (0.83)	-0.062	-0.013 (0.48)	-0.009	-0.030 (0.79)	-0.024
	2	-0.068 (0.92)		-0.004 (0.10)		-0.018 (0.58)	
70	1	-0.125 (0.85)	-0.093	-0.002 (0.01)	-0.008	-0.031 (0.58)	-0.030
	2	-0.060 (0.85)		-0.014 (0.57)		-0.030 (0.81)	

[a] r^2 ; [b] average inactivation rate for trials 1 and 2.

268

Temp	Trial	Phase-1 (log CFU/min)		Phase-2 (log CFU/min)		Full curve (log CFU/min)	
50	1	-0.005 (0.97)	-0.004	-0.002 (0.72)	-0.002	-0.003 (0.87)	-0.003
	2	-0.004 (0.78)		ND		-0.003 (0.79)	
55	1	-0.006 (0.93)	-0.006	-0.001 (0.66)	-0.002	-0.003 (0.85)	-0.004
	2	-0.006 (0.77)		-0.002 (0.36)		-0.004 (0.81)	
60	1	-0.020 (0.86)	-0.018	ND	-0.001	-0.014 (0.85)	-0.014
	2	-0.015 (0.81)		-0.001 (0.43)		-0.014 (0.90)	

65	1	-0.037 (0.97)	-0.036	-0.001 (0.15)	-0.004	-0.022 (0.83)	-0.023
	2	-0.036 (0.94)		-0.008 (0.60)		-0.025 (0.90)	
70	1	-0.040 (0.85)	-0.046	-0.011 (0.75)	-0.021	-0.032 (0.86)	-0.037
	2	-0.051 (0.88)		-0.031 (0.77)		-0.041 (0.87)	

269

270

271 Table 2. Average D-values (min) for phase-1, phase-2, and full curves for *S.* Mississippi M1 (top) and M14

272 (bottom).

Temperature	**Phase-1**	**Phase-2**	**Full curve**
50	133.5	756.7	332.5
55	100.0	477.1	211.3
60	28.7	74.1	48.4
65	16.7	175.7	44.3
70	12.3	235.9	33.3
Z-value	*17.7*	*27.8*	*18.8*

273

Temperature	**Phase-1**	**Phase-2**	**Full curve**
50	227.0	527.7	364.0
55	161.2	848.9	291.8
60	57.6	97.4	72.1
65	27.6	571.2	42.4
70	22.5	63.5	27.8

Z-value	*18.1*	*19.4*	*16.2*

274

Figure 1

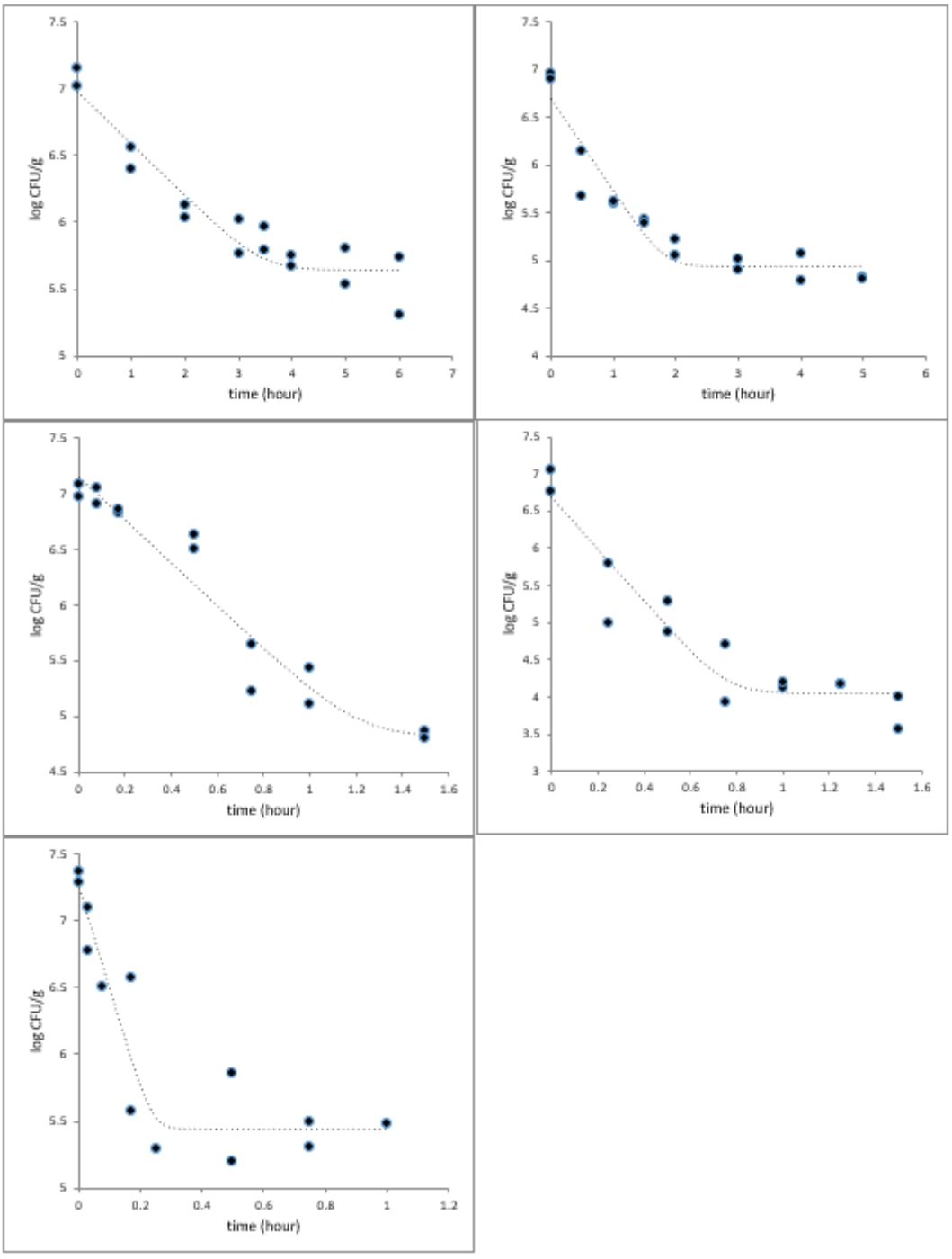
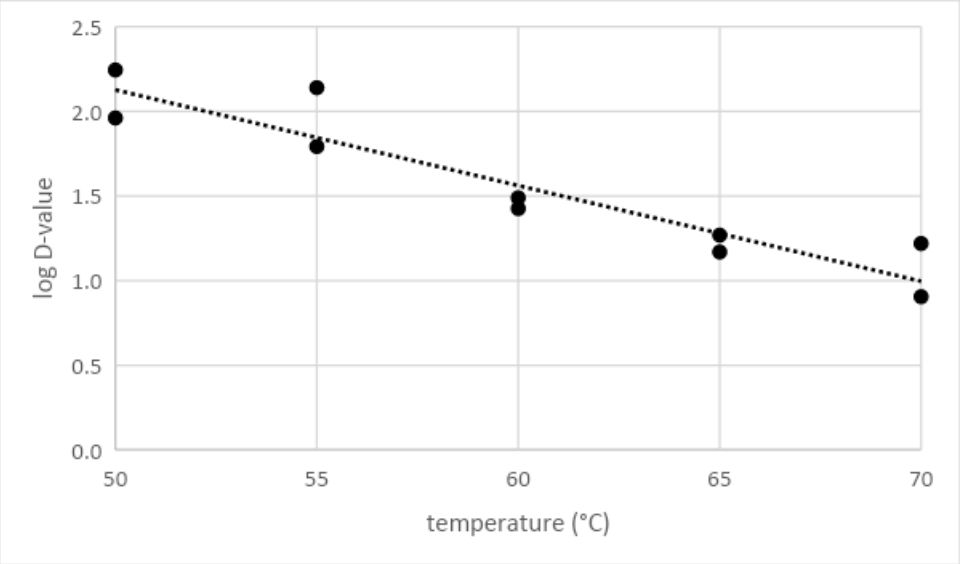


Figure 2

M1



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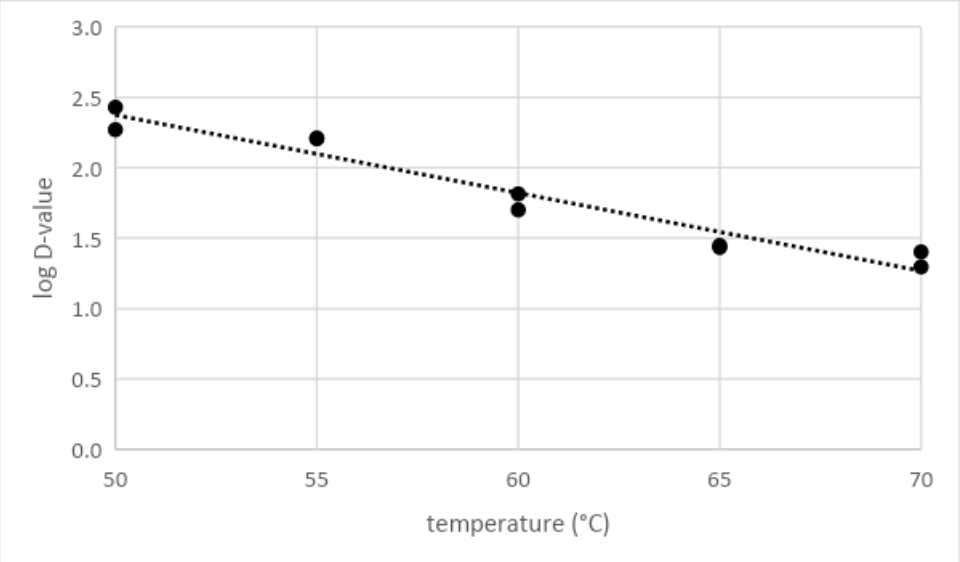
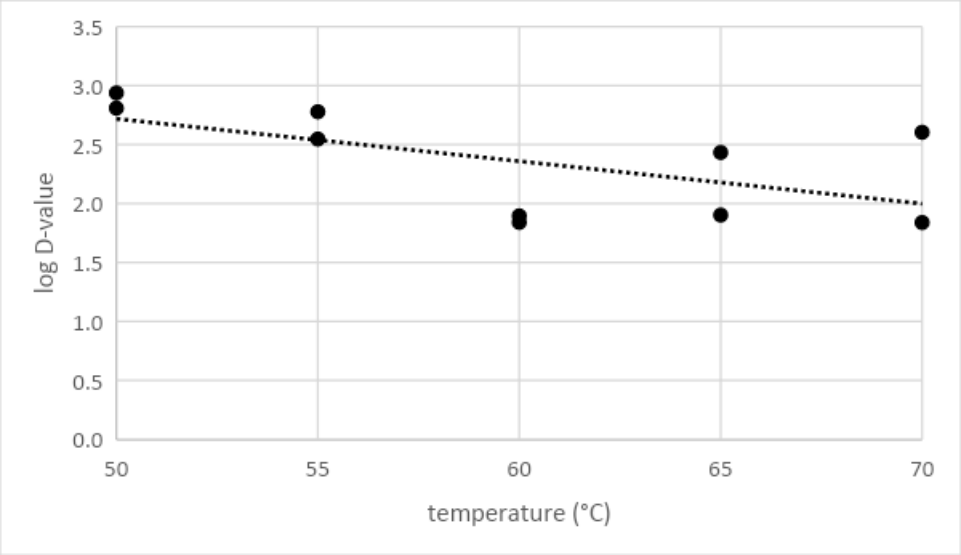


Figure 3

M1



M14

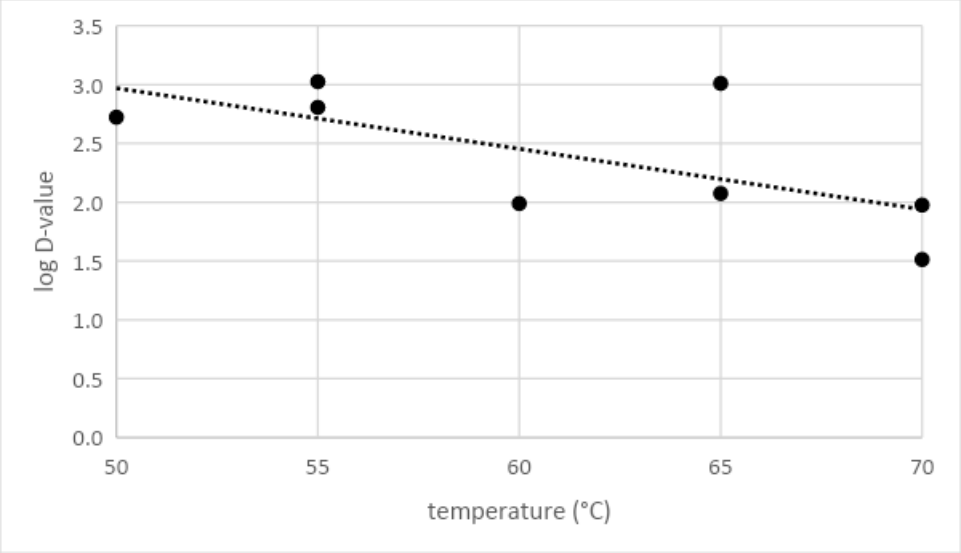
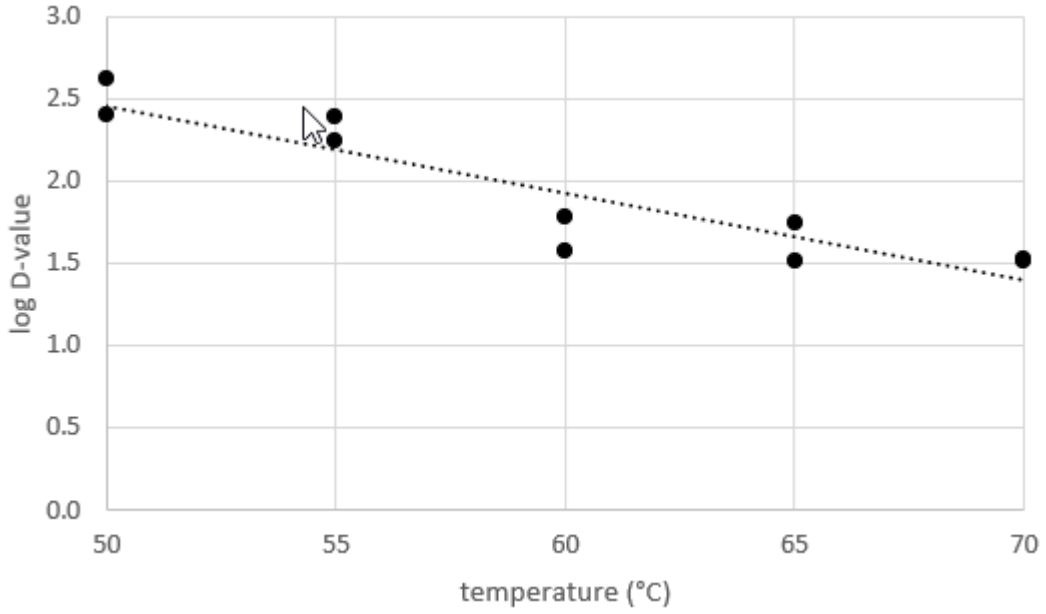


Figure 4

M1



M14