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4 Inactivation of a Norovirus by High Pressure Processing

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1 **Murine norovirus (strain MNV-1), a propagable norovirus, was evaluated for**
2 **susceptibility to high pressure processing. Experiments with virus stocks in Dulbecco's**
3 **Modified Eagle Medium (DMEM) demonstrated that at room temperature (20°C), the**
4 **virus was inactivated over a pressure range of 350 to 450 MegaPascals (MPa), with a 5-**
5 **minute 450-MPa treatment being sufficient to inactivate 6.85- \log_{10} PFU of MNV-1. The**
6 **inactivation of MNV-1 was enhanced when pressure was applied at an initial temperature**
7 **of 5°C; a 5-min pressure treatment of 350-MPa at 30°C inactivated 1.15- \log_{10} PFU of virus,**
8 **while the same treatment at 5°C resulted in a reduction of 5.56- \log_{10} PFU. Evaluation of**
9 **virus inactivation as a function of treatment times ranging from 0 to 150 s and 0 to 900 s at**
10 **5°C and 20°C respectively indicated that a decreasing rate of inactivation with time was**
11 **consistent with Weibull or log-logistic inactivation kinetics. The inactivation of MNV-1**
12 **directly within oyster tissues was demonstrated; a 5-min 400-MPa treatment at 5°C was**
13 **sufficient to inactivate 4.05- \log_{10} PFU. This work is the first demonstration that norovirus**
14 **can be inactivated by high pressure and suggests good prospects for inactivation of**
15 **nonpropagable human norovirus strains in foods.**

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1 Norovirus infection is a common worldwide problem, with approximately 40% of
2 infections in the U.S. being food-borne (30). Foods most frequently associated with norovirus
3 outbreaks are shellfish and produce, since these foods are often consumed uncooked. The
4 scientific literature is replete with food-borne norovirus outbreaks attributed to these foods (10,
5 23, 30, 34). Uncooked foods can become contaminated after exposure to contaminated irrigation
6 or propagation waters, or, in the case of produce, after human handling.

7 Research with human norovirus strains has been hampered by the lack of suitable
8 laboratory animals and the inability to propagate the virus *in vitro* (15). Consequently
9 genetically-related viruses, such as feline calicivirus (FCV; (14)) and, to a lesser extent, San
10 Miguel Sea Lion virus-17 (SMSV-17; (3)) have been used as research surrogates for non-
11 propagable human norovirus strains. Recently, murine norovirus (strain MNV-1), a virus that
12 can be propagated *in vitro*, has been identified (18, 38). Classified as a genogroup V virus,
13 MNV-1 has more biochemical, pathologic, and molecular similarities to human noroviruses than
14 other research surrogates used previously (39).

15 High pressure processing (HPP) has emerged as a promising non-thermal technology for
16 pasteurizing food products. The principal advantage of this technology is that foods retain an
17 uncooked character and taste. For the molluscan shellfish industry, HPP has particular utility,
18 since it facilitates the shucking process. Moreover, while organoleptic studies do describe some
19 changes in the character of pressure-treated oysters, HPP-treated oysters are well accepted by
20 consumers (13, 17, 28). Furthermore, HPP technology is currently used commercially to
21 eliminate *Vibrio* bacteria from U.S. Gulf Coast oysters (2, 12).

22 Beyond cooking, there currently is no adequate intervention for bivalve shellfish
23 contaminated with enteric viruses because these viruses can remain within shellfish for periods
24 beyond a few days (16, 24, 27, 35). Although some non-enveloped viruses are less sensitive, or

1 even completely resistant to pressures as high as 600 MPa (19, 22, 37), HPP has shown promise
2 for inactivation of hepatitis A virus (HAV) and norovirus surrogate viruses. Kingsley et al. (23)
3 demonstrated that 5-min room temperature treatments of 275 Megapascals (MPa) and 460 MPa
4 were sufficient to inactivate 7- \log_{10} PFU virus stocks of FCV and HAV, respectively.

5 Subsequent studies demonstrated the utility of HPP for the inactivation of HAV within oysters
6 and produce (4, 21). Characterizing inactivation of the norovirus surrogate FCV by HPP as a
7 function of treatment time indicated that while increased treatment time yielded greater
8 inactivation, there was a diminishing increase in inactivation consistent with non-linear Weibull
9 or log-logistic inactivation kinetics (8). In evaluating HPP inactivation of FCV as a function of
10 treatment temperature, it was noted that the inactivation increased by as much as 3- \log_{10} when
11 treatments were performed at refrigeration temperatures, or above 30°C, as compared to room
12 temperature (8).

13 In this publication, we evaluate HPP inactivation of the norovirus, MNV-1. This virus
14 displayed enhanced inactivation when HPP is performed at refrigeration temperatures.
15 Evaluation of HPP inactivation as a function of treatment time indicates a diminishing increase
16 in virus inactivation consistent with Weibull or log-logistic kinetics. Lastly, we demonstrate that
17 high pressure treatment can inactivate the virus directly within MNV-1 contaminated oysters
18 (*Crassostrea virginica*).

20 MATERIALS AND METHODS

21
22 **Virus and oysters.** Working stocks of MNV-1 were prepared using confluent monolayers
23 of mouse monocyte/macrophage cells RAW 264.7 (American Type Culture Collection,
24 Manassas, VA) cultured in high glucose Dulbecco's Modified Eagle media (DMEM; Gibco-

1 Invitrogen Co., Grand Island, NY) supplemented with 25 mM HEPES buffer, 10% fetal bovine
2 serum (FBS; Gibco-Invitrogen), 2 mM Gluta-MAX-1 (Gibco-Invitrogen), 100 U of penicillin,
3 and 100 µg/ml of streptomycin sulfate (Gibco-Invitrogen) essentially as described by Wobus et
4 al. (38).

5 Eastern oysters (*Crassostrea virginica*) were harvested from an approved area in Mobile
6 Bay, AL. After culling and sorting, 200 commercial-size oysters were placed into a depuration
7 flume at the US FDA Gulf Coast Seafood Laboratory, Dauphin Island, AL. Oysters were
8 maintained for > 2 wk prior to being transferred to a flume which utilized single-pass UV-treated
9 natural seawater maintained at 21-30°C. Salinities ranged from 5-28 ppt.

10 Three days before virus accumulation, 26 oysters were placed in the accumulation tank to
11 acclimate the shellfish to 10°C, as described previously (4). MNV-1 (5.6×10^9 PFU for trials 1
12 & 2 and 2.12×10^8 PFU for trial 3) was added to 4 L of sterile RO (reverse osmosis) water and
13 continually mixed at 4°C. Peristaltic pumps were adjusted to give a calculated overall
14 concentration within the accumulation tank of approximately 7000 PFU/ml for trial 1 and 2 and
15 660 PFU/ml for trial 3. After 24 h, the oysters were divided into eight groups of three oysters
16 each and shucked into sterile cups. The total weight for each group was approximately 30 g.

17 **High pressure treatment of oysters and MNV-1.** For temperature and direct inactivation
18 experiments, 1.5 ml aliquots of MNV-1 in DMEM with 10% FBS were placed in 2 × 3 inch
19 pouches modified from 4 × 6 inch sterile 3 mm thick polyethylene Stomacher pouches (Fisher
20 Scientific International, Ontario, Canada), heat sealed (Tilia Foodsaver Vac 540, San Francisco,
21 CA), then placed in 6 × 8 inch 3 mm thick nylon/polyethylene vacuum pouches (Prime Source
22 Packaging, Ltd., Spring, TX) and vacuum/heat sealed (Multivac, Inc. model A 300/16, Kansas
23 City, MO). Pouches were then subjected to pressures ranging from 250 MPa to 450 MPa at
24 specified starting temperatures. For MNV-1 contaminated oysters, shucked samples were

1 transferred into 4.5-mm pouches (Kapak Co., Minneapolis, MN) and heat-sealed using an
2 Impulse Food Sealer (American International Electric Co., Whittier, CA) according to the
3 manufacturer's instructions. An over pack 2-mm pouch was sealed over the inner pouch.
4 Refrigerated, shucked oyster samples were packed in accordance with International Air
5 Transport Association (IATA) Dangerous Goods Shipping Regulations in a biohazard shipping
6 container (SAF-T-PAK, STP 100, Alberta, Canada) and enclosed in an insulated carton with
7 "blue ice" packs to insure that the temperature remained $< 10^{\circ}\text{C}$ during shipping. Shipments
8 were by overnight carrier to the Food Science and Technology Department, Virginia Polytechnic
9 Institute and State University, Blacksburg, VA for processing. Pressurization of oyster samples
10 was performed for 5 min using a Quintus 35-L food press (QFP 35L-600; Flow International
11 Corporation, Avure Technologies Inc., Kent, WA). Samples were pressurized at 300, 325, 350,
12 375, and 400 MPa for 5-min at $\sim 5^{\circ}\text{C}$. Come-up times to reach final pressures were
13 approximately 75 s to 300 MPa and 90 s to 400 MPa. Pressure-release time was < 3 s.
14 Adiabatic heating during pressurization ranged from 10°C at 300 MPa to 13°C at 400 MPa.
15 After processing, the refrigerated samples were shipped overnight to the USDA Microbial Food
16 Safety Research Unit at Dover, DE, for virus extraction and assay.

17 **Virus extraction and plaque assays.** For MNV-1 stock virus in DMEM, all samples were
18 assayed using confluent 6-well dishes (Fisher Biotech, Fairlawn, NJ). Inoculation with 0.5 ml of
19 MNV-1 was for 2 h at 37°C followed by overlay with 2 ml of Modified Eagle medium (Gibco-
20 Invitrogen) containing 1.5% low melt agarose (Fisher Biotech) with 5% FBS, 2 mM Gluta-
21 MAX-1, 100 U of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin sulfate (Gibco-Invitrogen). After
22 three days incubation, plaques were visualized by staining with 0.03% neutral red (Fisher
23 Biotech) for 2 h at 37°C .

1 For MNV-1 extraction from contaminated oysters and plaque assay, three shellfish per
 2 treatment group were removed from pressurized sealed pouches, and placed in 50 ml conical
 3 tubes, and briefly centrifuged in a table top centrifuge to facilitate separation of oyster meat from
 4 oyster liquor. Non-pressurized (0 MPa) MNV-1-contaminated (positive) controls were also
 5 tested. Virus extractions were performed essentially as described by Calci et al. (4) by adding
 6 oyster meats (three oysters per treatment group) and phosphate buffer (0.15 M Na₃PO₄, pH 9.5,
 7 4°C) up to 100 ml, and homogenizing oyster meats in a laboratory blender (Waring Inc. New
 8 Hartford, CT) for 3-min at maximum speed. Homogenized extracts were centrifuged for 15 min
 9 at 15,000 × g and the supernatant was neutralized with 2 N HCl. Ten-fold serial dilutions were
 10 made in Earle's balanced salt solution (EBSS; Gibco-Invitrogen) and plaque assays were
 11 performed in triplicate using confluent monolayers of RAW cells (38). For undiluted and 1:10
 12 diluted oyster homogenates, two ml of extract or two ml of dilution were used to infect 100 mm
 13 confluent tissue culture dishes. Due to oyster debris remaining in the oyster homogenates, the
 14 100 mm dishes were washed with 2 ml of EBSS after inoculation and incubation for 2 hrs. For
 15 homogenate dilutions of 1:100 or greater, 0.5 ml was used to infect individual wells of a 6-well
 16 dish and washing was not performed. Plaques were visualized using neutral red staining 3 days
 17 post-infection.

18 **Modeling of survival curves.** The linear model assumes that all the cells or spores in a
 19 population have identical resistance to lethal treatments and their inactivation is governed by
 20 first-order kinetics (36).

$$21 \quad \log \frac{N}{N_0} = -\frac{t}{D} \quad (1)$$

22 Where N_0 = the initial number of virus (PFU/ml)

23 N = the number of survivors after an exposure time t (PFU/ml)

1 D (decimal reduction time) = the time (min) required to destroy 90% of the virus and
 2 is a measure of the resistance of a virus to lethal treatments

3 t = treatment time (min)

4 This Weibull model assumes that cells and spores in a population have different resistances and a
 5 survival curve is just the cumulative form of a distribution of lethal agents.

$$6 \quad \log \frac{N}{N_0} = -bt^n \quad (2)$$

7 where b and n are the scale and shape factors (33). The Weibull distribution corresponds to a
 8 concave upward survival curve if $n < 1$, concave downward if $n > 1$, and linear if $n = 1$. The
 9 log-logistic model was originally proposed by Cole et al. (11) and later was modified by Chen
 10 and Hoover (6) to avoid the use of different initial numbers and to reduce the number of
 11 parameters in the equation from four to three:

$$12 \quad \log \frac{N}{N_0} = \frac{A}{1 + e^{4\sigma(\tau - \log t)/A}} - \frac{A}{1 + e^{4\sigma(\tau + 6)/A}} \quad (3)$$

13 Where A = upper asymptote-lower asymptote (\log_{10} PFU/ml)

14 σ = the maximum rate of inactivation (\log_{10} (PFU/ml)/ \log_{10} min)

15 τ = the \log_{10} time to the maximum rate of inactivation (\log_{10} min)

16 The MSE values were used to compare the two models. The smaller the MSE values, the better
 17 the model to fit the data (31).

$$18 \quad MSE = \frac{\sum(\text{predicted} - \text{observed})^2}{n - p} \quad (4)$$

19 where n is the number of observations and p is the number of parameters to be estimated.

1 Survival curves were fitted using the PROC REG procedure of SAS (Release 9.1, SAS Institute
2 Inc., Cary, NC) for the linear model and PROC NLIN procedure of SAS for the nonlinear
3 models.

4 RESULTS

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6
7 The effect of high pressure on 1.5 ml aliquots of MNV-1 in DMEM tissue culture media
8 was evaluated using 5-min treatments at 20°C. A logarithmic reduction in virus titer was
9 observed from 325 MPa through 450 MPa (Fig. 1). To evaluate the effect of temperature on
10 virus inactivation, 5-min 350-MPa pressure treatments of virus stocks were performed at 5, 10,
11 20, and 30°C (Table 1). Results indicated that a starting temperature of 5°C, when pressure was
12 applied, gave the greatest degree of inactivation, reducing virus titer by 5.56- \log_{10} , while warmer
13 temperatures of 20 and 30°C gave appreciably less inactivation of 1.79 and 1.15- \log_{10} ,
14 respectively.

15 Inactivation of MNV-1 was also evaluated as a function of pressure level and treatment time
16 (Fig. 2). To characterize high pressure treatments as a function of time, time course analyses
17 were performed on virus stocks. Since there was considerable disparity in the response to
18 pressure as a function of temperature, pressures of 375 MPa at 20°C and 325 MPa at 5°C were
19 performed. As pressure magnitudes and/or treatment times increased, virus inactivation also
20 increased. The shapes of the inactivation curves were characterized by a rapid initial drop in
21 viral counts followed by tailing caused by a diminishing inactivation rate. Visual inspection of
22 these survival curves indicated that a nonlinear model, rather than a linear model, would better
23 describe these data. Fitting the survival data with the linear and nonlinear models (Fig. 2) and
24 comparing the goodness-of-fit of the models using mean square error (*MSE*) values confirmed

1 this observation. Among the three models compared, the log-logistic model had the lowest *MSE*
2 values, indicating that it produced the best fit to the inactivation curves. The linear model
3 produced the poorest fit. The mean *MSE* values for the linear, Weibull, and log-logistic models
4 were 1.76, 0.16, and 0.14, respectively (Table 2). The greater rate of inactivation observed at the
5 lower temperature (5°C) and pressure (325 MPa) as compared with treatments at higher
6 temperature (20°C) and pressure (375 MPa), reinforces the finding that cooler temperatures result
7 in substantially greater inactivation of MNV-1.

8 To demonstrate that high pressure treatment could inactivate MNV-1 within shellfish
9 tissues, live oysters (*C. virginica*) were exposed for 24 hrs to high levels of MNV-1 in seawater
10 to mimic environmental accumulation of enteric virus in shellfish growing waters. Three
11 different accumulations using three oysters per experiment were performed. Extraction of
12 MNV-1 from each untreated group of three oysters yielded 1.47×10^5 , 2.7×10^5 , and 1.8×10^4
13 PFU respectively, confirming that MNV-1 was accumulated to high levels after exposure. For 5-
14 min 5°C-HPP treatments of MNV-contaminated oysters, onset of inactivation was observed at
15 325 MPa, with a 400-MPa treatment being sufficient to inactivate 4.05-log₁₀ (Fig. 3).

17 DISCUSSION

18
19 This is the first direct demonstration that a norovirus, rather than a norovirus surrogate, can
20 be inactivated by high pressure. This demonstration suggests good prospects for inactivation of
21 human norovirus strains by high pressure, but should also be viewed with caution since different
22 viruses, and even different strains of viruses, can differ in susceptibility to HPP (19). It should
23 be noted that murine norovirus was less sensitive to pressure than the norovirus surrogates FCV

1 or SMSV-17, which were reduced by $> 7 \log_{10}$ PFU/ml after a 5-min 300-MPa treatment and $>$
2 2.49 \log_{10} PFU/ml after a 1-min 300-MPa treatment at 10°C, respectively (22, 3).

3 Given that FCV inactivation is enhanced by cooler temperatures of $< 20^{\circ}\text{C}$ (8), it is not
4 entirely surprising that HPP inactivation of MNV-1, also a calicivirus, is enhanced by cooler
5 temperatures. Cooler temperatures may maximize the protein denaturation under pressure due to
6 increased density of water molecules in the protein solvation cage (1, 26). This temperature
7 effect is not restricted to the caliciviruses, since the picornavirus, foot-and-mouth disease virus,
8 has also been shown to have enhanced inactivation at cooler temperatures (32). It should be
9 noted that HAV, another picornavirus, is an exception to this general rule, since cooler
10 temperatures do not enhance HAV inactivation (20).

11 Treatment time inactivation curves at both 5°C and 20°C were consistent with log-logistic
12 and Weibull kinetics rather than linear first-order kinetics. Nonlinear inactivation curves as a
13 function of constant pressure and time have been observed previously for other viruses such as
14 FCV and HAV (8, 20) as well as for bacteriophage (9) and a host of bacterial species (6, 7). Our
15 observation is consistent with curves obtained for other microorganisms, and may be a general
16 property of high pressure inactivation.

17 For oysters, results indicate that MNV-1 inactivation is feasible at ≤ 400 MPa with 400
18 MPA being the probable upper limit for commercial HPP treatment. Since greater inactivation
19 was observed for stock virus at 5°C than at 20°C, treatment of oysters was performed at the
20 cooler temperatures. It was important to perform studies with contaminated oysters since virus
21 inactivation by HPP is influenced by the environment in which the virus is found. How viruses
22 sequester themselves within shellfish meats is not well understood. Conceivably, MNV-1 could
23 be sequestered in a number of different anatomical structures within the oyster including mucous
24 membranes, within digestive gland contents, within phagocytic cells or free floating within the
25 open circulatory system of the oyster. For oysters, it is conceivable that physiologic,

1 environmental and seasonal factors, such as water salinity, may also alter HPP effectiveness.
2 Shellfish are osmoconformers, meaning their internal ionic strength mimics the ionic strength of
3 the waters in which they reside. Oysters used in this study were harvested from Mobile Bay, AL,
4 an estuary with salinity ranging from 5- 28 ppt. These oysters were harvested in late winter to
5 early spring prior to spawning.

6 If human and murine noroviruses have similar susceptibility to high pressure, we believe
7 that HPP would be a viable processing intervention, particularly if pressure treatment could be
8 performed at refrigeration temperatures. It is generally accepted that less than 100 virions are
9 sufficient to initiate illness (10). While shellfish can accumulate norovirus to high levels as
10 demonstrated in this study, it is probable that shellfish legally harvested from approved growing
11 areas would not be grossly contaminated. Presumably, a 4- \log_{10} reduction in PFU would
12 correlate with a similar reduction in infectious doses present within contaminated shellfish. It is
13 important to note that cooking has not always been sufficient to prevent shellfish-borne virus
14 transmission (5, 25, 29). Consequently, high pressure may also prove useful for reducing
15 infectious virus in bivalves prior to cooking.

16 In summary, this study demonstrates that MNV-1 can be readily inactivated by high
17 pressure, which may be a practical intervention for foods potentially contaminated with
18 noroviruses, such as shellfish. This inactivation is enhanced at temperatures below 20°C. HPP
19 inactivation of MNV-1 as a function of time reveals inactivation kinetics consistent with Weibull
20 and log-logistic kinetics. Inactivation of MNV-1 directly within oysters has also been
21 demonstrated.

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1 TABLE 1. Five-min treatments at 350 MPa of MNV-1 stock in Dulbecco's modified Eagle
2 medium with 10% fetal bovine sera at different initial temperatures

Initial temperature (°C)	Log reduction* +/- S.E.
30	1.15 +/- 0.33
20	1.79 +/- 0.53
10	4.77 +/- 0.56
5	5.56 +/- 0.81

3

4 * mean of three trial assayed in triplicate (n=9)

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FIGURE LEGENDS

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3 FIG. 1. Effect of pressure treatment of murine norovirus. Virus samples in Dulbecco's
4 modified Eagle medium with 10% FBS were treated for 5 min at 20°C. Data are the mean of
5 three replicates. Error bars represent standard error.

6
7 FIG. 2. Inactivation curves of murine norovirus. Time course analysis of pressure
8 treatment of MNV-1 stock virus was performed at A) 325 MPa and 5°C B) 375 MPa and 20°C.
9 Data are the means of three replicates and were fitted with linear, Weibull, and log-logistic
10 functions. Error bars represent ± 1 standard error. Pressurization time did not include the
11 pressure come-up or release times.

12
13 FIG. 3. Effect of pressure treatments on MNV-contaminated oysters. MNV-contaminated
14 oysters were administered 5-min treatments at 5°C. Average extractable MNV-1 reductions are
15 displayed graphically as a logarithmic reduction from three trials. Error bars represent standard
16 error for titer reductions from three trials.

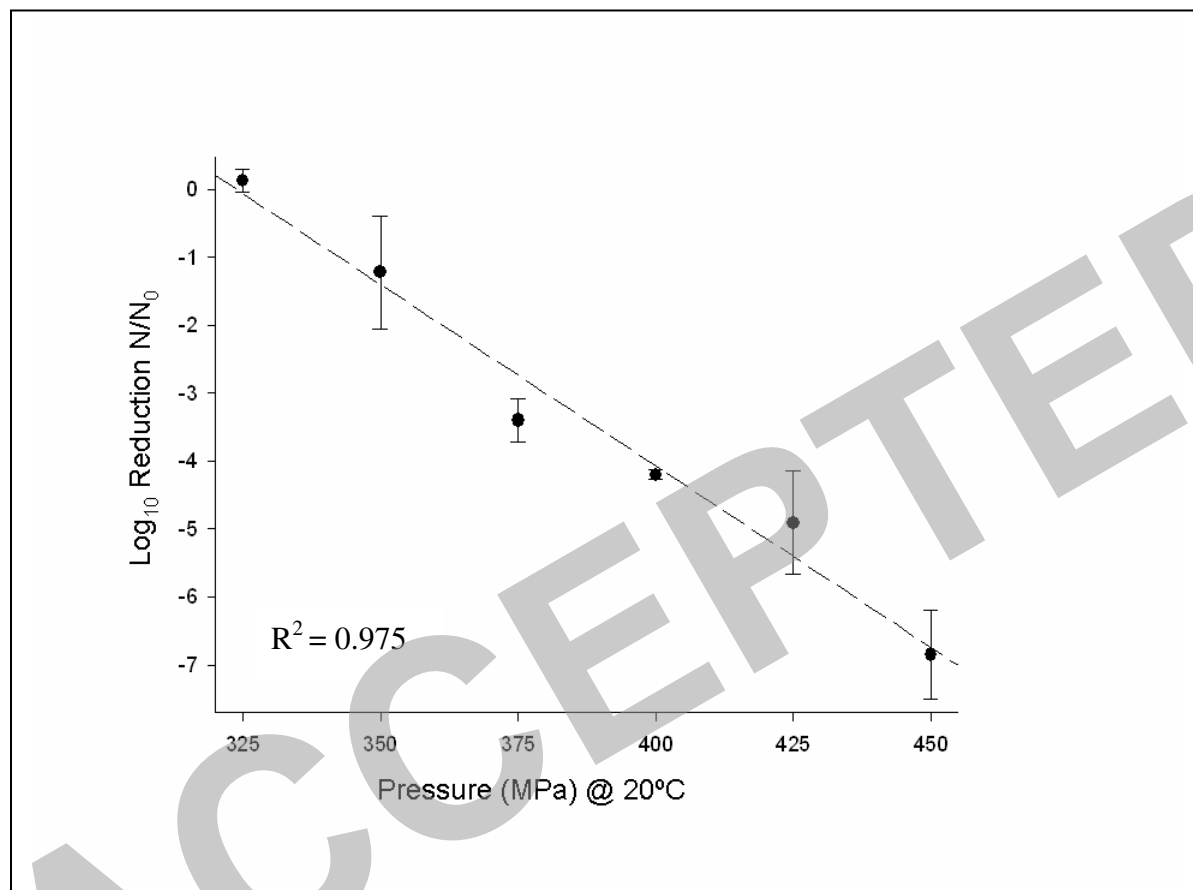
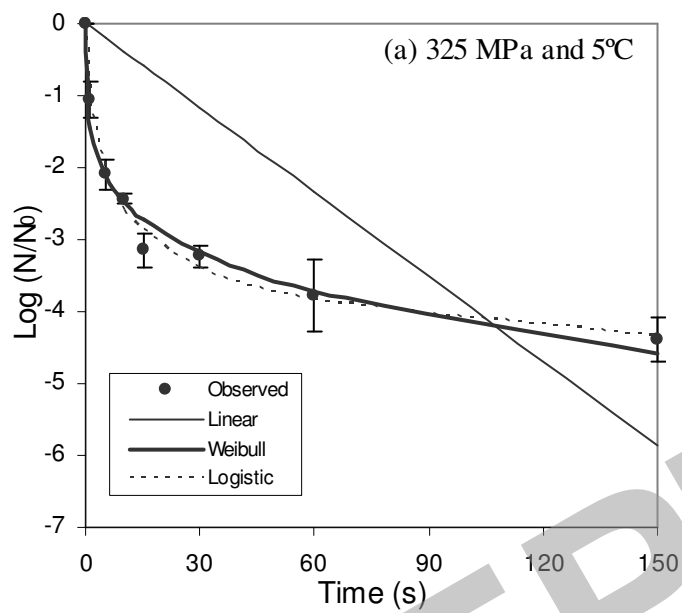


FIG. 1.

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1 Fig 2A.

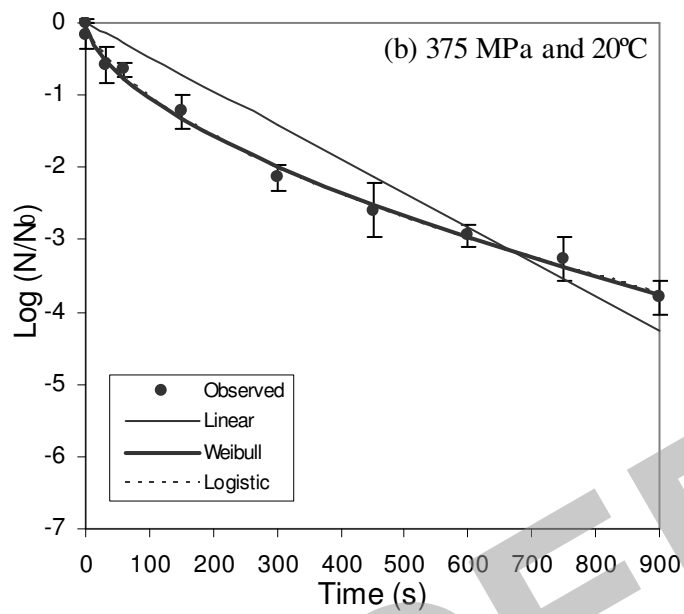
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FIG. 2B.

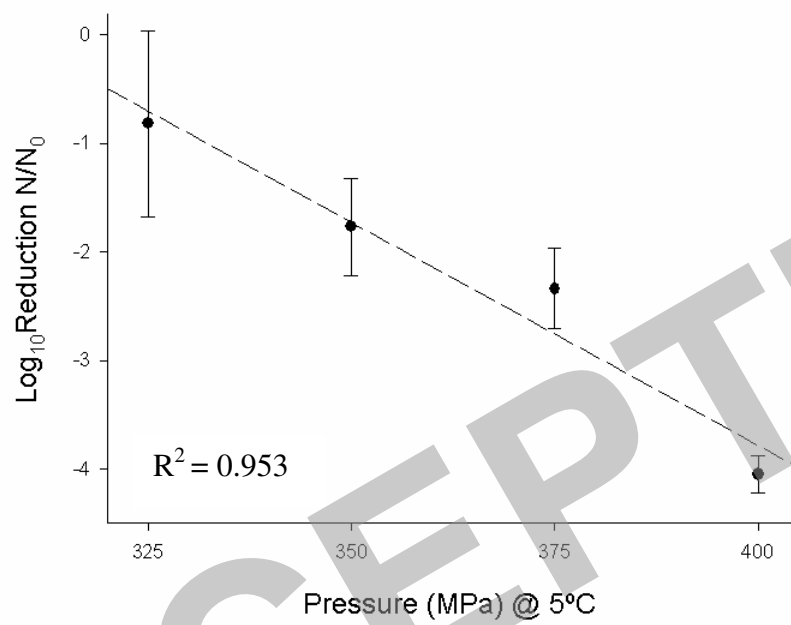


FIG.. 3.

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