Substantia
An International Journal of the History of Chemistry

Firenze University Press
www.fupress.com/substantia

OPEN ACCESS

Citation: A. Garrido Sanchis, B.W. Ninham (2020) Very High Efficiency of *E. coli* Inactivation by Body Temperature CO_2 Bubbles: in Pursuit of Mechanism. *Substantia* 4(2) Suppl.: 57-67. doi: 10.36253/Substantia-1030

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Very High Efficiency of *E. coli* Inactivation by Body Temperature CO_2 Bubbles: in Pursuit of Mechanism

Adrian Garrido Sanchis[2,*], Barry W. Ninham[1]

[1] *Department of Applied Mathematics, Research School of Physical Sciences, The Australian National University, Canberra, Australia*
[2] *School of Sciences, University of New South Wales, Canberra, Australia*
*Corresponding author: a.garridosanchis@adfa.edu.au

Abstract. A CO_2 bubble column (CBC) has been developed as a body-temperature lab-scale water sterilization process for the inactivation of pathogens. Both CO_2 and combustion gas bubbles inactivated *Escherichia coli* C-3000 (ATCC15597) with extraordinary efficiency in solutions with low alkalinity. The mechanisms of inactivation were not known. To characterise the phenomena a new first-order kinetic equation that correlates *E. coli* inactivation rates with a total alkalinity of the solutions has been developed as a first step towards understanding. This leads us to propose a new mechanism of inactivation.

Keywords: *E. coli*, water reuse, carbon dioxide, combustion gas, alkalinity.

1. INTRODUCTION

Water-borne pathogens remain one of the leading risks to public health worldwide. The World Health Organisation (WHO) estimated that over 30 million cases of diseases and millions of deaths could be caused by pathogen-infected water sources, globally, each year.[1]

The inactivation of microorganisms like bacteria and viruses in aqueous solutions is a perpetual challenge. For example, pathogenic bacteria present in water used in food or pharmaceutical manufacture can cause product contamination. In a bigger scenario wastewater from agricultural or industrial uses or water obtained from environmental flows, contains pathogenic microorganisms that need to be inactivated before the water is used for industrial or agricultural purposes or drinking water. This is an existential problem for humanity.

Present methods for inactivating viruses, bacteria, and other microorganisms include heat, irradiation (e.g. ultraviolet treatment), treatment with chemicals (e.g. ozone), high-pressure treatment, and filtration (e.g. membrane filtration). Many of these methods, especially, heat treatment, are energy-intensive. More energy-efficient treatment technologies are desperately needed.

High density and high-pressure CO_2, known as dense phase carbon dioxide (DPCD), is being used as a non-thermal disinfection alternative.[2,3]

Our process is different. We have previously studied CO_2 sterilization effects at atmospheric pressure in a bubble column on *E. coli* and viruses. The process has been implemented commercially. We found that for both pathogenic groups the sterilization effects increase with the temperature.[4] It is of much interest that inactivation becomes optimal above 38 degrees, body range temperature.

Carbon dioxide, being a greenhouse gas, is assigned some responsibility for global warming. But it is also literally vital for plant growth. Recently it has been shown likely to be Nature's way of controlling viral infection in the human body, following from these CO_2 antiviral properties a recent study has identified the use of body temperate humidified CO_2 as a possible early therapy to mitigate infection in COVID-19 patients.[5, 6] Many alternatives to reduce CO_2 emissions have been proposed, apart from planting trees and controlling bushfires. Geological sequestration in depleted gas and oil reservoirs, ocean storage, seaweed production, CO_2 mineralization are just a few.

Sterilisation by CO_2 has been widely used throughout history. After sodium chloride, natron, sodium bicarbonate is probably the first industrial-scale chemical used by mankind. Natron, in ancient Egypt, was essential for mummification. The still and little understood peculiar hydration properties of the bicarbonate ion in contact with air affects both tissue dehydration and sterilization against bacterial degradation.

The magic of the bicarbonate ion has remained, with the still unexplained problem of Roman and other cement formation. The foundation of physical chemistry can arguably be marked by Berthollot's observation that reactions are temperature dependent: He saw soda-lime, sodium carbonate rocks instead of the calcium carbonate on the banks of the Nile River during Napoleon's scientific expedition to Egypt in 1792. The normal precipitation of calcium carbonate from a sodium chloride calcium carbonate solution is reversed at high temperatures of the drying Nile flood in summer. The astute chef and detergent companies know that wet sodium bicarbonate is extremely effective in cleaning. It cuts peptide bonds of proteins bound to saucepans or dishes. But why?

1.1. Nature of the Present Studies

The work here presented studies the *E. coli* inactivation effect of CO_2 at different alkalinity levels at 38 °C (human body temperature range) and atmospheric pressure.

Alkalinity is defined as the ability of water to resist changes in pH when acids or bases are added to the solution or the capacity of water to neutralize acids. The CO_2-carbonate system describes which of the four forms of total inorganic carbon (molecular aqueous CO_2, Carbonate ion, CO_3^{-2}, Bicarbonate ion, HCO_3^-, or Carbonic acid, H_2CO_3) is the dominant species as a function of pH.[7,8]

We have analysed our results within the boundaries of the accepted theory of physical chemistry of solutions. This allowed the development of a new first-order kinetics equation that correlates *E. coli* inactivation rates with total alkalinity when bubbling CO_2 or combustion gases in a bubble column at atmospheric pressure and body temperature. But while it fits the data it does not account for why the process is efficient. We are then led to such an explanation outside the confines of classical theory.

2. MATERIALS AND METHODS

2.1. Coliform bacteria

Escherichia coli is a gram-negative bacterium with a straight cylindrical rod shape of 1.0-2.0 μm size.[9] *E. coli* are harmless, can be found in the intestines of healthy humans and animals but some are pathogenic to the host. As a result of fecal contamination, it can be found in water and soil. Its presence in water is used as an indicator to monitor water quality.[10]

The strain *E. coli* C-3000 (ATCC15597) is a biosafety Level-1 organism[11] which was selected as a representative model for bacterial contamination in water.[12,13]

Spinks at al.[14] demonstrated that pathogenic bacteria are inactivated in a temperature range of 55 to 65 °C. Other studies found that *E. coli* presents the first signs of thermal inactivation at temperatures over 55 °C, with high inactivation rates at 60 °C.[15]

An overnight *E. coli* C-3000 (ATCC 15597) culture was grown in 10 mL broth (see section 2.3 for more details) without agar at 37 °C for 18–20 hours in a Labtech digital incubator, model LIB-030M, while shaken at 110 rpm by a PSU-10i orbital shaker.

2.2. Experimental solutions

Solutions with different alkalinity levels (see Table 1) were prepared and sterilized by autoclaving in an Aesculap 420 at 15 psi and 121–124 °C, for 15 minutes.[16]

A standard **secondary-treated synthetic sewage** medium (Organisation for Economic Co-operation and

Development (OECD) medium) was prepared according to water-quality guidelines and standards.[17,18] The official Journal of the European Community for Secondary-treated Water Quality has the following requirements for discharges from urban wastewater-treatment plants: 125 mg/L of COD; 2 mg/L of total phosphorus; and 15 mg/L of total nitrogen.[19] This solution was designed to meet the European standards by using the following ingredients in a liter of boiled tap water or Milli-Q water (depending on the experiment): 120 mg of peptone; 90 mg of meat extract (replaced here by Bovril® according to recommendations in Biology of Wastewater Treatment[20]); 30 mg of urea; 13 mg of dipotassium hydrogen phosphate; 7 mg of sodium chloride; 2 mg of calcium chloride dehydrate; and 2 mg of magnesium sulfate heptahydrate.

Two NaCl solutions with concentrations of 0.001 M and 0.17 M NaCl (NaCl ≥ 99% purity, obtained from Sigma-Aldrich) in Milli-Q water, a 0.01 M $CaCl_2$ solution ($CaCl_2$.≥ 99% purity, obtained from Sigma-Aldrich) in Milli-Q water and tap water. These concentrations were chosen to coincide roughly with the physiological concentration of humans and to typical environmental conditions faced by the bacterium.

To ensure the full elimination of chlorine from tap water it was previously boiled for 30 minutes, therefore *E. coli* (C3000) was not affected by the disinfecting action of chlorine.

2.3. E. coli growth media

The plate count method[21] is commonly used for the detection of *E. coli* in, both wastewater and marine water. The degree of contamination in water is assessed based on the ability of bacteria to propagate forming colonies in a layer of agar.[22]

For each bacterial-growth experiment, two broths were prepared (A and B).

Broth A was prepared with 1 g of yeast extract, 6 g of NaCl, 13 g of tryptone, and 1,000 mL of Milli-Q water. This medium was aseptically distributed into two vessels containing 1.41% agar and no agar, respectively; the agar used in the experiments was molecular-biology grade from Sigma-Aldrich. The medium was heated to boiling to dissolve the agar and sterilized by autoclaving for 15 minutes in an Aesculap 420 at 15 psi and 121–124 °C.

Broth B was used to improve the viability of the bacteria. It was prepared by adding 0.010 g of thiamine and 1 g of glucose to 50 mL of Milli-Q water and filtered through a 0.22 μm filter for its sterilization. Once cooled to 50 °C, it was added aseptically to broth A in a proportion of 1:19.

The resulting 1.34% agar medium was poured into 100mm × 15mm Petri dishes and dried above a Bunsen burner to maintain local environmental sterility until the agar was not too dry nor too moist.[23]

2.4. CO_2 Bubble column (CBC) process

The CBC process used two different gases: CO_2 and combustion gases. A flow of 24 L/min of CO_2 was pumped through the electrical heater that maintained the gas temperature just above the sinter surface at 38°C for the CO_2 experiments. When using combustion gases, the exhaust pipe of an LPG gas generator (Greenpower 2.5 kW) was attached to an isolated metal pipe with a valve that provided an exhaust-gas flow rate of 27 L/min through the bubble column reaching a temperature of 58 °C just above the sinter surface. The base of the bubble column evaporator was fitted with a glass sinter (type 2) of 135 mm diameter and pore size 40–100 μm.

Once the experimental solutions (0.17 M NaCl, 0.01 M $CaCl_2$, 0.001 M NaCl, secondary treated synthetic sewage made with Milli-Q water and tap water) were poured into the column, the temperature of the solution was measured with a thermocouple in the center of the column. Hot bubbles of both gases passed through the sinter into the 300 ml solution, inactivating *E. coli*.

The sterilization effects of other gases like air, argon, and nitrogen on *E. coli* have been discussed[24], they are not significant compared with CO_2 bubbles, especially at low temperatures.

2.5. Experimental setup and procedure

The CBC treated 0.17 M NaCl, 0.01 M $CaCl_2$, 0.001 M NaCl, secondary treated synthetic sewage made with Milli-Q water, and tap water containing *E. coli* C-3000 (ATCC15597) at inlet CO_2 temperature of 38 °C, and combustion exhaust gas from an LPG generator at 60 °C.

The plate count method[25,26,27] was used to assess the viability of *E. coli*. Samples were collected from 10–15 mm above the central area of the bubble column. Each sample of 0.1 mL was spotted in triplicate.

Alkalinity measures the acid-neutralizing ability of dissolved substances in water and is equivalent to the amount of strong acid required to lower the pH of the solution from an initial value to about 4.2. The total alkalinity of each solution, in mg/L $CaCO_3$, was determined by the amount of 0.02 M HCl added in a titration until a pH electrode (ThermoFisher Scientific) measured an end pH of 4.2. The initial pH of the sample of each of the 6 solutions was less than 8.3 therefore the alkalin-

ity was calculated with the carbonate ion concentration multiplied by a factor of two since each [CO_3^{-2}] ion neutralised two protons[28], see Table 1.

2.6. Data analysis

The linear decay model was used to study the inactivation of *E. coli* over time in CBC.[24,29,30] The evaluation of viable *E. coli* in the samples was performed using the plate count agar (PCA) method.[23,26,31] Plate counts numbers indicate the number of *E. coli* colonies that grow on the surface of a solid and translucid agar media in a Petry dish. The mean and standard deviation of each triplicated sample was obtained using bacteria survival factor, $\log_{10}(CFU/\ CFU_0)$, where CFU_0 is the initial number of colony-forming units (CFU) per sample and CFU is the number of *E. coli* colonies per sample after a set exposure time to the CO_2 bubbles.[32]

When analysing the data we assumed that the inactivation of *E. coli* by CO_2 in different alkaline solutions follows first-order kinetics. We proposed a model that uses the decimal reduction time (D-value) to measure how the alkalinity of a given solution can influence *E. coli* inactivation. The D-Value is defined as the time, in minutes, needed to inactivate 90% (i.e. 1-log) loss of *E. coli*.

D-values were calculated using a linear exponential decay model.

$$\log(CFU_t)=\log(CFU_0)-\frac{t}{D} \quad [1]$$

$$\log\left(\frac{CFU_t}{CFU_0}\right)=-\frac{t}{D} \quad [2]$$

Here, is the number of *E. coli* colonies at time t, = the initial number of *E. coli* colonies, *D*= the decimal reduction time, -(1/D) = the slope of the curve.

3. RESULTS AND DISCUSSION

3.1. CBC process inactivating E. coli at 38 °C and one atmosphere

Zhong et. al.[33] observed that when bubbling CO_2 in different electrolyte solutions, their pH dropped until it reached a steady value in less than 10 minutes; this value was different for each solution. Similar behavior was observed when CO_2 gas at 38 °C and combustion gases were bubbled for 10 minutes through six different solutions (0.17 M NaCl, 0.01 M $CaCl_2$, 0.001 M NaCl, secondary treated synthetic sewage made with Milli-Q water and tap water) in the bubble column (see Table 1). The absorption of CO_2 into different solutions increases when CO_2 gas is bubbled through the sintering area in a bubble column; many bubbles are produced with a large CO_2-liquid contact surface that is continually produced.

When CO_2 dissolves in water 99% stays as the dissolved molecular gas and less than 1% as carbonic acid (H_2CO_3 (equations 1 and 2) which reduces the pH of the solution (see Table 1). Carbonic acid dissociates into bicarbonate ion (HCO_3^-) and carbonate ion (CO_3^{2-}) (see equations 3 and 4).[34]

$$CO_2(g)\rightarrow CO_2(aq) \quad [3]$$

$$CO_2(aq)+H_2O(l)\leftrightarrows H_2CO_3(aq) \quad [4]$$

$$H_2CO_3(aq)\leftrightarrows H^+ + HCO_3^-(aq) \quad [5]$$

$$HCO_3^-(aq)\leftrightarrows H^+ + CO_3^{2-}(aq) \quad [6]$$

This is the reason why the pH of every solution dropped (see Table 1) and was different from that of the other solutions.[34]

When a 24 L/min flow of CO_2 passes through a single glass tube immersed in a beaker containing *E. coli* in 300 mL of 0.17 M NaCl the big bubbles of 1-3 cm diameter do not inactivate the pathogens.[4] However, when the same flow of CO_2 gas at the same temperature in the same solution is bubbled through a sintering area in a bubble column *E. coli* inactivation occurs. The bubble column produces many small CO_2 bubbles (1-3 mm diameter). The consequent CO_2 dissolution rate increases due to the large CO_2-liquid contact surface that is constantly produced. Mass transfer from the CO_2 to the liquid depends highly on the interfacial area (α).[4] This increases the amount of CO_2 dissolved in the solution and produces a similar sterilization effect to what can be achieved by raising the pressure in dense phase carbon dioxide (DPCD) processes. *The overwhelming advantage of the bubble column delivery is that only atmospheric pressure is required.*

A possible mechanism previously described[4], could explain in part why *E. coli* (doesn't work for viruses) can be inactivated by bubbling CO_2 at body temperature in a bubble column. It is that the pH decreases when CO_2 dissolves in a solution and this acidification of the solution increases the binding of bicarbonate ion to bacterial membrane phospholipid head groups. The resulting change in the head group area and membrane structure to an open mesh phase then allows free diffusion of CO_2 through the bacterial membranes. The CO_2 inside the cell produces an intracellular pH decrease that exceeds the cell's buffering capacity, resulting in cell inactiva-

tion.[35,36] Orij et al. observed that *Saccharomyces cerevisiae* cell division rate is controlled by intracellular pH changes and low intracellular pH could reduce the cell division.[37] This idea has a degree of plausibility and may well contribute to bacterial inactivation. However, it does not explain the inactivation of viruses.

When CO_2 was bubbled at 38 °C through 0.17M NaCl and 0.001 M NaCl solutions (made with Milli-Q water) in the bubble column evaporator, for both solutions, 0.6-log of *E. coli* inactivation was observed after 10 minutes due to the high CO_2 surface area in the solution (see Fig. 1). This occurred even though the two solutions had very different bubble densities. When using secondary-treated synthetic sewage and 0.01 M $CaCl_2$ (both made with Milli-Q water), a 0.2-log and 0.3 log reduction of *E. coli* was achieved respectively after 10 minutes (Fig. 1a and Table 1). However, when the same experiment was conducted with boiled tap water and with secondary-treated synthetic sewage (made with boiled tap water), no inactivation was observed after 11 minutes (Fig. 1a).

When combustion gas from a generator (with 12.5–14% of CO_2)[38] at 60°C was used in the bubble column experiments an *E. coli* inactivation rate of 2.6-log in 0.17 M NaCl solutions (made with Milli-Q water) was observed after 10 minutes.[24] However, when using secondary-treated synthetic sewage (made with boiled tap water) no inactivation was detected after the same period (Fig. 1b). This is consistent with the hypothesis that reduced pH buffering capacity aids inactivation. Within the combustion gas, other products than CO_2, H_2O, and N_2 could be present due to minor components and impurities in the fuel and different fuel/air ratios. These gases are carbon monoxide (CO), hydrogen (H_2), sulfur oxide (SO_2), and mono-nitrogen oxides (NO*x*) such as NO and NO_2.[38] Even with the presence of these gases, no inactivation was observed.

The tap water used in this work had a total alkalinity of 87.4 mg $CaCO_3$/L (Table 1), and an initial pH of 8 and therefore can act as a buffer solution. After bubbling CO_2 the pH was reduced to 4.9 for the pure tap water and 5.4 for the secondary-treated synthetic sewage, with an alkalinity of 94.5 mg $CaCO_3$/L (Table 1). This pH is too high to allow the penetration of the CO_2 through the membranes, with a consequent lack of *E. coli* inactivation when using the CBC process in solutions with high alkalinity.

Milli-Q-water-based solutions had a reduced buffer capacity of only 6 mg $CaCO_3$/L (Table 1), reaching low pH values of 4 for NaCl and $CaCl_2$ based solutions. For Milli-Q-water based secondary-treated synthetic sewage the pH reached 4.4 and the total alkalinity value was 27.7 mg $CaCO_3$/L (Table 1); this solution contains different salts that can slightly increase its alkalinity, with a consequent reduction in inactivation.

3.2. Effect of alkalinity on E. coli inactivation when bubbling CO_2 and combustion gases

Decimal reduction times (D-values) (Table 1), calculated from inactivation values, have been used to understand the effect of alkalinity on *E. coli* inactivation. To do this, we compared *E. coli* inactivation performance of the CBC when using body temperature CO_2 with that of combustion gases for six solutions (0.17 M NaCl, 0.01 M $CaCl_2$, 0.001 M NaCl, secondary treated synthetic sewage made with Milli-Q water and tap water) with different alkalinity values, see Fig. 2.

The correlation between the log of the D-values and the corresponding total alkalinity is shown in Fig. 2 and Table 1. A D-value is a time needed to inactivate 90% (i.e. 1-log) of the *E. coli*. To measure the impact of alkalinity on *E. coli* inactivation when bubbling CO_2 we have proposed

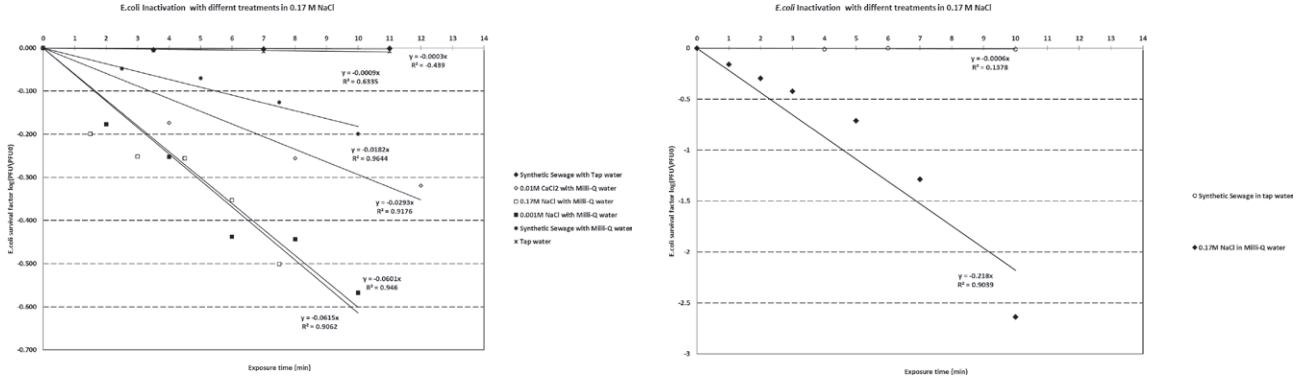

Figure 1. (a) Comparison of *E. coli* inactivation with CO2 at 38 °C in a bubble column in 6 different solutions; (b) *E. coli* inactivation with combustion gas at 60 °C in a bubble column in 2 different solutions.

Table 1. Initial and final pH values after bubbling CO_2 and combustion gas in 6 different solutions, amount of 0.02 M HCl to reduce the pH of the solutions to 4.2, alkalinity in mg/L of $CaCO_3$ and D-values.

Solution	pH initial	pH final	ml of HCl 0.02 M to reach pH 4.2	ml of HCl 0.02 M to reach final pH	Total Alkalinity (mg/L of $CaCO_3$) (to reach a pH of 4.2)	Equivalent (mg/L of $CaCO_3$) (to reach final pH)	D-value (min)	Log10 (D-value, min)
CO_2 gas at 38°C								
0.001M NaCl in Milli-Q water	5.78	4	0.9	1.3	6	8.7	16.3	1.21
0.17M NaCl in Milli-Q water	5.86	4	0.9	1.3	6	8.7	16.6	1.22
0.01M $CaCl_2$ in Milli-Q water	5.94	4	1	1.5	6.7	10	34.1	1.53
Tap water	8	4.9	13.1	12	87.4	80.1	2,000	3.30
Secondary-treated synthetic sewage made with tap water	7.63	5.4	14.3	10.5	95.4	70.1	3,333	3.52
Secondary-treated synthetic sewage made with Milli-Q Water	6.83	4.4	4	2.6	26.7	17.3	54.9	1.74
Combustion gas at 60 °C								
Secondary-treated synthetic sewage made with tap water	7.63	6.5	14.3	5.4	95.4	36	1,666	3.22
0.17M NaCl in Milli-Q water	5.86	4	0.9	1.3	6	8.7	4.6	0.66

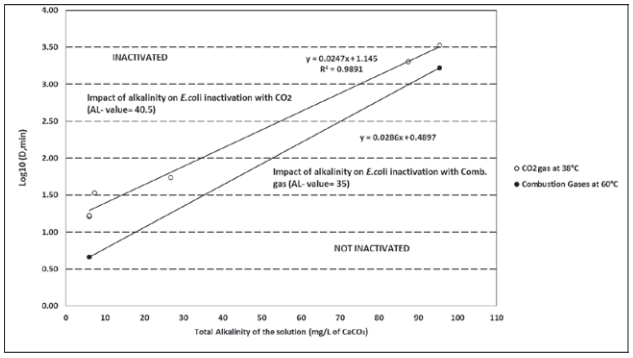

Figure 2. Minimum CO_2 bubbling times at different alkalinity values to achieve 1-log bacteria inactivation in 6 different alkaline solutions. Above and to the right of the lines the pathogens will be sterilized by 1-log.

the new AL-value. The AL-value gives the total alkalinity required to change the D-value by a factor of 10 and reflects the alkalinity impact on *E. coli* inactivation. The smaller the AL-value, the greater the sensitivity to low alkalinity.

Figure 2 compares the impact of alkalinity on *E. coli* inactivation between CO_2 and combustion gases. Above and to the right of the lines *E. coli* will be inactivated by at least 1-log.

From the AL-values obtained with CO_2 (AL=40.5) and combustion gases (AL=35), it can be seen that the inactivation for both gases showed a similar alkalinity-dependent behavior (Fig. 2). A reduced AL-value of 35 to 40.5 mg/L of $CaCO_3$ suggests that when bubbling both gases inactivation rates of *E. coli* will highly depend on the alkalinity of the solution, see Table 1 and Figure 1.

For alkalinity values in the range, 5–95 mg $CaCO_3$/L, combustion gases presented higher *E. coli* inactivation rates than when using pure CO_2, with D-values of 16.3 min at 6 mg $CaCO_3$/L for pure CO_2 and 4.6 min at 6 mg $CaCO_3$/L for combustion gases. At higher alkalinity values both gases needed longer times to achieve 1 log *E. coli* inactivation, combustion gases required 1,666 min at 95.4 mg $CaCO_3$/L, pure CO_2 needed 3,333 min at 95.4 mg $CaCO_3$/L (Fig. 2 and Table 1).

For both gases, *E. coli* inactivation can be increased by reducing the alkalinity of the solution. Solutions with a low buffer capacity, low alkalinity, after bubbling CO_2, or combustion gases in the bubble column for 10 minutes significantly reduced their pH as observed in our experiments (Table 1). This reduced pH could affect microbial cell inactivation, since cell membranes stop protons from penetration but also make them more permeable to other substances, like CO_2, due to the chemical modification on the phospholipid bilayer of the membranes.[35,36] When CO_2 penetrates inside of the bacteria dissolves in the intracellular fluid producing carbonic acid (H_2CO_3) what triggers an intracellular pH decrease that exceeds the cell's buffering capacity, resulting in cell inactivation.[35,36]

3.3. Development of an inactivation model based on alkalinity

Based on the evidence (see Figure 3) that *E. coli* inactivation in different alkaline solutions when bubbling 38

°C CO_2 and combustion gases follows first-order kinetics we proposed a new *E. coli* inactivation equation that considers the alkalinity of different solutions.

3.4 Disinfection Kinetics model

The disinfection efficiency of bubbling CO_2 or combustion gases at low temperatures through a solution with *E. coli* depends on the total alkalinity of the solution. A new model that includes alkalinity based on the Chick-Watson first-order equation has been developed in this work.

E. coli inactivation follows the first-order kinetics, thus the model results in the following equation:

$$\frac{dCFU}{dt} = -k\ CFU \quad [7]$$

where the *E. coli* population (CFU) varies with processing time (t) at a constant rate (k).

The New Model based on the decimal reduction time (D-value) and the new AL-value explains how the alkalinity of a given solution can influence *E. coli* inactivation when CO_2 or combustion gases, that contain CO_2, are bubbled through at atmospheric pressure. The D-value is defined as the time, in minutes, needed to inactivate 90% (i.e. 1-log) of *E. coli*.

D-values were calculated using a linear exponential decay model.

$$\log S(t) = \log\left(\frac{CFU_t}{CFU_0}\right) = -\frac{t}{D} \quad [8]$$

where $S(t)$ is the survival fraction after a time (min), is the number of *E. coli* colonies at time t, = the initial number of *E. coli* colonies, D= the decimal reduction time, -(1/D) = the slope of the curve (see Figure 3).

For this work, we have defined a new parameter, the AL-value, that is the reduction in alkalinity needed to reduce the D-value by 1-log when bubbling CO_2 in a bubble column at body temperature (38°C) and atmospheric pressure. It measures the impact of alkalinity on *E. coli* inactivation.

Thus:

$$\log D_1 = \frac{1}{AL}(A_1 - A_2) + \log D_2 \quad [9]$$

$$AL = \frac{A_1 - A_2}{\log D_1 - \log D_2} \quad [10]$$

Where, A_1 first alkalinity of the interval, A_2 second alkalinity of the interval, and D_1 and D_2 are the D-values at A_1 and A_2.

Equation 5 is obtained from Fig. 3, where A is alkalinity.

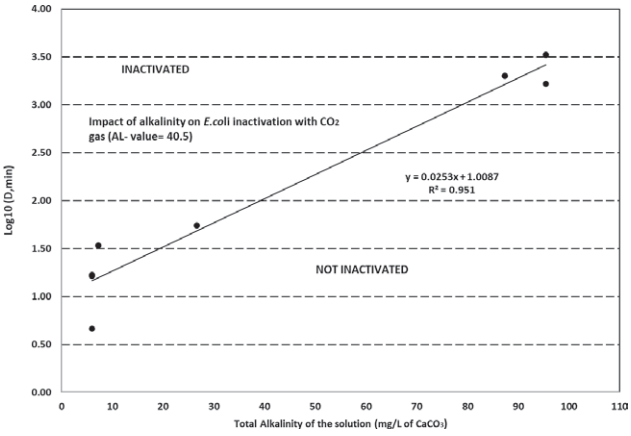

Figure 3. Minimum CO_2 and combustion gas bubbling times at different alkalinity values to achieve 1-log bacteria inactivation in 6 different alkaline solutions. Above and to the right of the lines the pathogens will be sterilized by 1-log.

$$\log D = 0.0253A + 1.0087 \quad [11]$$

From quations 2 and 5.

$$\log\left(\frac{CFU_t}{CFU_0}\right) = \frac{-t}{e^{(0.0253\ A\ +\ 1.0087)}} \quad [12]$$

Where, CFU_t is the number of *E. coli* at time t, CFU_0= the initial number, A= is the alkalinity of the solution in mg/L of $CaCO_3$ (see Figure 3).

4. POSSIBLE MECHANISMS OF STERILISATION

4.1. Negotiating the Quagmire: Nanobubbles

We began this and previous works[4,24] with the remarkable discovery that CO_2 bubbles at body temperatures are extremely efficient sterilising agents for both bacteria and viruses. The technology is being implemented at industrial scales. Potential applications are legion. The use of recycled water in cities has been prohibited in large part because viruses could not be easily removed from drinking water. A recent discovery in physiology has revealed the structure and function of the ubiquitous endothelial surface layer (ELS), unknown for 150 years.[39]

It is a micron thick foam of carbon dioxide nanobubbles. CO_2 is being produced by metabolism. and passes through a molecular frit that is the polymeric glycocalyx to form nanobubbles. This mimics our bubble column apparatus and vice versa. The ESL foam repels red cells and protects organs from invasion by bacteria, neutrophils, low-density lipoproteins, and a legion of

other invaders, good and bad. Viruses like COVID-19 have to run the gauntlet of this layer of nanobubbles on their way out to the adjoining bloodstream and exit with CO_2 nanobubbles via the lungs. The issue of CO_2 sterilisation takes on a wider import as Nature's gift to protect us from the disease. The question of what factors combine to account for the mechanism of sterilisation then takes on a decided imperative. The results of the experiments we have been able to subsume in the single first-order kinetic equation of Section 3.3. We now have to see if we can extract any sense and hints at mechanism from this. In attempting to do so we have negotiated our way through a swamp of misinformation. By this, we mean the classical foundations of physical chemistry that inform our intuition suffer from serious sins of omission and of commission. [40] These are due to the omission of specific ion (Hofmeister) effects, of dissolved gas: and the organization and reactivity of nanobubbles. Their universal presence has been a hidden variable that controls reactivity in solution. They are the energy source via free radicals that drive in polymerization[41], and enzymatic reactions.[42,43] Their presence has been revealed and studied extensively by laser spectroscopy over many years.[44-46] Nanobubbles even appear on mechanical shaking and contain highly reactive hydrogen peroxide and of course on sonication.[47-49]

4.2. Available Evidence

With these complications of classical theory in mind we marshall what further evidence we can that could illuminate the issue of mechanism.

Other gases: argon, air, and nitrogen have no deleterious on pathogens at low temperatures. At very high temperatures they do explore in a succeeding paper.[24] Our problem is peculiar to CO_2.

4.3. Sodium bicarbonate.

The astute chef and detergent companies know that the removal of food proteins adhering to utensils is not easily accomplished. It is by using reasonably warm concentrated sodium bicarbonate solution. This involves active nanobubbles nucleated at the surface that cut peptide bonds. It is also aided by Berthelot's 1795 observations on the Nile of precipitation of sodium carbonate rather than calcium carbonate. The ion exchange at and above 50 degrees removes calcium and weakens adhesive bonds. This occurs with the destruction of proteins and their adhesion in the protective coats of both bacteria and viruses.

With bacteria the lipids of the exterior membranes of both gram-positive and gram-negative types fall apart above 50-60 degrees because of the same change in head group hydration with temperature – apart from the obvious change in hydrocarbon chain packing.

4.4. pH changes with salt and salt concentration

We have already remarked on apparently peculiar specific differences in pH with different salts with bubbling CO_2 through the solution. This artifact occurs for solutions with CO_2 and those without. They are because the classical theory of pH and buffers assumes that electrostatic forces alone are operating. Specific Hofmeister effects arise when the theory is corrected to include dispersion force acting ions, anion cations, and buffer ions. Further complications of buffers show up explicitly in[42]. But also listed below of course all classical measurements ignore dissolved gas that also affects matters like the interpretation of surface potentials (pH). Reversal of the Hofmeister series for pH is seen with buffers nominally at the same pH, e.g., phosphate vs cacodylate, nominally pH 7.[50-52]

These are serious complications around which we have to tread carefully. See e.g. for an explicit illustration of obstacles to interpretation caused by the use of classical theory.[53] This reports studies of growth rates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* as a function of salt concentration for a large range of anions spanning the Hofmeister series. The variations are enormous and not explainable in the usual framework of colloid science.[53]

4.5. Finally a Mechanism of Sterilisation

So armed for pitfalls of theory, we can tread carefully towards an understanding of what is going on.

Essentially this: Nanobubbles are formed through the turbulent passage of CO_2 through the bubble column frit. They are stabilised by the adsorption of ions and proteins from the bubble column solution. The nanobubbles will have a dynamic highly reactive surface containing bicarbonate and carbonate ions in amounts depending on alkalinity that determines the proportion of each.

Carboxylates form due to surface reactions with solutions depending on solution constituents. If in high enough concentration these will react with peptides of bacterial (and viral) coats, resulting in destruction. This is consistent with the astute chef and detergent companies cleaning recipe.

5. CONCLUSIONS

Pure CO_2 gas or combustion gases can be used in the bubble column to effectively inactivate *E. coli* C-3000 (ATCC15597) in the water at atmospheric pressure and ambient temperatures. The efficiency of the process appears to depend on the alkalinity of the solution.

CO_2 bubbles will have a dynamic highly reactive surface containing bicarbonate and carbonate ions in amounts depending on the alkalinity of the solution that determines the proportion of each. Carboxylates form due to surface reactions with solutions on solution constituents. If in high enough concentration, especially in low alkalinity solutions, this will react with peptides of bacterial (and viral) coats, resulting in their inactivation.

We have developed a new first-order kinetics equation that predicts *E. coli* inactivation rates from solutions with different alkalinity when bubbling CO_2 or combustion gases in a bubble column at atmospheric pressure.

6. ADDENDUM

Since this work was written we discovered an excellent paper from 1948 dealing with the Surface Inactivation of Bacterial Viruses and of Proteins by Mark H.Adams.[54] It explores the effects of shaking with different gases but not CO_2 on viruses and proteins. It even compares with the effect of bubbling through a frit. This and references contained therein go back to the beginnings of genuine molecular biophysics and predate the molecular biology DNA revolution. It would be remiss of us not to refer the reader to the beginnings of quantification of the physical effects of gas and nanobubbles, long-forgotten and missing from contemporary physical chemistry.

7. CONTRIBUTIONS

Adrian Garrido Sanchis (principal author) carried out the lab experiments, performed analysis on all samples, interpreted data, and lead the writing of the manuscript.

Barry Ninham acted the part of devil advocate and critic and led his disciple back to the paths of correctness.

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