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Virus and bacteria inactivation by CO_2 bubbles in solution

Adrian Garrido Sanchis[1], Richard Pashley[1] and Barry Ninham[2]

The availability of clean water is a major problem facing the world. In particular, the cost and destruction caused by viruses in water remains an unresolved challenge and poses a major limitation on the use of recycled water. Here, we develop an environmentally friendly technology for sterilising water. The technology bubbles heated un-pressurised carbon dioxide or exhaust gases through wastewater in a bubble column, effectively destroying both bacteria and viruses. The process is extremely cost effective, with no concerning by-products, and has already been successfully scaled-up industrially.

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INTRODUCTION

Wastewater usually contains human enteric viruses like hepatitis and rotavirus and bacteria like *Escherichia coli*. If this water is to be reused it has to be disinfected. Collivignarelli et al.[1] found that ultraviolet (UV) irradiation and chemical treatments using chlorine, chlorine dioxide, peracetic acid or ozone were the most used technologies for wastewater disinfection. However, all these water disinfection technologies have limitations. For example, chlorine and chlorine dioxide react with organic compounds and form reactive chlorinated organic compounds that are hazardous to humans. In addition, chlorine needs at least 30 min contact time and is not able to eliminate *Cryptosporidium*. Chlorine dioxide has high management costs and is very unstable. Other disinfection methods such as ozone and UV irradiation are complex to operate and maintain. Rotavirus can be resistant to UV treatments and its efficiency is affected by the dissolved organic and inorganics in the wastewater, as well as its colour and turbidity.[2] Paracetic acid increases chemical oxygen demand (COD) and biochemical oxygen demand (BOD) due to the formation of acetic acid.[1] Therefore, a major challenge exists to develop new, energy-efficient technologies to address these problems.

Here we report on one such candidate technology for sterilisation that seems to do the job. It uses atmospheric pressure bubbles of CO_2 in a new device (ABCD). If this process successfully inactivates MS2 virus (ATCC15597-B1) and *E. coli* C-3000 (ATCC15597), that are surrogates for enteric pathogens, then this technology will be able to inactivate real waterborne viruses and bacteria for water reuse without the need for (high energy) boiling.

In preceding work[3,4] we conducted different experiments where the bubble diameter of 1–3 mm was measured using high speed cameras. An earlier variant we called the hot bubble column evaporator (HBCE) process.[5–7] It used hot air bubbles of 1–3 mm diameter and was operated in the temperature range of 150–250 °C. The bubbles transferred heat to surrounding water and thermally inactivated dispersed viruses and bacterial cells. At the same time, low, steady-state solution temperatures in the range of 42–55 °C were maintained.[8] An instantaneous transient hot surface layer must also form around the rising, initially hot, air bubbles. The inactivation process clearly involves collisions of bacteria or viruses with the hot air bubbles[5,6] and the surrounding heated layers.[7] Other gases (air, N_2, O_2 and Argon) achieved similar inactivation results, at 200 °C inlet gas temperatures for viruses and at 150 °C for bacteria.[9] However, CO_2 gas, at the same inlet gas temperature, is far superior with much higher inactivation rates at lower temperatures than with other gases.[9] Hence, we here embark on a more thorough study of the effects of CO_2 bubbling on viral and bacterial inactivation in pure sodium chloride solutions, using the HBCE device at atmospheric pressure with the acronym ABCD.

Many waste disposal industries like landfills, bio-gas plants and coal power plants emit large amounts of CO_2. Hence, the potential use of CO_2 bubbles in water treatment processes to sterilise water at atmospheric pressure offers an attractive new technology at the very least. Earlier we showed[9] too that the heat generated in exhaust combustion gases that contain CO_2 can also be used to increase the performance of this new sterilisation treatment. That we will also take further.

The process is very different to others that involve CO_2. Thus, many authors[10] have shown that pressurised CO_2 in a range of 5 to 1000 atm can achieve viral and bacterial inactivation.

High-pressure carbon dioxide has been proposed as a cold pasteurisation alternative for more than 25 years.[11] The new ABCD reactor, described here, achieves equivalent or better results but without the need for pressurisation, i.e., at just 1 atm. The process has been patented by the University of New South Wales as Australian Patent Application No. 2017904797.

RESULTS AND DISCUSSION

Negative hypothesis experiments: effect of pH and temperature

The inactivation of *E. coli* and MS2 virus using the ABCD shows promising results in pure sodium chloride solutions, since they provide a more controlled environment and this CO_2 inactivation effect can be easily studied. To establish a baseline for pH, temperature and type of gas and discard possible confounding variables when inactivating these model pathogens in the bubble

[1]School of Physical, Environmental and Mathematical Sciences, University of New South Wales, Canberra, Northcott Drive, Campbell, Canberra, ACT 2610, Australia and [2]Department of Applied Mathematics, Research School of Physical Sciences, The Australian National University, Canberra, ACT, Australia
Correspondence: Richard Pashley (r.pashley@adfa.edu.au)

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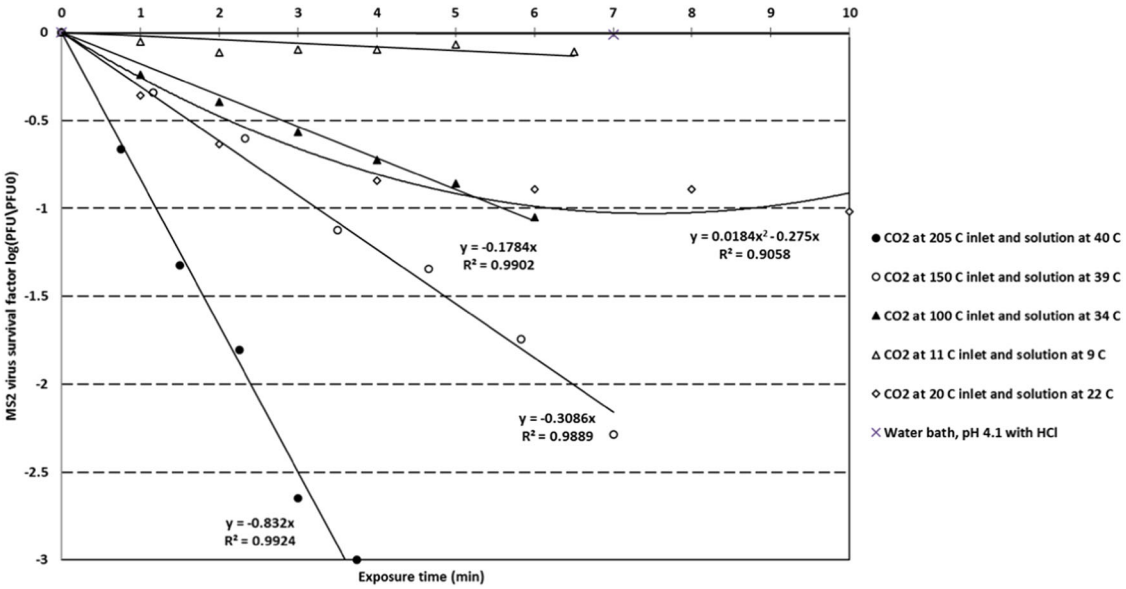

Fig. 1 Inactivation of MS2 viruses at different CO_2 inlet temperatures in ABCD

column process, a series of experiments were carried out based on our earlier work.[5,8,9]

Significantly reduced pH of 4.1 (observed in our experiments) could have an effect on virus and microbial cell inactivation, since cell membranes not only 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.[12,13] Cheng et al.[11] believed that CO_2 molecules could enter virus capsids much easier than H^+. They observed almost no inactivation change for three different viruses (MS2, Qβ and ϕX174) under four different pH conditions (pH 4, 4.5, 5 and 5.5).

In our previous work,[9] when bubbling room temperature CO_2 through a glass tube in 0.17 M NaCl solution, the pH dropped from 5.9 to 4.1 and the *E. coli* and MS2 viruses added to the solution were found to be unaffected. The same lack of inactivation was observed in this study when two experiments were conducted, one with MS2 virus and another one with *E. coli*, in a stirred beaker with 0.17 M NaCl at pH 4.1. These clearly prove that reduced pH had little or no effect on MS2 viruses, with just 0.018-log inactivation and for *E. coli* with only 0.05-log inactivation, after 14 min (see results in Figs.1 and 2a).

Figure 2a shows the results of bubbling air at 41 °C through a 0.17 M NaCl solution containing *E. coli* cells. These results indicate that at 41 °C the heated bubbles and any slightly heated layer around the bubbles did not produce any collisional, thermal inactivation.

In earlier studies,[5,8] no MS2 virus inactivation was observed in water bath experiments using 0.17 M NaCl solution heated to a typical equilibrium temperature of the bubble column (in this case, 54 °C). These results confirmed that the viruses did not become inactivated at the equilibrium, steady-state, temperature of the water in the bubble column.

At low inlet gas temperatures, cool CO_2 gas bubbles do not show any sterilisation properties. For example, when CO_2 was cooled down and bubbled through the 0.17 M NaCl solution at 9 °C, only a 0.1-log MS2 virus reduction was observed after 6.5 min (see Fig. 1). Also, at an inlet CO_2 temperature of 7 °C, no *E. coli* inactivation was appreciable, with only 0.04-log reduction after 13 min of bubbling (see Fig. 2a).

The role of the bubble coalescence inhibition effect in the ABCD inactivation process

In the ABCD process, a solution of 0.17 M NaCl produces a high density of bubbles (of 1–3 mm diameter)[3,4] due to the bubble coalescence inhibition phenomenon. The phenomenon of bubble–bubble interactions in electrolytes was explored by us 30 years ago.[14,15] Gas passing through a frit produces bubbles. Passing up a column (cf. a fish tank), the bubbles collide and become larger. The column stays clear. As the background salt concentration increases, at physiological concentration, 0.17 M, suddenly the bubbles no longer fuse. The column becomes dense with smaller bubbles. The same inhibition of fusion occurs for a single bubble–bubble interaction. In these experiments, 0% coalescence was observed for 0.17 M NaCl and 87% for 0.001 M NaCl.[15]

Over 10 min of run time in the bubble column, the MS2 virus survival factor for both solutions, with a 22 °C inlet CO_2 temperature, was compared for the ABCD system and the results are given in Fig. 3a. The results showed that the addition of 0.17 M NaCl had an effect, with inactivation rates of 1.018-log after 10 min of treatment. This result indicated that the virus inactivation rate using 0.17 M NaCl was about twice as efficient as when using 0.001 M NaCl solution, with a 0.40-log reduction, after 10 min of treatment. CO_2 bubbles are able to reduce bubble coalescence in both solutions but using 0.17 M NaCl solution further enhances this effect and apparently this caused the difference in the observed inactivation rates.

In our previous work,[5] when using 0.001 M NaCl solution with an inlet air temperature of 150 °C, the virus reduction was found to be just 0.12-log after 90 min. In the current work with the same solution but using pure CO_2 at 22 °C inlet temperature, the inactivation rate increased up to 0.40-log, after just 10 min (Fig. 3a). These results indicate that the high bubble density of CO_2 produced in the ABCD process can effectively inactivate viruses independently of the solution and the bubble coalescence effect but if 0.17 M of NaCl is added then the inactivation will be greater than when using 0.001 M NaCl.

By comparison, *E. coli* inactivation in the ABCD process with CO_2 inlet gas at 38 °C, for three different NaCl solutions (i.e., 0.17 M, 0.001 M and secondary treated synthetic sewage), produced almost 0.60-log reduction for the NaCl solutions and 0.20-log for

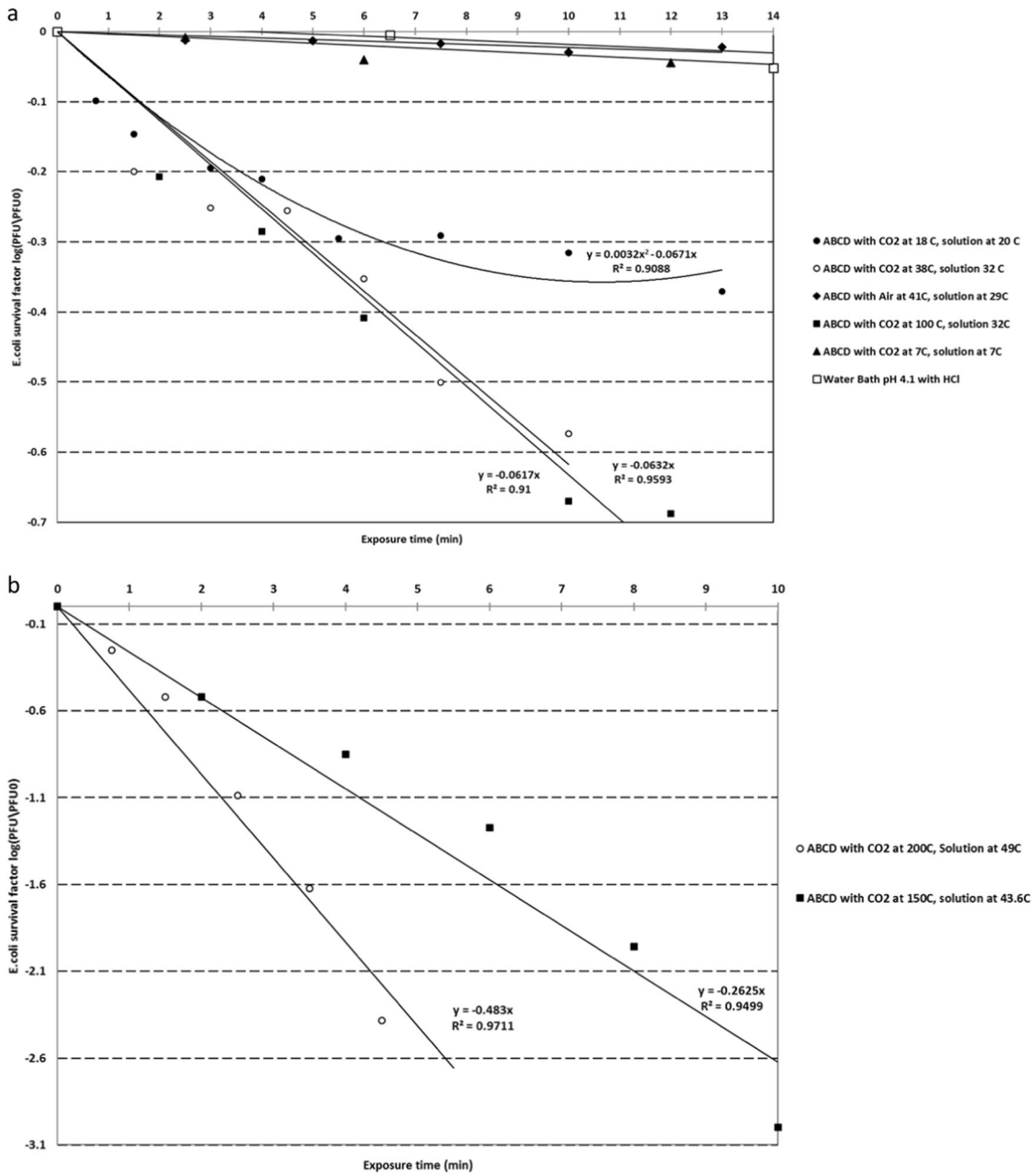

Fig. 2 **a** *E. coli* low temperature CO_2 inactivation in 0.17 M NaCl solution. **b** *E. coli* high temperature CO_2 inactivation in 0.17 M NaCl using ABCD process

the secondary treated synthetic sewage after 10 min of treatment. These results indicate that at body temperature CO_2 inlet gas (i.e., at 38 °C), *E. coli* inactivation occurred at a faster rate in simple NaCl electrolyte solutions than in secondary treated synthetic sewage (see Fig. 3b).

Effect of CO_2 inlet bubble temperatures on virus inactivation rates Cheng et al.[11] propose an inactivation mechanism for bacteriophages MS2 and Qβ based on the penetration of CO_2 inside the capsid under pressure, with subsequent expansion when depressurised, so damaging the capsid. CO_2-protein binding could also damage the capsid inactivating the virus. Dense phase carbon dioxide treatment (DPCD) has effectively inactivated viruses possibly by CO_2 chemical reactions and interactions, which partially or totally alter the virus protein–protein and protein–lipid structure.[16] With the ABCD process, it is possible that the hot CO_2 penetrates inside the MS2 virus capsid due to the high density of CO_2 produced by the continuous CO_2–liquid contact surface area. Then, the CO_2 can bind inside the capsid proteins through acid/base interactions[17] producing the high virus inactivation rates that we have observed (Fig. 1).

In the HBCE process, when hot air bubbles form on the surface of the sinter, a thin layer of heated water will be created around the surface of the bubbles, and the thickness and temperature of this thin, transient layer is likely to be important in virus inactivation.[5,8] This is because collisions between these hot air bubbles and virus have been established as the fundamental inactivation mechanism.[5]

When CO_2 bubbles at room temperature are produced within the ABCD process, 1-log virus reduction was achieved in just 10 min (Fig. 1). However, if the temperature of the inlet CO_2 gas is

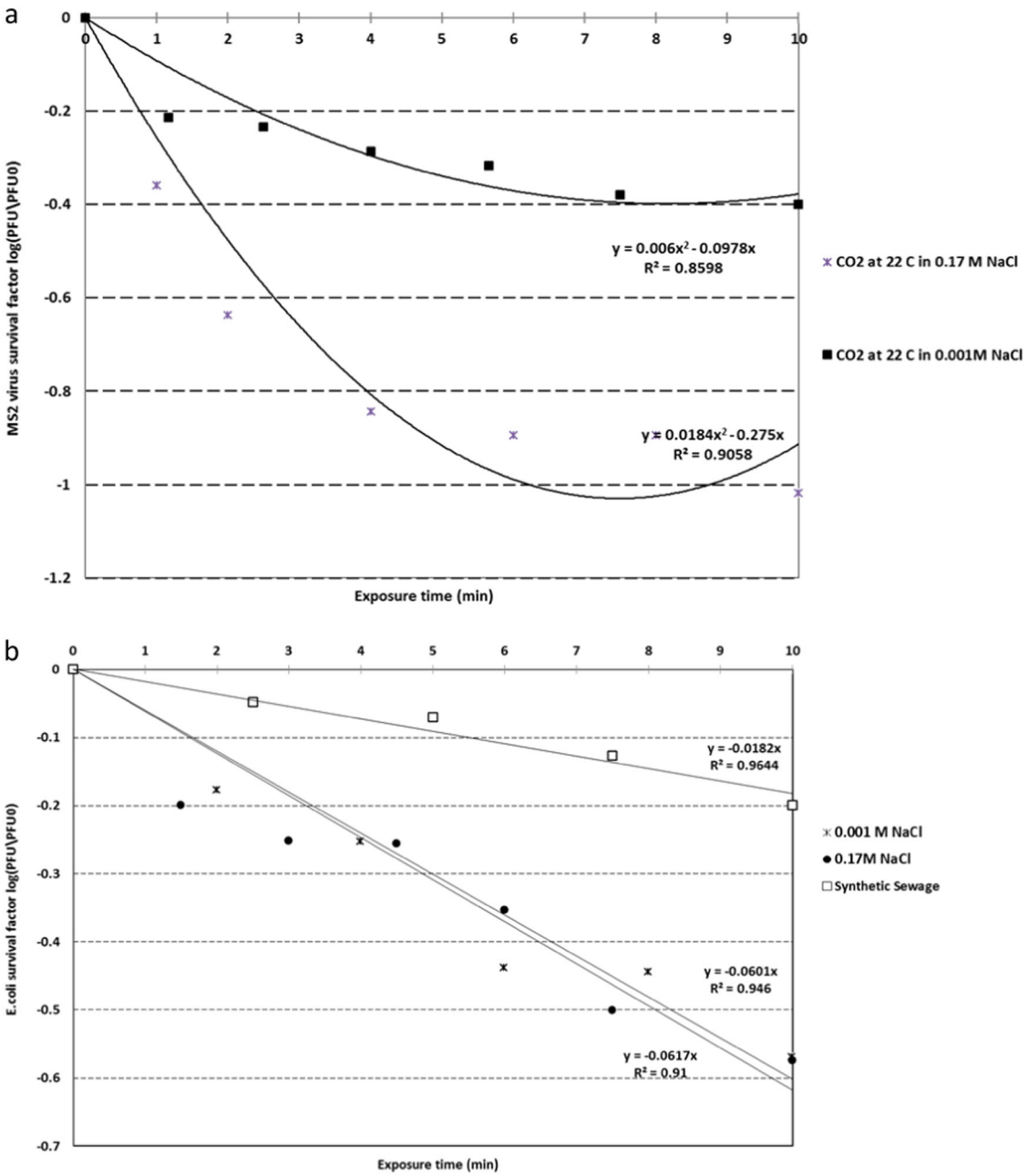

Fig. 3 **a** Virus inactivation in ABCD at 22 °C CO_2 inlet gas temperature with two different solutions: 0.17 M NaCl and 0.001 M NaCl. **b** *E. coli* inactivation in ABCD at 38 °C CO_2 inlet gas temperature in three different solutions: 0.17 M NaCl, 0.001 M NaCl and secondary treated synthetic sewage

increased, virus inactivation rates also increase, achieving a 3-log reduction at 205 °C after only 3.8 min (see Fig. 1).

In our theoretical model, the temperature and the thickness of the transient hot water layer around the surface of a 1 mm diameter CO_2 bubble can be roughly estimated for a range of inlet CO_2 temperatures using these formulae:

$$T_{avg} = \frac{100 + T_c}{2}, \quad (1)$$

where T_{avg} (in °C) is the average (transient) temperature of the hot water layer surrounding the CO_2 bubble and T_c (°C) is the equilibrium temperature of the solution in the ABCD, assuming that the hot CO_2 bubbles had cooled from their initial inlet temperature to 100 °C.

The thickness of the transient, heated layer can then be estimated by balancing the heat supplied by the cooling bubble with the heat required to raise the film to this average. Thus, the volume of the heated film V is given by: $V = 4\pi r^2 z$, where r is the bubble radius with a constant value of 0.001 m, and z the heated film thickness around the bubble, where $r >> z$.

Then, the thermal energy balance is given by:

$$C_p \Delta T V = C_{water} \Delta t 4\pi r^2 \rho_w z, \quad (2)$$

where C_p and C_{water} are air and water heat capacities, respectively, ρ_w is the liquid water mass density, ΔT is the cooling of the air bubble (from its inlet temperature to 100 °C) and Δt is the transient temperature increase in the water layer, relative to the column solution temperature.

Table 1. Summary of studies of inactivation of *E. coli* and MS2 virus with different technologies

Pathogen	Treatment	Log 10 reduction	Time (s)	Solution	pH	Solution temp, °C	Source
E. coli	Thermal inactivation 60 °C	6 log	1800	Sewage water	8	60	[38]
	2.0 mg O_3/l	1.3 log	300	Tap water	7.6	23	[39]
	2.0 mg Cl_2/l	2 log	300	Tap water	7.6	23	[39]
	UV (0.78 mW/cm^2) at 295–400 nm	3.8 log	300	Natural water	7	Room temp.	[40]
	DPCD, CO_2 at 197 atm and 34 °C	2.5 log	600	Sterilised water		34	[41]
	Bubble column, CO_2 at 200 °C, 1 atm	2.3 log	300	0.17M NaCl	6	49	Fig. 2b, this study
MS2 virus	0.1 mg O_3/l	1.2	180	Ultrapure water	7	22	[42]
	1.0 mg H_2O_2/l	0.001 log	90	0.01 M Phosphate buffer	6-10	3–10	[43]
	30 mg Cl_2/l	1 log	300	Primary sewage effluent	8	15	[44]
	UV (0.19 mW/cm^2)	3.5 log	180	Ultrapure water	7	22	[42]
	Bubble column, CO_2 at 200 °C, 1 atm	3 log	230	0.17 M NaCl	6	49	Fig. 1, this study
	Bubble column, air at 200 °C, 1 atm	0.17 log	300	0.17 M NaCl	6	52	[8]

In practice, we might expect that roughly half of the heat supplied by the cooling bubble will be used in evaporating water into the CO_2 bubble and hence the calculated, roughly estimated, film thicknesses should be halved.

For an inlet CO_2 gas temperature of 150 °C, the average temperature and the thickness of the heated water layer around the bubble would be roughly around 70 °C and 44 nm, respectively, and under these conditions the inactivation rate observed in the ABCD for the MS2 virus was a 2.3-log reduction in 0.17 M solution (Fig. 1) after 7 min of treatment. When the inlet gas temperature was increased to 205 °C, the average temperature of the transient water layer around the bubble should slightly increase (to around 73.5 °C and the thickness to around 100 nm), which appeared to increase the inactivation rates for MS2 viruses, up to 3-log reduction (see Fig. 1) after only 3.8 min of treatment.

At 100 °C inlet CO_2 temperatures, little or no heated water layer would be formed around the bubble, since the heat would be mostly lost to the evaporating water collected into the bubble, and therefore the 1-log reduction after 6 min must be only due to the CO_2 virus disinfection effect (that is, rather than a temperature effect) (Fig. 1). This observation is further supported by the 0.9-log inactivation (Fig. 1) obtained when running the ABCD at 22 °C inlet CO_2 temperature, where the inactivation can only be produced by the CO_2 inactivation effect.

After 6.5 min at 9 °C (inlet CO_2 temperature and equilibrium water temperature) only 0.1-log MS2 virus reduction was achieved (Fig. 1). At low temperatures (less than 18 °C), it appears that CO_2 is not able to penetrate through the capsid of the viruses, and therefore no inactivation was observed.

When the CO_2 temperature is in the range of 18–100 °C, CO_2 penetrates the capsid of the viruses producing the CO_2 inactivation effect. For CO_2 inlet temperatures over 100 °C, virus inactivation is most likely due to the combination of CO_2 inactivation effect and the virus collision with the hot water layer around the bubble.

Effect of CO_2 inlet bubble temperatures on *E. coli* inactivation with the ABCD process

Many studies have used *E. coli* C-3000 (ATCC15597) as a representative model for bacteria in water.[18,19] Different mechanisms have been suggested to explain the antibacterial effect of dissolved CO_2. In Chapter 4 of the book "Dense Phase Carbon Dioxide", Erkmen[12] describes, in great detail, the different steps proposed for the bacterial inactivation mechanism for pressurised CO_2. When pressurised CO_2 first dissolves in the solution, its pH decreases, and this acidification of the solution increases the penetration of CO_2 through the membranes. The CO_2 inside of the cell will produce an intracellular pH decrease that will exceed the cell's buffering capacity, resulting in cell inactivation.[12,13]

As for viruses, the collisions between the hot air bubbles and the dispersed coliforms were earlier proposed as the source of the mechanism for the coliform inactivation observed.[6]

At 100 °C inlet CO_2 temperatures, little or no heated water layers would be formed around the bubble, and therefore the 0.67-log reduction observed after 10 min was most likely only due to the CO_2 *E. coli* disinfection effect (Fig. 2a). This observation is supported by the 0.58-log inactivation (Fig. 2a) obtained when running the ABCD at 38 °C (temperature of the human body) inlet CO_2 temperatures, where the inactivation was, again, only produced by the CO_2 inactivation effect. With CO_2 bubbles at 18 °C, only a 0.37-log *E. coli* reduction was achieved in just 10 min (Fig. 2a).

When the inlet CO_2 gas temperature increased to 150 °C so did the *E. coli* inactivation rate with a 3-log reduction in 10 min (Fig. 2b). For an inlet CO_2 gas temperature of 200 °C, the average temperature and the thickness of the heated water layer around the bubble was estimated to be roughly 73.5 °C and 100 nm, respectively, and the inactivation rate achieved in the ABCD for the *E. coli* was 3-log reduction in 0.17 M solution (Fig. 2b), after less than 5 min of treatment.

Isenschmid et al.[20] proposed that at temperatures over 18 °C, the concentration of dissolved compressed CO_2 is the key parameter behind the observed cell death rate. This could explain why no CO_2 inactivation effect was appreciable, with only 0.04-log reduction after 13 min at 7 °C inlet CO_2 temperature (Fig. 2a). If the CO_2 temperature rises over 18 °C, the penetration of the CO_2 through the membrane of the cells increases with the consequent CO_2 effect on bacterial inactivation.

At 7 °C CO_2 inlet temperature and the same column 0.17 M NaCl solution temperature, CO_2 was not able to penetrate through the *E. coli* membrane, and therefore no inactivation was observed. When the CO_2 inlet temperature was in the range of 18–100 °C, a CO_2 inactivation effect due to CO_2 penetration through the cell membranes appears most likely. *E. coli* inactivation rates increased when the CO_2 inlet gas temperature went over 100 °C. At these temperatures it seems that the CO_2 *E. coli* inactivation effect was present, as well as a thermal inactivation effect, due to the *E. coli* collisions with the hot water layer around the bubbles and the hot gas bubbles themselves.

Inlet gas (air vs CO_2) thermal inactivation comparison

In our previous research[8] it was shown that MS2 virus inactivation in the HBCE can be improved by increasing the inlet air temperatures from 150 °C to 250 °C. The thermal inactivation

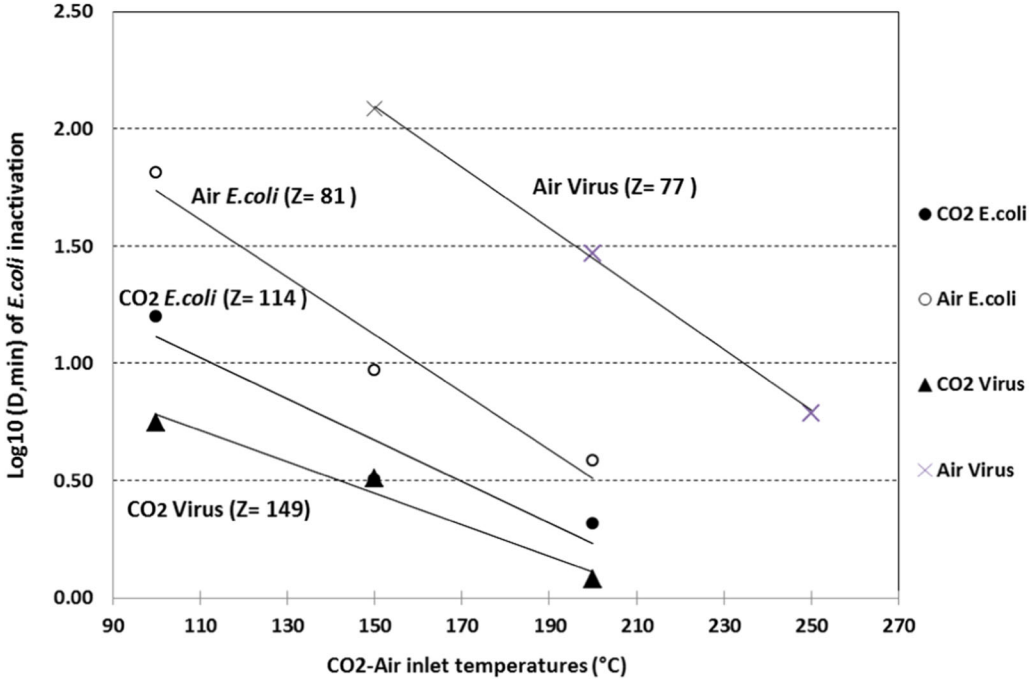

Fig. 4 Impact of temperature on *E. coli* and MS2 virus inactivation in 0.17 M NaCl solution in a bubble column

effect improves when the inlet air temperature increases probably by creating a thicker and hotter transient heated water layer around the rising air bubble surface.[8] *E. coli* and viruses will be thermally inactivated by the collisions with this layer. However, when using hot CO_2, this inactivation effect can be highly improved (Table 1).

To understand the gas effect (air vs CO_2) for thermal inactivation of pathogens (MS2 virus and *E. coli*), decimal reduction times (D-values) at three inlet gas temperatures, at intervals of 50 °C, were obtained, and the correlation between log of the D-values and the corresponding temperature is represented in Fig. 4. A D-value is the time needed to inactivate 90% (i.e., 1-log) of the pathogens. To measure the heat resistance of a microorganism, *Z*-values (Fig. 4) have been calculated. This value gives the temperature change required to change the D-value by a factor of 10 and reflects the temperature impact on a pathogen (*E. coli* and MS2 virus in our study). The smaller the *Z*-value, the greater the sensitivity to heat.

Figure 4 shows the minimum CO_2 and air bubbling times at different temperatures to achieve 1-log pathogen (virus and bacteria) inactivation in 0.17 M NaCl solutions. Above and to the right of the lines, the pathogens will be sterilised by 1-log.

At CO_2 inlet temperatures below 150 °C, MS2 viruses are inactivated in half of the time than *E. coli*, and therefore MS2 viruses are more sensitive to hot CO_2 than *E. coli*, in the range of 100° to 150 °C. However, when the inlet CO_2 temperature reaches 150 °C, viruses and bacteria present the same D-value of 3 min (Fig. 4).

For inlet gas temperatures in the range of 100 to 250 °C, CO_2 inactivates viruses much faster than air, with a D-value of 3.2 min for MS2 virus for CO_2 at 150 °C and a D-value of 122 min when using air at 150 °C. With *E. coli*, CO_2 presents faster inactivation rates than air. Especially at lower temperatures, 100 °C to 150 °C, with D-values of 16 min for CO_2 and 62 min for air at 100 °C and 3.2 min for CO_2 and 9.3 min for air at 150 °C. When inlet gas temperatures reach 200 °C, the D-values for both gases are similar but still CO_2 presents better inactivation rates than air; with 2.1 min and 3.8 min respectively (Fig. 4).

For viruses (*Z*-value = 149) and *E. coli* (*Z*-value = 114), inactivation with hot CO_2 bubbles is less temperature dependent that when using hot air bubbles with viruses (*Z*-value = 77) and *E. coli* (*Z*-value = 81) (Fig. 4). Low CO_2 inlet gas temperatures already present virus and *E. coli* inactivation effects. These effects can be incremented by increasing the CO_2 temperature. The combined effect of CO_2 sterilisation and CO_2 thermal inactivation at atmospheric pressure increases the sterilisation properties of CO_2 and makes it less temperature dependent and more effective than other gases, such as air, by an order of magnitude.

Hence, CO_2 offers an additional sterilisation process beyond that of other "inert" gases such as air. This effect is more appreciable for viruses than for bacteria, and at temperatures over 200 °C, *E. coli* presents similar inactivation rates for both gases. For inlet gas temperatures in the range of 100° to 200 °C, CO_2 presents clear advantages over air for both pathogens.

Comparison of the ABCD process with other technologies

Table 1 compares the *E. coli* and MS2 virus inactivation rates achieved using the ABCD process with different studies of the most common disinfection technologies in different types of water. For both pathogen groups, ABCD and UV technologies presented the best inactivation results, with 3-log inactivation after 230 s and 3.5-log after 180 s respectively when inactivating MS2 viruses. For the bacterium a 2.3-log inactivation was achieved after 300 s for ABCD and 3.8-log after 300 s for UV when inactivating *E. coli*. Ozone and chlorination sterilisation rates could be improved by increasing the dosage but at the concentrations used in these studies they present less or similar inactivation rates than the ABCD process (Table 1).

Current water disinfection technologies have several limitations.[2] The new ABCD technology could become a new disinfection technology candidate able to compete with the existing ones. The fact that the process can use heated CO_2 gas instead of heated water and the possibility of reusing exhaust gas from combustion processes makes the ABCD process potentially more energy efficient. If pure CO_2 or combustion gas from gas

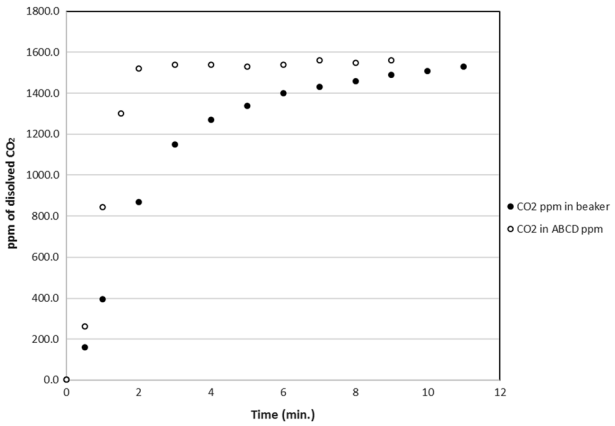

Fig. 5 Comparison of the absorption of CO_2 in NaCl 0.17 M solution with the ABCD and a single glass tube supplying CO_2 gas into a stirred beaker

generators is used, the only by-product that the system will generate will be 1% of carbonic acid at pH 4.1.

Absorption of carbon dioxide into 0.17 M NaCl solution

When CO_2 gas is bubbled through the sinter area, many bubbles are produced with the consequent CO_2 dissolution rate increment due to the large CO_2–liquid contact surface that is continually produced. Mass transfer from the CO_2 to the liquid phase is obviously a key process in the ABCD apparatus that depends highly on the interfacial area (α).

This increases the amount of CO_2 dissolved in the solution and produces a similar sterilisation effect to what can be achieved by raising the pressure in DPCD processes, but with the advantage that only atmospheric pressure is required. The high CO_2 inactivation effect is probably related to its high solubility in water.

The absorption of CO_2 into 0.17 M NaCl solutions, including its effect on pH, was studied in two experiments. An initial concentration of 2.8 ppm of CO_2 in 0.17 M solution at 22 °C was measured in both experiments. In the first experiment, high CO_2 bubble density was produced when bubbling through the sinter surface in a bubble column, and the CO_2 saturation point of 1570 ppm was reached in less than 2 min (see Fig. 5). In the second experiment, low CO_2 bubble density was produced when bubbling through a glass tube in a stirred beaker. In this experiment the same saturation point of 1570 ppm was reached after 11 min (Fig. 5). It was also found that the pH typically dropped from 5.9 to 4.1, in less than 45 s, once bubbling began.

When small CO_2 bubbles are produced continuously through a sinter surface, a high interfacial area (α) is generated in the solution, increasing the solubility of the gas in the solution and therefore the sterilisation effect even at atmospheric pressure for MS2 virus and *E. coli*. However, when bubbling CO_2 through a single glass tube in the same solution big bubbles are produced, a small interfacial area is generated, with the consequent lack of inactivation for the same pathogens.[9]

This study has shown that CO_2 gas bubbles can be used in the ABCD process to inactivate MS2 virus and *E. coli* in different NaCl solutions at atmospheric pressure, even at ambient temperatures. The efficiency of the process appears to depend on the use of CO_2 and its specific properties.

When CO_2 inlet gas temperatures are in the range of 18–100 °C, the precise mechanism that drives inactivation in the ABCD process is unknown. We can speculate that the penetration of CO_2 molecules into the virus capsid and bacterial membrane, due to the high density of CO_2 produced by the continuous CO_2–liquid contact surface area, plays a central role. At temperatures under 18 °C, these mechanisms appear not to be appreciable for viruses or bacteria.

At inlet CO_2 temperatures greater than 100 °C, the combined effect of CO_2 sterilisation and CO_2 thermal inactivation increases inactivation rates for both pathogen groups and this leads to the expectation that the new ABCD disinfection technology should be well able to compete with existing ones.

METHODS

Experimental solutions

Three different solutions were prepared and sterilised by autoclaving in an Aesculap 420 at 15 psi, and 121–124 °C for 15 min.[21] The first solution comprised 0.17 M NaCl (≥99% purity, obtained from Sigma-Aldrich) in 300 ml of Milli-Q water. Salt at such a concentration or higher is necessary to prevent bubble coalescence and increase the performance of the ABCD process by producing a higher CO_2–water interfacial area.[15] The second solution used was 0.001 M NaCl, in 300 ml of Milli-Q water. Bubble coalescence is not prevented at this low salt concentration.

To study the performance of the ABCD process with sewage water, a third solution, secondary treated synthetic sewage, was prepared according to water quality guidelines and standards.[22,23] This synthetic sewage Organization for Economic Co-operation Development (OECD medium) presents a mean dissolved organic carbon concentration of 100 mg/l and a COD of 300 mg/l in the influent (OECD reference). The official Journal of the European Community for secondary treated water quality has the following requirements for discharges from urban waste water treatment plants: 125 mg/l of COD, 2 mg/l of total phosphorus and 15 mg/l of total nitrogen.[24] Our secondary treated synthetic sewage was designed to meet the European standards by using the following ingredients: 120 mg of peptone, 90 mg of meat extract (we have replaced meat extract by Bovril® according to the recommendations in Biology of Wastewater Treatment[25]), 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 sulphate heptahydrate in 1000 ml of Milli-Q water.

Bacterial strain

E. coli C-3000 (ATCC15597) is a biosafety level 1 organism[26] and was used as a representative model for bacteria in water[18,19] for the *E. coli* inactivation experiments. It can be used as a MS2 virus host.[27] That is why it was selected for this work.

For a successful plaque assay, the *E. coli* C-3000 (ATCC 15597) must be in an exponential growth phase. This was achieved by growing two separate bacterial cultures: an overnight culture and a log phase culture.[21,27,28] The overnight culture was grown in 10 ml of the media without agar at 37 °C for 18–20 h in a Labtech digital incubator, model LIB-030M, while shaking at 110 rpm with a PSU-10i orbital shaker. The overnight culture resulted in high numbers of bacteria in the culture and this was used as a reference standard.

Viral strains

The MS2 bacteriophage (ATCC 15597-B1)[29,30] was chosen as the model virus to evaluate the efficiency of thermal inactivation by the ABCD

process. MS2 is used as a surrogate for enteric viruses since it is inactivated only at temperatures above 60 °C, is resistant to high salinity and susceptible only to low pH.[31]

A freeze-dried vial of MS2 bacteriophage was acquired from the American Type Culture Collection. Bacteriophage MS2 (ATCC 15597-B1) was replicated using *E. coli* C-3000 (ATCC 15597) according to the International Standard ISO 10705-1[21] and the Ultraviolet Disinfection Guidance Manual of the United States Environmental Protection Agency.[32] MS2 activity is usually quantified by counting infectious units via a standard plaque assay.

The atmospheric bubbling with CO_2 device process

In the ABCD process used in these experiments CO_2 gas was pumped through an electrical heater that maintained the gas temperature just above the sinter surface, from which the gas was released, over a range of 7° to 205 °C, depending on the experiment. The base of the bubble column evaporator was fitted with a 40–100 µm pore size glass sinter (type 2) of 135 mm diameter.

Once the experimental solutions were poured into the column, the temperature of the solution was measured with a thermocouple in the centre of the column solution. The hot CO_2 gas bubbles inactivated MS2 viruses or *E. coli*, in separate experiments.

The World Health Organisation (WHO) in their guidelines for drinking-water quality[33] compared thermal inactivation rates for different types of bacteria and viruses in hot liquids. They concluded that water temperatures have a higher impact on bacterial inactivation than on viruses. This is the reason why we have selected different target temperatures. Viruses and bacteria are two different pathogenic groups with different inactivation response to temperature.

Disinfection experiments

Experiments were performed using 0.17 M NaCl, 0.001 M NaCl aqueous solutions and secondary treated synthetic sewage, with the temperatures of the CO_2 inlet gas set at 7°, 22°, 38°, 100°, 150° and 200 °C.

The evaluation of bacteriophage and *E.coli* viability was performed by the plaque assay method.[28,34,35]

Once the solutions with known concentrations of coliphage and *E. coli* were prepared, two rounds of experiments were conducted in the ABCD to study the inactivation of MS2 virus and then for *E. coli*. Samples of 1.3 ml were collected from 10 to 15 mm above the central area of the sinter. Each sample of 0.07 ml was spotted in triplicate following the double layer plaque assay technique.[32]

Carbon dioxide absorption experiments

In two different CO_2 water saturation experiments (one in a bubble column and the other one in a stirred beaker) the dissolved CO_2 in water was measured with an Orion™ 9502BNWP carbon dioxide ion selective electrode. The probe was calibrated with a 1000 ppm standard, obtaining a slope of 55.5 mV/decade.[36] In order to stop CO_2 bubbles from being trapped at the tip of the electrode, the probe was placed at a 20° angle from the vertical[36] inside a small beaker.

Data analysis

The linear and second order polynomial decay models have been used to study the inactivation of viruses and bacteria in the bubble column evaporator with time. Plaque counts were performed for all 19–21 plates from each of the experiments.[5,37] The mean and the standard deviation of each triplicated sample was obtained using a virus or bacteria survival factor: $\mathrm{Log}_{10}(\mathrm{PFU}/\mathrm{PFU}_0)$, where PFU_0 is the initial number of plaque-forming units (PFU) per sample and PFU is the PFU per sample after an exposure time in min.[31]

To measure the heat resistance of a microorganism, we have used the decimal reduction time (D-value) that is the time needed to inactivate 90% (i.e., 1-log) of the pathogens to compare the temperature impact on a pathogen. The *Z*-value is the temperature change required to change the D-value by a factor of 10. The smaller the *Z*-value, the greater the sensitivity to heat.

D-values and *Z*-values were calculated using a linear exponential decay model or Thermal Death Model.[37]

$$\log(N_t) = log(N_0) - \frac{t}{D}, \tag{3}$$

$$\log\left(\frac{N_t}{N_0}\right) = -\frac{t}{D}, \tag{4}$$

where N_t is the number of microorganisms at time t, N_0 is the initial number, D is the decimal reduction time and $-(1/D)$ is the slope of the curve.

The *Z*-value is the increase in temperature needed to reduce the D-value by 1-log unit. It measures the impact of a change in temperature on pathogen inactivation. Thus:

$$Z = \frac{T_1 - T_2}{\mathrm{logD}_1 - \mathrm{logD}_2}, \tag{5}$$

where T_1 is first temperature of the interval, T_2 is second temperature of the interval and D_1 and D_2 are the D-values at T_1 and T_2.

DATA AVAILABILITY

The data that support the findings of this study are available as supplementary information.

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AUTHOR CONTRIBUTIONS

A.G.S. (principal author) carried out the lab experiments, performed analysis on all samples, interpreted data and wrote manuscript. R.P. (co-author) supervised development of the work, helped in data interpretation, manuscript preparation and acted as corresponding author. B.N. (co-author) supervised development of work, helped in data interpretation and manuscript preparation.

ADDITIONAL INFORMATION

Supplementary information accompanies the paper on the *npj Clean Water* website (https://doi.org/10.1038/s41545-018-0027-5).

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