Virus and bacteria inactivation by CO₂ bubbles in solution

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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.

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 wastewater 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 inactivation 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 CO₂ 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) 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 CO₂ 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
column process, a series of experiments were carried out based on our earlier work.\textsuperscript{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\textsubscript{2}, due to the chemical modification on the phospholipid bilayer of the membranes.\textsuperscript{12,13} Cheng et al.\textsuperscript{11} believed that CO\textsubscript{2} molecules could enter virus capsids much easier than H\textsuperscript{+}. They observed almost no inactivation change for three different viruses (MS2, Q\textsubscript{B} and ϕX174) under four different pH conditions (pH 4, 4.5, 5 and 5.5).

In our previous work,\textsuperscript{5} when bubbling room temperature CO\textsubscript{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, after 0.17 min of bubbling (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 collateral, thermal inactivation.

In earlier studies,\textsuperscript{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\textsubscript{2} gas bubbles do not show any sterilisation properties. For example, when CO\textsubscript{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\textsubscript{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)\textsuperscript{5,8} due to the bubble coalescence inhibition phenomenon. The phenomenon of bubble–bubble interactions in electrolytes was explored by us 30 years ago.\textsuperscript{4,15} 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.\textsuperscript{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\textsubscript{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\textsubscript{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,\textsuperscript{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\textsubscript{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\textsubscript{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\textsubscript{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...
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 Q$_\beta$ 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
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 CO₂ bubble can be roughly estimated for a range of inlet CO₂ temperatures using these formulae:

\[
T_{\text{avg}} = \frac{100 + T_c}{2},
\]

(1)

where \( T_{\text{avg}} \) (in °C) is the average (transient) temperature of the hot water layer surrounding the CO₂ bubble and \( T_c \) (°C) is the equilibrium temperature of the solution in the ABCD, assuming that the hot CO₂ 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 \( r \gg z \).

Then, the thermal energy balance is given by:

\[
C_p \Delta T V = C_{\text{water}} \Delta t 4\pi r^2 \rho_w z,
\]

(2)

where \( C_p \) and \( C_{\text{water}} \) are air and water heat capacities, respectively, \( \rho_w \) is the liquid water mass density, \( \Delta T \) is the cooling of the air bubble (from its inlet temperature to 100 °C) and \( \Delta t \) is the transient temperature increase in the water layer, relative to the column solution temperature.
In practice, we might expect that roughly half of the heat supplied by the cooling bubble will be used in evaporating water into the CO₂ bubble and hence the calculated, roughly estimated, film thicknesses should be halved.

For an inlet CO₂ 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 CO₂ 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 CO₂ 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 CO₂ temperature, where the inactivation can only be produced by the CO₂ inactivation effect.

After 6.5 min at 9 °C (inlet CO₂ 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 CO₂ is not able to penetrate through the capsid of the viruses, and therefore no inactivation was observed.

When the CO₂ temperature is in the range of 18–100 °C, CO₂ penetrates the capsid of the viruses producing the CO₂ inactivation effect. For CO₂ inlet temperatures over 100 °C, virus inactivation is most likely due to the combination of CO₂ inactivation effect and the virus collision with the hot water layer around the bubble.

Effect of CO₂ 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. Different mechanisms have been suggested to explain the antibacterial effect of dissolved CO₂. In Chapter 4 of the book “Dense Phase Carbon Dioxide”, Erkmen describes, in great detail, the different steps proposed for the bacterial inactivation mechanism for pressurised CO₂. When pressurised CO₂ first dissolves in the solution, its pH decreases, and this acidification of the solution increases the penetration of CO₂ through the membranes. The CO₂ inside of the cell will produce an intracellular pH decrease that will exceed the cell’s buffering capacity, resulting in cell inactivation.

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. At 100 °C inlet CO₂ 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 CO₂ 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 CO₂ temperatures, where the inactivation was, again, only produced by the CO₂ inactivation effect. With CO₂ bubbles at 18 °C, only a 0.37-log E. coli reduction was achieved in just 10 min (Fig. 2a).

When the inlet CO₂ 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 CO₂ 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. proposed that at temperatures over 18 °C, the concentration of dissolved compressed CO₂ is the key parameter behind the observed cell death rate. This could explain why no CO₂ inactivation effect was appreciable, with only 0.04-log reduction after 13 min at 7 °C inlet CO₂ temperature (Fig. 2a). If the CO₂ temperature rises over 18 °C, the penetration of the CO₂ through the membrane of the cells increases with the consequent CO₂ effect on bacterial inactivation.

At 7 °C CO₂ inlet temperature and the same column 0.17 M NaCl solution temperature, CO₂ was not able to penetrate through the E. coli membrane, and therefore no inactivation was observed. When the CO₂ inlet temperature was in the range of 18–100 °C, a CO₂ inactivation effect due to CO₂ penetration through the cell membranes appears most likely. E. coli inactivation rates increased when the CO₂ inlet gas temperature went over 100 °C. At these temperatures it seems that the CO₂ 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 CO₂) thermal inactivation comparison

In our previous research 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

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

| Pathogen | Treatment | Log Reduction | Time (s) | Solution | pH | Solution Temp, °C | Source |
|----------|-----------|---------------|---------|----------|----|-------------------|--------|
| E. coli  | Thermal inactivation 60 °C | 6 | 1800 | Sewage water | 8 | 60 | 38 |
|          | 2.0 mg O₂/l | 1.3 | 300 | Tap water | 7.6 | 23 | 39 |
|          | 2.0 mg Cl₂/l | 2 | 300 | Tap water | 7.6 | 23 | 39 |
|          | UV (0.78 mW/cm²) at 295–400 nm | 3.8 | 300 | Natural water | 7 | Room temp. | 40 |
|          | DPCD, CO₂ at 197 atm and 34 °C | 2.5 | 600 | Sterilised water | 34 | 41 |
|          | Bubble column, CO₂ at 200 °C, 1 atm | 2.3 | 300 | 0.17M NaCl | 6 | 49 | Fig. 2b, this study |
| MS2 virus | 0.1 mg O₂/l | 1.2 | 180 | Ultrapure water | 7 | 22 | 42 |
|          | 1.0 mg H₂O₂/l | 0.001 log | 90 | 0.01 M Phosphate buffer | 6-10 | 3-10 | 43 |
|          | 30 mg Cl₂/l | 1 log | 300 | Primary sewage effluent | 8 | 15 | 44 |
|          | UV (0.19 mW/cm²) | 3.5 log | 180 | Ultrapure water | 7 | 22 | 42 |
|          | Bubble column, CO₂ 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 |
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. E. coli and viruses will be thermally inactivated by the collisions with this layer. However, when using hot CO₂, this inactivation effect can be highly improved (Table 1).

To understand the gas effect (air vs CO₂) 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 CO₂ 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 CO₂ inlet temperatures below 150 °C, MS2 viruses are inactivated in half of the time than E. coli, in the range of 100° to 150 °C. However, when the inlet CO₂ 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, CO₂ inactivates viruses much faster than air, with a D-value of 3.2 min for MS2 virus for CO₂ at 150 °C and a D-value of 122 min when using air at 150 °C. With E. coli, CO₂ presents faster inactivation rates than air. Especially at lower temperatures, 100 °C to 150 °C, with D-values of 16 min for CO₂ and 62 min for air at 100 °C and 3.2 min for CO₂ 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 CO₂ 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 CO₂ 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 CO₂ inlet gas temperatures already present virus and E. coli inactivation effects. These effects can be incremented by increasing the CO₂ temperature. The combined effect of CO₂ sterilisation and CO₂ thermal inactivation at atmospheric pressure increases the sterilisation properties of CO₂ and makes it less temperature dependent and more effective than other gases, such as air, by an order of magnitude.

Hence, CO₂ 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, CO₂ 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. 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 CO₂ 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 CO₂ or combustion gas from gas
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 CO₂ gas is bubbled through the sinter area, many bubbles are produced with the consequent CO₂ dissolution rate increment due to the large CO₂–liquid contact surface that is continually produced. Mass transfer from the CO₂ 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 CO₂ 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 CO₂ inactivation effect is probably related to its high solubility in water.

The absorption of CO₂ into 0.17 M NaCl solutions, including its effect on pH, was studied in two experiments. An initial concentration of 2.8 ppm of CO₂ in 0.17 M solution at 22 °C was measured in both experiments. In the first experiment, high CO₂ bubble density was produced when bubbling through the sinter surface in a bubble column, and the CO₂ saturation point of 1570 ppm was achieved in less than 2 min (see Fig. 5). In the second experiment, low CO₂ bubble density was produced when bubbling through a bubble column and the CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ and its specific properties.

When CO₂ 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 CO₂ molecules into the virus capsid and bacterial membrane, due to the high density of CO₂ produced by the continuous CO₂–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 CO₂ temperatures greater than 100 °C, the combined effect of CO₂ sterilisation and CO₂ 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.9 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 CO₂–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 Treatment25), 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 organism26 and was used as a representative model for bacteria in water.27,18,19 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.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 10705-153 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 CO2 device process

In the ABCD process used in these experiments CO2 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 17°C 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 CO2 gas bubbles inactivated MS2 viruses or E. coli, in separate experiments. The World Health Organisation (WHO) in their guidelines for drinking-water quality33 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 CO2 inlet gas set at 7°C, 22°C, 38°C, 100°C, 150°C 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 CO2 water saturation experiments (one in a bubble column and the other one in a stirred beaker) the dissolved CO2 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 CO2 bubbles from being trapped at the tip of the electrode, the probe was placed at a 20° angle from the vertical 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.3,5,7 The mean and the standard deviation of each triplicated sample was obtained using a virus or bacteria survival factor: \( \log_{10}(\text{PFU}/\text{PFU}_0) \), where PFU0 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

\[
\log(N_t) = \log(N_0) - \frac{t}{D},
\]

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.

\[
\log \left( \frac{N_t}{N_0} \right) = -\frac{t}{D}
\]

\[
Z = \frac{T_2 - T_1}{\log D_2 - \log D_1}
\]

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

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REFERENCES

1. Collivignarelli, M., Abbà, A., Benigna, I., Sorlini, S. & Torretta, V. Overview of the main disinfection processes for wastewater and drinking water treatment plants. Desalination 351, 236–242 (2014).
2. Shahid, M., Fan, C. & Pashley, R. M. Insight into the bubble column evaporator and its applications. Int. Rev. Phys. Chem. 35, 143–185 (2016).
3. Garrido, A., Pashley, R. M. & Ninham, B. W. Low temperature MS2 (ATCC15597-B1) virus inactivation using a hot bubble column evaporator (HBCE). Colloids Surf. B Biointerfaces 151, 1–10 (2016).
4. Xue, X. & Pashley, R. M. A study of low temperature inactivation of fecal coliforms in electrolyte solutions using hot air bubbles. Desalination Water Treat. 57, 9444–9454 (2016).
5. Shahid, M., Pashley, R. & Rahman, A. F. M. Use of a high density, low temperature, bubble column for thermally efficient water sterilization. Desalination Water Treat 52, 4444–4452 (2014).
6. Sanchis, A. G., Shahid, M. & Pashley, R. M. Improved virus inactivation using a hot bubble column evaporator (HBCE). Colloids Surf. B Biointerfaces 165, 293–302 (2018).
9. Garrido, A., Pashley, R. M. & Ninhm, B. W. Water sterilisation using different hot gases in a bubble column reactor. J. Environ. Chem. Engineer. 6, 2651–2659 (2018).
10. Garcia-Gonzalez, L. et al. High pressure carbon dioxide inactivation of microorganisms in foods: the past, the present and the future. Int. J. Food Microbiol. 117, 1–28 (2007).
11. Cheng, X. et al. Inactivation of bacteriophages by high levels of dissolved CO2. Environ. Technol. 34, 539–544 (2013).
12. Erkmen, O. Dense Phase Carbon Dioxide. Food and Pharmaceutical Applications. 67–97 (Wiley-Blackwell, Chichester, 2012).
13. Lin, H. M., Yang, Z. & Chen, L. F. Inactivation of Saccharomyces cerevisiae by supercritical and subcritical carbon dioxide. Biotechnol. Prog. 8, 458–461 (1992).
14. Vincent S. J. Craig, Barry W. Ninhm, and Richard M. Pashley. The effect of electrolytes on bubble coalescence in water. The Journal of Physical Chemistry 97, 10192–10197 (1993).
15. Craig, V. S. J., Ninhm, B. W. & Pashley, R. M. Effect of electrolytes on bubble coalescence. Nature 364, 317–319 (1993).
16. Ferrentino, G., Calix, T., Poletto, M., Ferrari, G. & Balaban, M. O. Dense Phase Carbon Dioxide 37–66 (Wiley-Blackwell, Chichester, 2012).
17. Thomas R. Cundari, A. K. W. et al. CO2-formatics: how do proteins bind carbon dioxide? J. Chem. Inf. Model. 49, 2111–2115 (2009).
18. Yang, X. A Study on Antimicrobial Effects of Nanosilver for Drinking Water Disinfection 1–12 (Springer, Singapore, 2017).
19. Gaska, I. et al. Deep UV LEDs for public health applications. Int. J. High. Speed Electron. Syst. 23, 1450018 (2014).
20. Isenschmid, A., Marison, I. W. & von Stockar, U. The influence of pressure and temperature of compressed CO2. J. Biotechnol. 39, 229–237 (1995).
21. ISO 10705-1. Water Quality Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-Specific RNA Bacteriophages, (World Health Organization, Geneva, 1995).
22. ISO 11733. Water Quality – Determination of the elimination and biodegradability of organic compounds in an aqueous medium – Activated sludge simulation test (World Health Organization, Geneva, 2004).
23. OECD. Simulation Test - Aerobic Sewage Treatment: 303 A: Activated Sludge Units - 21. ISO 11733-1. Water Quality – Determination of the elimination and biodegradability of organic compounds in an aqueous medium – Activated sludge simulation test (World Health Organization, Geneva, 2004).
24. OECD. Simulation Test - Aerobic Sewage Treatment: 303 A: Activated Sludge Units - 303 & Biofilms (Organization for Economic Co-operation Development, Paris, 2001).
25. EEC. Vol. (91/271/EEC) (ed. European Union Council) 13 (EEC, Of the European Communities, EU publications. 1991)https://publications.europa.eu/en/home.
26. ATCC. Eschrichia coli bacteriophage MS2 (ATCC 15597-B1). Product sheet. (2015)available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/D15856~.pdf. (2009).
27. ATCC. Eschrichia coli (Migula) (ATCC 15597). Product sheet. (2015)https://www.atcc.org/en/Products/Quality_Control/Strains/Water_Environmental/15597-B1.aspx#documentation.
28. USEPA-a, Method 1602: Male-specific (F+) and somatic coliphage in Water by Single Agar Layer (SAL) procedure. Office of Water, USEPA, Washington, DC, EPA 821-R01-030, 2001 https://www.epa.gov/sites/production/files/2015-12/documents/method_1601_2001.pdf.
29. Hryniszyn, A., Skonieczna, M. & Wsianozski, J. Methods for detection of viruses in water and wastewater. Adv. Microbiol. 3, 442–449 (2013).
30. World Health Organization. Evaluating Household Water Treatment Options: Health-Based Targets and Microbiological Performance Specifications (World Health Organization, Geneva, 2011).
31. Seo, K., Lee, J. E., Lim, M. Y. & Ko, G. Effect of temperature, pH, and NaCl on the inactivation kinetics of murine norovirus. J. Food Prot. 75, 533–540 (2012).
32. Pirnie, M., Linden, K. G., & Malley, J. P. J. (2006). Ultraviolet disinfection guidance manual for the final long term enhanced surface water treatment rule (pp. 1–436). Washington: United States Environmental Protection Agency, https://ocw.tudelft.nl/wp-content/uploads/guide_lt2_uvguidance.pdf.
33. World Health Organization. Guidelines for Drinking-Water Quality, 4th; World Health Organization: Geneva, Switzerland, 2011.
34. Clokie, M. R. J. & Kropinski, A. M. (eds) Bacteriophages Vol. 501 (Springer, New York, 2009).
35. Cormier, J. & Janes, M. A double layer plaque assay using spread plate technique for enumeration of bacteriophage MS2. J. Virol. Methods 196, 86–92 (2014).
36. Thermo Fisher Scientific Inc. Carbon Dioxide Ion Selective Electrode. User guide. Available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/D15856~.pdf. (2009).
37. Smelt, J. & Brul, S. Thermal inactivation of microorganisms. Crit. Rev. Food Sci. Nutr. 54, 1371–1385 (2014).
38. Mocoe-Livina, L., Muniesa, M., Pimenta-Vale, H., Lucena, F. & Jofre, J. Survival of bacterial indicator species and bacteriophages after thermal treatment of sludge and sewage. Appl Environ Microbiol 69, 1452–1456 (2003).
39. de Souza, J. B. & Daniel, L. A. Synergism effects for Escherichia coli inactivation applying the combined ozone and chlorine disinfection method. Environmental Technology 32, 1401-1408, 10.1080/09593330.2010.537373 (2011).
40. Mamane, H., Shemer, H. & Linden, K. G. Inactivation of E. coli, B. subtilis spores, and MS2, T4, and T7 phage using UV/H2O2 advanced oxidation. Journal of Hazardous Materials 146, 479–486 (2007).
41. Spilimbergo, S., Deighani, F., Bertucco, A. & Foster, N. R. Inactivation of bacteria and spores by pulse electric field and high pressure CO2 at low temperature. Biotechnology and Bioengineering 82, 118–125 (2003).
42. Fang, J., Liu, H., Shang, C., Zeng, M., Ni, M. & Liu, W. E. coli and bacteriophage MS2 disinfection by UV, ozone and the combined UV and ozone processes. Frontiers of Environmental Science & Engineering 8, 547–552 (2014).
43. Hall, R. M. & Sobsey, M. Inactivation of hepatitis A virus and MS2 by ozone and ozone-hydrogen peroxide in buffered water. Vol. 27 (1993).
44. Tree, J. A., Adams, M. R. & Lees, D. N. Chlorination of indicator bacteria and viruses in primary sewage effluent. Applied and Environmental Microbiology 69, 2038–2043 (2003).

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