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In situ generation of highly localized chlorine by laser-induced graphene electrodes during electrochemical disinfection

Ju Zhang, Le Cheng, Liqing Huang, Pok Him Ng, Qianjun Huang, Ana Rita Marques, Brett MacKinnon, Libei Huang, Yefeng Yang, Ruquan Ye, Sophie St-Hilaire

HIGHLIGHTS

• An array of mechanisms works synergistically to inactivate bacteria close to LIG electrodes.
• The RCS was likely responsible for the predominant cause of antibacterial effects in the bulk solution.
• At a low applied voltage (3 V), highly localized RCS on the LIG surface contributed to efficient disinfection.
• LIG with low voltages inactivated bacteria without high levels of oxidants, suggesting a safe electro-disinfection.

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ABSTRACT

Laser-induced graphene (LIG) has gained popularity for electrochemical water disinfection due to its efficient antimicrobial activity when activated with low voltages. However, the antimicrobial mechanism of LIG electrodes is not yet fully understood. This study demonstrated an array of mechanisms working synergistically to inactivate bacteria during electrochemical treatment using LIG electrodes, including the generation of oxidants, changes in pH—specifically high alkalinity associated with the cathode, and electro-adsorption on the electrodes. All these mechanisms may contribute to the disinfection process when bacteria are close to the surface of the electrodes where inactivation was independent of the reactive chlorine species (RCS); however, RCS was likely responsible for the predominant cause of antibacterial effects in the bulk solution (i.e., ≥100 mL in our study).

Furthermore, the concentration and diffusion kinetics of RCS in solution was voltage-dependent. At 6 V, RCS achieved a high concentration in water, while at 3 V, RCS was highly localized on the LIG surface but not measurable in water. Despite this, the LIG electrodes activated by 3 V achieved a 5.5-log reduction in Escherichia coli (E.coli) after 120-min electrolysis without detectable chlorine, chlorate, or perchlorate in the water, suggesting a promising system for efficient, energy-saving, and safe electro-disinfection.

Abbreviations: LIG, Laser-induced graphene; RCS, Reactive chlorine species; UV, Ultraviolet; ROS, Reactive oxygen species; E.coli, Escherichia coli.

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1. Introduction

Waterborne pathogens remain one of the primary environmental concerns threatening public health (Ramírez-Castillo et al., 2015). As water resources become more scarce, efficient and environmentally-friendly water disinfection techniques are becoming more critical to allow for its safe reuse (Yuan et al., 2016). Conventional disinfection methods like chlorination, ultraviolet (UV) radiation, ozonation, and biocides have limitations, such as high energy consumption and safety issues (Von Gunten, 2003), transportation and storage of equipment (Moreno-Andrés et al., 2018), and the short duration of efficacy with UV-disinfection (Oguma et al., 2001).

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Recently, electrochemical disinfection has been considered as a green technology (Huang et al., 2016; Bruguera-Casamada et al., 2017), and attracted attention as an alternative to traditional water treatment technologies (Poyatos et al., 2010; Cano et al., 2012; Ding et al., 2017). It provides multiple advantages, including low energy consumption, amenability to automation, high efficiency in removing a wide range of pathogens, and the unnecessary use of additional chemicals (Barrera-Díaz et al., 2003; Vecitis et al., 2011; Huang et al., 2016; Monasterio et al., 2017; Liang et al., 2018; Mao et al., 2018; Zhang et al., 2018).

Two primary antibacterial mechanisms are involved in electrochemical disinfection, including direct and indirect oxidation (Bergmann et al., 2014; Bruguera-Casamada et al., 2016). Direct oxidation occurs when pathogens come into contact with the electrodes and then lose electrons, causing damage to their cell membranes (Long et al., 2015). Indirect oxidation is mediated by strong oxidizing agents generated on-site when an electric current is passed through the electrode (Rahmani et al., 2019). Oxidants such as reactive chlorine species (RCS) (Cl2) can be formed by oxidizing chlorine ions on the anode (Bonfatti et al., 2000). Other oxidants like ozone also can be formed on the anode when oxygen is oxidized (Panizza and Cerisola, 2009). In contrast, oxidants like H2O2 are generated from oxygen reduction, which occurs in the cathode (Brillas and Martínez-Huitle, 2015). Commonly reported antibacterial oxidants produced during the in-direct oxidation process on electrodes include RCS (i.e., [Cl2], [HOCI], [ClO−], [Cl−], [CTCl]), reactive oxygen species (ROS) (i.e. [OH•], [O2•], [H2O2•], [O2•]), and other products such as SO4•−, C2O6•− and P2O8•− (Canizares et al., 2005; Jeong et al., 2009; Huang et al., 2016; Bruguera-Casamada et al., 2017; Rahmani et al., 2019). Different electrode materials favour different oxidizing agents and have different disinfection mechanisms (López-Gálvez et al., 2012; Huang et al., 2016). For example, in materials such as carbon nanotube sponges and carbon fibre felt (Liang et al., 2018; Ni et al., 2020), disinfection occurs primarily via direct oxidation; while for materials such as boron-doped diamond (BDD), Ti/RuO2, Pt, Ti/IrO2, an indirect oxidation mechanism seems to play a larger role (Jeong et al., 2009).

Although different electrode materials may act via different inactivation mechanisms, many exhibit excellent antimicrobial properties. Most of these materials, however, are limited to small-scale applications due to their high energy consumption, high cost, generation of toxic compounds, and unwanted oxidants (Ferreira et al., 2006; Lin et al., 2013; Schaefer et al., 2015; Wu et al., 2016). Graphene and its derivatives are the next-generation materials for wastewater remediation (Yin et al., 2020a). Among them, laser-induced graphene (LIG) has been described using linear sweep voltammetry (LSV) curves, cyclic voltammetry (CV) curves, and chronocoulometry (CA) curves. All measurements were conducted using the CHI 760 E electrochemical workstation (CHI Instruments, Shanghai, China). A Three-electrode configuration was used for this purpose, consisting of an Ag/AgCl reference electrode (3 M KCl), a platinum (Pt) wire counter electrode, and a LIG working electrode (1 cm × 8 cm with a working area of 1 cm × 5 cm). Electrochemical measurements were carried out in an undivided system with both NaCl and Na2SO3 aqueous electrolyte solutions separately. All applied potentials were converted to the reversible hydrogen electrode (RHE) scale via the following equation:

\[ E (\text{vs. RHE}) = E (\text{vs. Ag/AgCl}) - 0.592 \text{ pH} + 0.230 \text{ V}. \]

The LSV and CV curves were evaluated across a range of voltages −5 to +5 V vs. RHE at a scan rate of 100 mV min⁻¹. The CA curves were recorded at 2.35 V vs. RHE and 2.29 V vs. RHE in NaCl and Na2SO3 solution, respectively. Applied voltages in the CA curves analysis were based on the LSV results of LIG electrodes when the working potential of 3 V was used.

2. Materials and methods

2.1. Fabrication and characteristics of LIG

A polyimide film (Zeman Tape Material Technology, China) with a thickness of 0.05 mm was irradiated with a 10.6 μm CO2 laser marking machine (MS-1380, Min Sheng Laser Co Ltd, China) to fabricate LIG. The laser power, speed, and line spacing were set at 1.8 W, 500 mm/s, and 700 mm, respectively. Raman spectra of the LIG were obtained with a LabRAM HR800 laser confocal micro-Raman spectrometer with a laser wavelength of 514.5 nm. SEM images of the LIG were performed using a QUATTRO S scanning electron microscope from Thermo Fisher (Ramé-Hart model 190). TEM characterizations of LIG were performed using a 200 kV JEOL 2100 Field Emission Gun TEM.

The electrochemical characterizations of LIG electrodes were described using linear sweep voltammetry (LSV) curves, cyclic voltammetry (CV) curves, and chronoamperometry (CA) curves. All measurements were conducted using the CHI 760 E electrochemical workstation (CHI Instruments, Shanghai, China). A Three-electrode configuration was used for this purpose, consisting of an Ag/AgCl reference electrode (3 M KCl), a platinum (Pt) wire counter electrode, and a LIG working electrode (1 cm × 8 cm with a working area of 1 cm × 5 cm). Electrochemical measurements were carried out in an undivided system with both NaCl and Na2SO3 aqueous electrolyte solutions separately. All applied potentials were converted to the reversible hydrogen electrode (RHE) scale via the following equation:

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2.2. Chemical and electrode system

Sodium sulfate (Na2SO4), sodium chloride (NaCl), and sodium thiosulfate (Na2S2O3) were purchased from Aldrich Co. (USA). All chemicals were of reagent grade and used without further purification. All supporting electrolytes and stock solutions were prepared with distilled and deionized water from a Millipore purification system (Barnstead NANO Pure, USA).

To investigate the primary antibacterial oxidants and elucidate the function of each LIG electrode during electrolysis (Jiang et al., 2010),
two different electrolytic systems were used: (1) a divided system (Figs. S1–B) with a Nafion 117 membrane separating the anode compartment from the cathode compartment and (2) an undivided system, where the electrodes were housed together (Figs. S1–A). The divided and undivided electrolytic systems are made from borosilicate glass (Jingke technology, China). In the undivided system, the total solution volume was 100 mL, while the total solution volume in the divided system was 400 mL. The LIG electrodes used in the electrolysis systems had a surface area of 1 cm × 8 cm with a working size of 1 cm × 5 cm. The LIG-paired electrodes had a 2 mm gap between the cathode surface and anode surface in the undivided system, while the anode was separated from the cathode by 17 cm in the divided system. Conductive graphite paper (Jinglong Co Ltd, Beijing, China) was attached to the top of LIG membranes using double-sided carbon conductive tape (Nisshin EM Co Ltd, Japan). Alligator clips were attached to the top of the graphite sheet to convey the electricity supplied from a direct current power source (UTP1306S, Uni-Trend Technology China Co Ltd, China) (Fig. S1). LIG electrodes were applied with 3 V or 6 V of electricity using a direct current power source.

In addition, recirculating water systems (Figs. S1–C) were constructed to assess further the antibacterial effects of LIG electrodes between chloride-containing (NaCl) and non-chloride-containing electrolyte solutions (phosphate buffer, NaCl). In the recirculating system, the LIG membranes were cut into two 2 cm × 8 cm strips and placed 2 mm apart in an external filtering box (PF-120, Shiruba, Taiwan, China) with a cotton filter (230A, XILONG, Guangdong, China) supporting the LIG membranes. The LIG membranes were suspended 5 cm below and 3 cm above the water line. Conductive graphite paper (Jinglong Co Ltd, Beijing, China) was attached to the top of LIG membranes using double-sided carbon conductive tape (Nisshin EM Co Ltd, Japan). Alligator clips were attached to the top of the graphite sheet to convey the 3 V of electricity supplied from a direct current power source (UTP1306S, Uni-Trend Technology China Co Ltd, China). Water was recirculated in the 2 L aquarium after it passed over the LIG strips at a flow rate of 120 L/h.

2.3. Bacterial preparation

A single colony of bacteria (E. coli) on a solid tryptic soy agar (TSA) (Sigma-Aldrich, USA) plate was transferred to 1 mL of tryptic soy broth (TSB) (Sigma-Aldrich, USA) in a shaking incubator (250 rpm) at 37 °C overnight. A 1% bacterial suspension was then transferred to new TSB (Sigma-Aldrich, USA) plate was transferred to 1 mL of tryptic soy broth (250 rpm) at 37 °C overnight. A 1% bacterial suspension was then transferred to new TSB (Sigma-Aldrich, USA) in a shaking incubator (250 rpm) at 37 °C overnight. A 1% bacterial suspension was then transferred to new TSB (Sigma-Aldrich, USA) in a shaking incubator (250 rpm) at 37 °C. After overnight growth, a 1% bacterial suspension was then transferred to new TSB (Sigma-Aldrich, USA) in a shaking incubator (250 rpm) at 37 °C. After overnight growth, a 1% bacterial suspension was then transferred to new TSB (Sigma-Aldrich, USA) in a shaking incubator (250 rpm) at 37 °C. After overnight growth, a 1% bacterial suspension was then transferred to new TSB (Sigma-Aldrich, USA) in a shaking incubator (250 rpm) at 37 °C.

The bacteria were quantified on eosin-methylene blue (EMB) medium using the plate counting method (Jeong et al., 2009; Ashby et al., 2020).

2.4. Bacterial inactivation by LIG electrodes

The bacterial inactivation experiments were done at room temperature in two different electrolyte solutions: (1) NaCl (Chloride-containing solution); and (2) NaClO4 (Non-chloride-containing solution). All electrolyte solutions were adjusted to 0.05 M before the start of the study. The voltages applied to the LIG electrodes varied between 0 V (control), 3 V, and 6 V. All treatment and control units were done in triplicates.

In brief, before turning on the DC power source, E. coli was added to the different electrolyte solutions to achieve a final concentration of ~5 × 10^5 CFU/mL. This mixture was stirred with a magnetic stirrer for 30 min to obtain a well-distributed cell suspension in the reactor before electrolysis. During electrolysis, we mixed the solutions using a sterilized glass pipette every 30 min to ensure the even distribution of bacteria. To assess the inactivation effect, we sampled 1 mL of the solutions to determine the bacteria counts at time 0 and after the electrochemical reaction (at 120 min). Samples were immediately quenched with excess Na2S2O3 (10 mM) to eliminate the residual oxidants before enumerating the viable bacteria (Jeong et al., 2009; Ashby et al., 2020).

The bacteria were then quantified on eosin-methylene blue (EMB) medium using the plate counting method (Jeong et al., 2006).

2.5. Inactivation by the electrolyzed water

To exclude the direct bacteria-killing effect of the electricity and evaluate the effect from the exfoliation of LIG, we further looked at the antibacterial effect of the electrolyzed water collected after the 120 min-electrolysis. In brief, the electrolyzed water samples from the undivided and divided systems were collected after 120 min of electrolysis and then incubated with E. coli. This differs from the experiments described above (section 2.4) because the bacteria were not in the containment units during the electrolysis process. After the electrolysis, a 1 mL sample of 3 V and 6 V treated 0.05 M NaCl was collected from the undivided system and each of the compartments of the divided system. Then, a stock concentration (10^6 CFU/mL) of E. coli was added to the electrolyzed water to reach the final E. coli concentration of ~5 × 10^3 CFU/mL. A 1 mL sample of the bacterial suspension in the electrolyzed water was withdrawn immediately (post-0 min) and after 120 min of incubation (post-120 min) at 37 °C. To eliminate the residual disinfectant effect before incubating E. coli, bacterial suspensions withdrawn were immediately combined with Na2S2O3 (10 mM) to neutralize the impact of any oxidants before bacteria were quantified on eosin-methylene blue (EMB) medium using the plate counting method (Jeong et al., 2009).

2.6. Oxidant measurements and water parameters

During the experiment, chlorine, ozone, and H2O2 were measured immediately when samples were withdrawn at each time point from the systems. Total residual chlorine was measured by Vacu-vials (K-7423, Oxidation technologies, USA) and chlorine test strips (HACH, USA), which both adopted the N, N-diethyl-p-phenylenediamine (DPD) colorimetric method (Kosaka et al., 1998; Norra et al., 2022). The production of H2O2 was assessed by Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, USA). Dissolved ozone was measured using a portable dissolved oxygen meter (DOZ-30, Dini Purification Equipment Co Ltd, China). The pH (ECO pH+, Trans Instruments (S) Pte Ltd, Japan) and ORP (YSI ro10, YSI Incorporated, USA) were measured at the beginning and end of the electrochemical disinfection procedure.

We also analyzed the concentration of chloride, chlorate, and perchlorate after 120 min of electrolysis in the undivided system when 3 V of electricity was applied to the LIG electrodes. Samples were diluted 100 times and quantified by ion chromatography (Dionex ICS-1100, Sunnyvale, CA) with AS18 Analytical Column, AG18 Guard Column, and Dionex ASRS300-4 mm Suppressor. Each sample was run twice for 60 min.

2.7. Statistical analysis

We compared the log-reduction of bacterial counts for different treatment groups at 120 min using ANOVA. All values were expressed as mean ± standard deviation (SD values) for the triplicate tanks in each treatment. Differences were considered significant if p < 0.05. Mean ± SD values of ORP between treatment groups were also analyzed similarly. All statistical analyses were performed in Stata software (Version 17, Stata Software, Texas, United States).
2.8. Visualization of LIG killing action using fluorescence microscopy

A separate trial was conducted to monitor the antibacterial activity of the LIG electrodes under a microscope. In brief, the LIG anode and LIG cathode sheets (1 cm × 1 cm each), separated by a 0.5 mm gap, were affixed on a microscope slide, and 100 μL of GFP-tagged E. coli suspension (~1 × 10^6 CFU/mL) in 0.05 M NaCl solution was placed on the electrodes, and these were covered with a glass coverslip. GFP-tagged E. coli was constructed according to Xu et al. (2021). The same setup was also done using 0.05 M Na_2SO_4 solution to compare the antibacterial efficacy of LIG electrodes in different solutions. LIG electrodes were activated by 3 V in this experiment. The bacterial cells were observed using an optical microscope (Nikon EclipseTi2) to capture the motion of GFP-E. Coli (2.5 fps and 2720 × 2720 pixels) during the 30 s treatment.

To confirm the viability of the bacteria after treatment, the surface of the LIG electrodes was stained using LIVE/DEAD BacLight Bacterial Viability and Counting Kit (Thermo Fisher, USA) containing SYTO 9 and propidium iodide (PI) dye, which reflects the permeability of the cell membrane and cell wall (Aronsson et al., 2005). The SYTO 9 dye can cross all bacterial cell membranes, while PI can only enter cells with disrupted membranes, allowing differentiation between live and dead cells based on their relative green fluorescence from the SYTO 9 staining. Dead cells displayed red fluorescence from PI staining (Robertson et al., 2019). Stained LIG electrode samples were stored in the dark for 15 min before observation with fluorescence microscopy. Live bacteria were observed using 494 nm as the excitation wavelength and 517 nm as the emission wavelength. Dead bacteria were assessed using 535 nm as the excitation wavelength and 617 nm as the emission wavelength.

3. Results

3.1. Characterisation and electrochemical measurements of LIG electrodes

The Raman spectra for the LIG surface had three peaks at ~1350, ~1580, and ~2700 cm⁻¹ (Fig. 1-A). Imaging the LIG surface using SEM revealed a highly porous foam-like structure (Fig. 1-B). Transmission electron microscopy (TEM) images of the LIG showed a rippled structure (Fig. 1-C).

The LSV analysis of the LIG electrodes showed that in the NaCl solution, the actual voltage on the LIG electrodes were 2.35 V and ~0.65 V when 3 V of electricity was used (Figs. S2-A). When 6 V of electricity was applied to the LIG electrode, the actual voltages on the LIG electrodes were 3.74 V and ~2.26 V (Figs. S2-A). Similarly, in the Na_2SO_4 solution, the actual voltages on the LIG were 2.29 V and ~0.71 V when 3 V of electricity was used (Figs. S2-B). When 6 V of electricity was applied, the actual voltages on the LIG electrodes were 3.65 V and ~2.35 V (Figs. S2-B).

The CV curves of LIG in NaCl and Na_2SO_4 solutions showed a tilted rectangular shape (Figs. S2-C). This was as expected based on the capacitive behaviour of LIG within the working potential of 3 V and 6 V (Peng et al., 2015). When 3 V of electricity was used on the LIG electrodes the CA curves suggested that the current in both NaCl and Na_2SO_4 solutions were relatively stable within a range of 1.5–2 mA during the entire 120 min of electrolysis (Figs. S2-D).

3.2. Antibacterial activity of LIG electrodes

Bacteria remained alive for undivided and divided systems when no electricity was applied to the LIG electrodes (control) (Fig. 2). After electricity was applied to LIG electrodes in our treatment groups, antibacterial effects were observed (Fig. 2); however, the antibacterial performance differed depending on the electrolyte solutions, voltages, and types of electrolysis systems.

In the undivided system with the NaCl solution, there were statistically significant reductions in E. coli (p < 0.05) (Fig. 2-A-C). In addition, the antibacterial effect in the NaCl solution was significantly enhanced (p < 0.05) when the voltage applied to the LIG was increased (Fig. 2-C). However, these reductions were not observed in the chloride-free electrolyte solutions (i.e., Na_2SO_4, phosphate buffer) in the undivided system or the recirculating system (Fig. S6 A and B), regardless of the voltages (3 V and 6 V) applied to the LIG electrodes (Fig. 2 A and B).

In the divided system, antibacterial performance in the anode compartment depended on the voltage applied and the electrolyte type (Fig. 2 D and E). No significant bacterial inactivation was in the anode compartment when 3 V was applied to the LIG electrode with NaCl as the electrolyte solution (Fig. 2-E). However, statistically significant reductions in E. coli (p < 0.05) were noticed in the anode compartment when 6 V was applied to the LIG electrodes (Fig. 2-D). In contrast, no significant bacterial reduction was noted in this compartment in the Na_2SO_4 solution, even when 6 V was applied to the LIG electrodes (Fig. 2-E).

Unlike the anode compartment of the divided cell, antibacterial performance in the cathode compartment was independent of the type of electrolyte solution and voltage used. There was complete E. coli inactivation (5–6 log-reduction) in both the NaCl and Na_2SO_4 solutions in the cathode compartment of the divided system (Fig. 2 D and E). In addition, a complete E. coli inactivation (5–6 log-reduction) was also observed in the cathode compartment when 3 V was applied to the LIG electrodes (Fig. 2-E).

3.3. Antibacterial effects of the electrolyzed water

In the undivided system, no bacterial inactivation was observed using the electrolyzed water from the 3 V-LIG treated NaCl solution (Fig. 3-A). However, the complete bacterial reduction occurred immediately when E. coli was exposed to the electrolyzed water collected from the 6 V-LIG treated NaCl solution (Fig. 3-A).

In the divided system, the complete bacterial reduction occurred when E. coli was exposed to the electrolyzed water collected from the 6

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Fig. 1. Characteristic of LIG. (A) Raman spectra; (B) SEM image; (C) TEM image.
V-LIG treated NaCl solution from the anode compartment (Fig. 4-A). However, electrolyzed water collected from the 6 V-LIG treated NaSO₄ solution in the anode compartment did not show significant bacterial reduction (Fig. 4-A). Unlike the antibacterial activity of electrolyzed water from the anode, where bacteria reduction was only observed when NaCl solution was used (Fig. 4-A), E. coli was entirely and immediately inactivated by electrolyzed water from the cathode compartment regardless of the type of electrolyte solution (Fig. 4-G).

3.4. Concentration of oxidants

3.4.1. Concentration of oxidants during the antibacterial assessment of LIG electrodes

The concentration of H₂O₂ was similar among the electrolyte solutions when the same solution was applied to the LIG electrodes in both the undivided and divided systems (Fig. S3 A, B, and E). The H₂O₂ concentration increased with the reaction time but did not appear to increase with voltage (i.e., 6V) (Fig. S3 C and D). In fact, it was lower in the 6 V-treated NaCl solution than in the 3 V–NaCl treated solution (Fig. S3 C and D). Despite the increase in H₂O₂ over time, the concentration was always below 1.12 mg/L (Fig. S3).

Unlike H₂O₂, ozone concentration increased with voltage applied to the LIG electrodes in NaCl solution in both the undivided and divided systems (Fig. S4 C and D). However, ozone concentration was highly variable, with only a few high readings observed during the electrolysis process (Fig. S4 C, D, and E). These readings were inconsistent across electrolyte solutions of replicate systems (Fig. S4). Total residual chlorine was measured when NaCl solution was used. In the undivided system, an average of 4.643 ± 0.451 mg/L chlorine was detected when 6 V was applied to the LIG electrodes (Fig. 5 B and C). This value was nearly 100 times greater than the chlorine concentrations when 3 V was used (0.047 ± 0.01 mg/L) (Fig. 5 A–C). The results of the ion chromatography showed no significant peaks for chloride and perchlorate, which were observed after 120 min of electrolysis in the divided system when 3 V was applied to the LIG electrodes. In the divided system, high chlorine levels (0.873 ± 0.956 mg/L) were detected when 6 V was applied to the anode compartment (Fig. 5 D and E). The chlorine concentration in the anode compartment of the divided system was approximately 2 times lower than in the undivided system with the same voltage (Fig. 5 B–E). There was no detectable chlorine in the anode of the divided system when 3 V was used (Fig. 5-D).

3.4.2. Concentration of oxidants in the electrolyzed water (section 2.5)

In the undivided system, there was no ozone and negligible chlorine concentration in the electrolyzed water when 3 V was used (Fig. 3 B and C). However, a high chlorine concentration was detected on the surface of the LIG anode by HACH strips at this voltage (Fig. 5-F). Increased concentrations of ozone and chlorine in the solution were noticed when 6 V was applied to the LIG electrodes (Fig. 3 B and C). A higher chlorine concentration was also detected on the anode surface when 6 V was used (Fig. 3-F).

In the divided system, there was a high ozone concentration in the anode compartment in both the electrolyzed NaCl and NaSO₄ solutions when 6 V treatment was used (Fig. 4-B). Chlorine was detected at an average of 0.91 mg/L in the NaCl solution (Fig. 4-C) when LIG electrodes were applied with 6 V; however, it was much lower than the chlorine concentration in the undivided systems (an average of 4.5 mg/L) when the same voltage was applied (Fig. 3-C and Fig. 4-C). The HACH strips also showed no colour change in the 6 V-treated electrolyzed NaCl solution collected from the anode compartment (Fig. 4-F). The divided system showed a lighter colour change on the anode surface than the colour changes on the anode in the undivided system when 6 V of electricity was used (Fig. 3-F and Fig. 4-F).

3.5. Water parameters

During the disinfection process by LIG electrodes (section 2.4), the ORP increased in NaCl and NaSO₄ solutions in the undivided system after 120 min of electrolysis by LIG electrodes (Fig. S5 A-C). Significantly higher ORP was observed in NaCl solution when 6 V was applied
to the LIG electrodes than when 3 V was used ($p < 0.05$) (Figs. S5–C).

Similarly, in the antibacterial study by the electrolyzed water (section 2.5), ORP increased with voltages in the electrolyzed NaCl solution (Fig. 3–D).

In the divided system, ORP in the anode compartment showed a similar trend as in the undivided system during the disinfection process by LIG electrodes (section 2.4) (Fig. S5 D and F). In the cathode of the divided system, although a significant increase of ORP ($p < 0.05$) was also noticed in the NaCl solution (Fig. S5 E), it was only a slight increase compared with the increase in the anode compartment (Fig. S5 D-G). ORP in the electrolyzed water experiment (section 2.5) also significantly increased ($p < 0.05$) in both the NaCl and Na$_2$SO$_4$ solutions after electrolysis in both the anode and cathode compartments (Fig. 4 D and H). This increase was also minor compared to the increase of ORP in the anode (Fig. 4 D and H).

In terms of the pH, in the undivided system, there was no noticeable variation in pH ($\sim 6.5 \pm 0.29$) in different electrolyte solutions after 120 min of electrolysis with low voltage (3 V) (Fig. 6 A and C). When 6 V was used, there was an increase in the pH when NaCl solution was used, turning it alkaline with an average pH of 9.3 ± 1.16 at 120 min (Fig. 6 B and C).

In the divided system, pH increased dramatically (10.8 ± 0.44 at 120 min) in the cathode compartment, regardless of the voltage or type of electrolyte solution used (Fig. 6 E and G). In contrast, the pH was low in the anode compartment, and this acidic effect was correlated with the increased electricity used on the electrodes (pH $\sim 3.63 \pm 0.46$) after 120 min (Fig. 6 D and F). A similar pH change was observed in the experiment that used electrolyzed water to inactivate bacteria (Fig. 3–E).

### 3.6. Visualization of LIG killing action using fluorescence microscopy

Our microscopy results were similar for both the NaCl and Na$_2$SO$_4$ solutions. We noticed the movement of GFP-E. coli toward the LIG electrodes and the subsequent disappearance of the bacteria (Supplementary video) under the fluorescence microscope. The formation of gas bubbles was also observed at the anode surface (Supplementary video). In the live dead staining assay, we observed more dead cells after the 30 s LIG treatment on the surfaces of the anode and cathode (Fig. 7 C, D, G, and Fig. S7) compared to the controls with no electricity (Fig. 7 A, B, E and F and Fig. S7).

### 4. Discussion

Our study revealed that LIG electrodes can disinfect water through multiple mechanisms, including the generation of oxidants, changes in pH—specifically high alkalinity associated with the cathode, and electro-adsorption on the electrodes themselves. All three mechanisms likely occurred to some extent as we observed bacterial inactivation in both the chloride-free electrolyte (i.e., Na$_2$SO$_4$) and chloride-containing electrolyte (i.e., NaCl) solutions during our fluorescent microscopy study; however, when we had experimental units with larger volumes of solution, the generation of oxidants, in particular, reactive chlorine species (RCS) may have played a more significant role in the bacterial reduction. We hypothesized that this inactivation mechanism was important as we only observed a significant bacterial reduction in our larger systems when there was a source of chloride ions in the aqueous solution. Several other studies have also suggested that oxidants generated during electrochemical reactions are important in bacterial
inactivation (Sopaj et al., 2015; Coria et al., 2016; Ghasemian et al., 2017).

In our systems that used more than 100 mL of NaCl solution, we detected RCS regardless of the voltage used. The quantity of RCS produced appeared to be voltage-dependent. Other researchers have also eluded to the idea that the creation of chlorine is voltage-dependent and requires the use of at least 1.4 V of electricity applied to LIG electrodes (Manderfeld et al., 2021). In our system, where 6 V of electricity was applied to the electrodes, we were able to measure RCS in solution at a concentration over 3 mg/L (Fig. 5B and C), which is well above the reported 1 mg/L disinfection level for chlorine (Galal-Gorchev, 1996; Furukawa et al., 2017). However, when we used 3 V of electricity, we only detected chlorine close to the anode (Figs. 3F, and Fig. 5A and C). It is possible that the concentration of chlorine generated by this level of electricity was too low to be detected in the solution. The highly porous foam-like structure of LIG may have trapped RCS close to the anode. Others have reported this with three-dimensional porous carbon nanotubes (Wang et al., 2020).

Reactive chlorine species are powerful oxidants which can damage bacterial cells through several mechanisms (Gray et al., 2013). Our study observed an RCS dose-dependent antibacterial effect (Fig. 2C, Fig. 3A and C, and Fig. 5C). This was observed in both the undivided and divided systems, as well as in the post-electrolyzed water. In the undivided systems with NaCl, the 6 V units had higher chlorine concentrations and higher inactivation of bacteria compared to the 3 V units. Further, solutions without the chloride ions (i.e., Na₂SO₄), which had no chlorine, had minimal evidence of disinfection (Fig. 2B) even when we increased the voltage to 6 V. The antibacterial effects from the electrolyzed water from the undivided systems further indicated the dose-response effect. For example, the RCS were not detectable in the 3 V-treated NaCl solution collected after the electrolysis process, and it showed no bacterial inactivation (Fig. 3A, C, and F). In contrast, the 6 V-treated NaCl solution had high levels of chlorine (Fig. 3C and F), and this solution immediately inactivated bacteria even after the electrolysis process (Fig. 3A).
In the divided system, chlorine was also detected in the anode compartment of the 6 V experiment (Fig. 5 D and E), and we observed significant bacterial inactivation (Fig. 2 D and E). Interestingly, chlorine was not detectable in the anode compartment of the 3 V experiment (Fig. 5 D), and we did not observe a decline in the bacterial concentration (Fig. 2 E). It is possible that RCS in the 3 V anode compartment of the divided system were not generated at the same concentration as in the undivided system due to the increased distance between the anode and cathode, which may have affected the strength of the current (Anglada et al., 2011; Fernandes et al., 2016). Comparing the chlorine levels in the 6 V undivided and divided systems (Fig. 3 F, Fig. 4 F, and Fig. 5 B, C, D and E) provided evidence for this hypothesis as there were higher chlorine levels in the undivided system.

The high pH found after 120 min of running the LIG electrodes with 6 V of electricity in the undivided system may also have contributed to bacterial inactivation. Although E. coli can survive at very low pH (i.e., pH = 3) (Geveke and Kozempel, 2003; Bruguera-Casamada et al., 2016), strong alkaline solutions such as what was measured in our study (Fig. 6 E and G) are detrimental to these bacteria (Mendonca et al., 1994; Guo et al., 2016). This increase in pH may limit the application of LIG; however, our study suggests that applying a lower voltage to the LIG electrodes in solutions with chloride ions can inactivate bacteria without changing the pH. This was observed in both the undivided (Figs. 2 A and Fig. 6 A) and the recirculating systems (Fig. S6 A, B, E, and F).

The other oxidants detected in this study were H₂O₂ and ozone. Both of these oxidants are relatively unstable and difficult to measure, which could account for the wide range of values within the same experimental groups in this study (Wu et al., 2007; Prabha et al., 2015). Regardless, even the highest levels of H₂O₂ and ozone detected in this study did not meet the reported disinfection threshold for the products (Loeb et al.,...
0.05 M NaCl without electricity, (B) 0.05 M Na
movement of GFP-tagged E. coli at 3 V, respectively; (F) 0.05 M NaCl with no electricity, (G) 0.05 M NaCl at 3 V, (H) 0.05 M NaSO₄ at 3 V, respectively. In each image of (I–P), the green dots represented live bacteria stained with SYTO9, and the red dots represented damaged cells stained with PI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 7. The viability of GFP-tagged E. coli after 30 s treatment of LIG (A–H). (A–D) SYTO9 and PI stain on GFP-tagged E. coli after 30 s treatment of LIG anode in (A) 0.05 M NaCl without electricity, (B) 0.05 M NaSO₄ without electricity, (C) 0.05 M NaCl at 3 V, (D) 0.05 M NaSO₄ at 3 V, respectively; (E–H) SYTO9 and PI stain (merged) on GFP-tagged E. coli after 30 s treatment of LIG cathode in (E) 0.05 M NaCl without electricity, (F) 0.05 M NaSO₄ without electricity, (G) 0.05 M NaCl at 3 V, (H) 0.05 M NaSO₄ at 3 V, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2012; Ding et al., 2019), no matter what voltages and what types of electrolyte solutions were used (Fig. S3 and Fig. S4). Other researchers have also reported low levels of H₂O₂ in their LIG experiments (Singh et al., 2017). Similarly, the concentration of H₂O₂ in our study did not appear to be sufficiently high to inactivate bacteria in the solution. This was evident in our experiments, which compared chloride-free electrolytes (i.e., Na₂SO₄, phosphate buffer) and the chloride-containing electrolyte (i.e., NaCl) (Fig. 2 A and B, and Fig. S6 A and B). In the systems with no chloride source, there was no bacterial inactivation. Notably, we found a reduction in bacteria in the cathode compartment of our divided systems which also had a low concentration of H₂O₂ (Fig. S3 D and E). This observation occurred in both Na₂SO₄ and NaCl solutions (Fig. 2 D and E). However, the antibacterial effects of H₂O₂ in the cathode compartment may have been confounded by a very high pH (Fig. 6 E and G). This change in pH would explain the high reduction in bacteria better than the low levels of H₂O₂ (Mendonca et al., 1994; Guo et al., 2016). Separating the effect of pH and H₂O₂ close to the cathode requires further study.

The last mechanism we observed for bacterial inactivation in this study was electro-adsorption. Using fluorescence microscopy, we witnessed the direct inactivation of bacteria on the surface of the LIG electrodes. This bacterial inactivation process appeared to be independent of RCS generation, as it was noted with both NaCl and Na₂SO₄ (Fig. 7). Others have also reported reduced bacterial viability on the surface of LIG electrodes in both the chloride-containing and chloride-free solutions (Manderfeld et al., 2021). The antibacterial mechanism of the LIG surface may be attributed to electrical, physical, and or chemical damage (Guyot et al., 2007; Huo et al., 2017; Singh et al., 2017; Manderfeld et al., 2021). First, the electrical charge on the electrodes may attract bacteria. This was evident in our study by the movement of GFP-E. coli towards the electrodes (Supplementary video). Close contact with the nanosized sharp edges of LIG could physically damage cell structures (Akhavan and Ghaderi, 2010; Hu et al., 2010; Tu et al., 2013; Perreault et al., 2015). Additionally, bacteria attached to the LIG surface may be oxidized directly due to the loss of electrons to the anode, resulting in irreversible membrane damage and death (Van Loey et al., 2001; Long et al., 2015; Pillet et al., 2016). The results of the LIVE/DEAD® staining of E. coli in our study suggested there were more bacteria with damaged cell membranes on the electrode surface after electrolysis than before the treatment (Fig. 7). There is also a possibility that higher levels of oxidants and more extreme pH are present close to the electrodes, which can further cause bacterial inactivation. Researchers have reported that anodes made of porous materials can trap hydrogen ions, resulting in localized low pH (Scaldone et al., 2009; Ghernaout et al., 2011), which can further favour chlorine evolution from the anode surface (Martínez-Huitle et al., 2015). Singh et al. (2017) hypothesized that there was also a higher concentration of H₂O₂ on the surface of LIG, resulting in an enhanced antibacterial effect. Furthermore, we observed the generation of bubbles during the electrolysis process at the anode surface (Supplemental video), which may promote electrochemical reactions (Vogel et al., 2020) and enhance disinfection; however, we were not able to confirm this in our study. Despite these limitations, it would appear that electrochemical disinfection using LIG can reduce E. coli significantly, and the process may be multifactorial.

Our study suggested that disinfection using LIG technology may be an environmentally-friendly way to treat water, especially when a low electrical voltage is applied to the system, as it does not generate high chlorine levels, toxic chlorine by-products (i.e., negative ion chromatography results), or high pH. However, although we explored the antibacterial mechanisms of LIG electrodes using several types of experiments, our study was limited to lab-based investigations with relatively clean water, and therefore, there remains gaps in our understanding of the antibacterial mechanism underpinning this process as well as its potential toxicity. For instance, water that has higher organic matter may have higher levels of toxic by-products after treatment with high voltage. We also could not rule out whether the
exfoliation of LIG was involved in the antibacterial effects when 6 V of electricity was used. However, we are confident that this did not happen in the experiments with 3 V of electricity because we did not observe a bacterial reduction in the treated aqueous solution from these studies. Additionally, the considerable variability in the disinfection efficacy across and within experiments (Fig. 2 A and C), and the slight decrease in currents after 120 min of electrolysis, as evident in our CA curves (Figs. S2–D) could be problematic if this technology is to be scaled up for use in a commercial setting because these results may indicate instability of LIG electrodes. Defining the duration of efficacy of this new material and finding ways to improve the longevity of the product will be the key to its commercialization. Future research on this topic is necessary before this technology can be considered for commercial use.

5. Conclusion

The antibacterial mechanism of electrochemical disinfection using LIG electrodes was investigated in this study. It was found that bacterial inactivation occurred through several mechanisms working synergistically. These included the generation of oxidants, electro-adsorption on the electrodes, and changes in pH. All these mechanisms may contribute to the disinfection process when bacteria are close to the surface of the electrodes; however, in larger systems (i.e., with >100 mL), the predominant cause of bacterial death appears to be from the generation of RCS, which was voltage dependent. When high voltages were used (i.e., 6 V), the RCS concentration and the pH changes in the solution may be problematic to aquatic animals. It may be possible to address some of these limitations using new resin technologies, which have been shown to reduce halogenated disinfection by-products (Yin et al., 2020a). However, when lower voltages were used to activate the LIG electrodes, many of these issues seem to be circumvented. A recent study demonstrated that rapid disinfection of saltwater could be achieved using LIG electrodes without negative health impacts on fish (Zhang et al., 2023).

Types of contribution

Ju Zhang: conducted most of the studies and wrote the manuscript, Le Cheng: Helped with the analysis of electrochemical characteristics of LIG electrodes, Pok Him Ng: Assisted with experimental set-up and materials preparation, Qianjun Huang: Assisted with data acquisition and manuscript revising, Liqing Huang: Assisted with experimental set-up and materials preparation, Libei Huang: Assisted with data acquisition and manuscript revising, Brett MacKinnon: Assisted with data acquisition and manuscript revising, Yefeng Yang: Assisted with statistical analysis, Ana Rita Marques: Assisted with statistical analysis, Ruquan Ye: Assisted with the conception of the study, Sophie St-Hilaire: Assisted with the conception of the study and manuscript revisions.

Author contributions

Ju Zhang, Ruquan Ye, and Sophie St-Hilaire contributed to the idea of the study. Ju Zhang conducted most of the experiment. Le Cheng helped with the analysis of the electrochemical characteristics of LIG electrodes; Liqing Huang and Ng Pok Him assisted with experimental set-up and materials preparation. Qianjun Huang, Brett MacKinnon, and Libei Huang were involved in data acquisition and manuscript revising. Yefeng Yang and Ana Rita Marques contributed to the statistical analysis.

Submission declaration and verification

The work described has not been published previously and it is not under consideration for publication elsewhere. This publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or any other language, including electronically without the written consent of the copyright-holder.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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