(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 February 2016 (18.02.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/025934 A1

(51) International Patent Classification:
A61L 2/20 (2006.01)

(21) International Application Number:
PCT/US2015/045434

(22) International Filing Date:
15 August 2015 (15.08.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/038,171 15 August 2014 (15.08.2014) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) **Title**: SELF-CONTAINED PORTABLE OZONE STERILIZER FOR SMALL MEDICAL EQUIPMENT

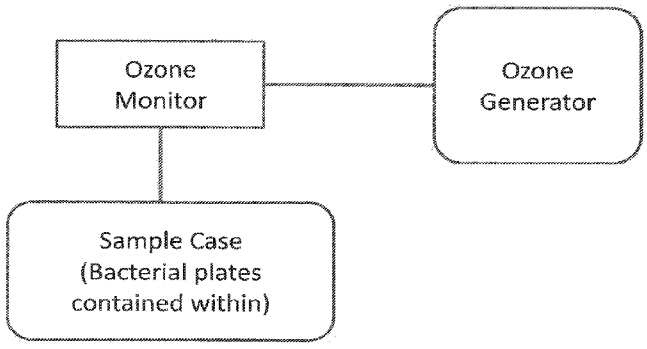

FIG 1

(57) **Abstract**: This invention is directed to a portable lightweight, easy-carry, reusable, and self-contained assembly for sterilizing contaminated equipment using ozone and humidity generated in situ without requiring external electricity, fuels or other exogenous energy sources for operation and a method of use thereof.

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SELF-CONTAINED PORTABLE OZONE STERILIZER FOR SMALL MEDICAL EQUIPMENT

CROSS-REFERENCE OF RELATED APPLICATION

[001] This application claims priority to U.S. Provisional Application No. 62/038,171 filed 15 August 2014.

BACKGROUND OF THE INVENTION

Field of the Invention

[002] The present invention relates to a lightweight, portable, self-contained sterilization apparatus whose operation does not require external power sources. The invention, more specifically, relates to an apparatus that uses self-generated ozone for sterilization and disinfection of small medical, dental and surgical instruments. The apparatus is designed to function in field conditions where water, electrical power, and/or fuel sources may be absent, unavailable, or of limited availability.

Description of the Prior Art

[003] Military clinicians and dentists often face austere environments in both combat zones and during humanitarian missions. In these remote locations, instrument sterilization techniques typically employed by medical treatment facilities are often unavailable or unfeasible due to limited resources in the field. Currently, contaminated medical instruments are often transported with patients via aircraft to distant hospitals for sterilization in large steam autoclaves, and then returned enclosed in sterile packaging to the surgical teams via aircraft for re-use. Hospital steam autoclaves typically have 18-20 cubic foot chambers, weigh several hundred pounds, and require at a minimum

permanent steam lines, water lines, and 110V electricity for operation.

[004] Field medical facilities sometimes also are equipped with electric autoclaves onsite. Although, these electric autoclaves are smaller than hospital steam autoclaves, they still require special installation (such as 120 and 208-240 volt electrical lines), and consume significant amounts of water and power per sterilization cycle. For example, a typical sterilization cycle in an electric autoclave runs for 50-60 minutes and consumes about 1.5 gallons of potable water and approximately 12 kilowatts of power (at 236 volts). They are also difficult to transport in vehicles due to their weight (>100 lbs.), bulky dimensions, and reliance on generators or other power sources for operation. Examples of current ruggedized sterilization system developed for use outside of traditional medical treatment facilities, such as M-138 portable steam sterilizer (a.k.a. Big Bertha), which weights 312 lbs. (141.52 kg) and require water and a large external power source. The newer systems, such as STERILUCENT™ hydrogen peroxide sterilizer system, which weights roughly 440 lbs (200Kg) and requires external power and hydrogen peroxide. Though effective at sterilization, these systems are cumbersome, require additional consumable resources, which present logistical issues in a primitive environment. The development of a true portable, self-contained sterilizer system remains a critical need for forward-deployed care providers.

[005] Currently, three common methods were used for sterilization medical/surgical/dental instruments: conventional moist heat method, radiation method, or chemical sterilization method. Conventional moist heat sterilization requires bulky autoclaves and significant amounts of power to generate steam. The high temperature used in some of these systems are often damaging to specialized components of nearby heat-sensitive tools and equipment. Radiation sterilization also requires special power supply, and often needs to be housed in shielded enclosures. Chemical sterilization has the potential to be conveniently portable, which has led to the claims of portable

disinfection, but not complete sterilization. The chemical methods often cannot achieve sterilization/disinfection in convenient times.

[006] Furthermore, most chemical method achieves sterilization via controlled chemical processes killing microbial contaminants. This requires either i) direct transportation of biocidal chemical agents to location in safety carriers, or ii) transportation of the appropriate chemical precursors and heavy-duty chemical equipment to location, and chemically generating biocidal agents or sterilization conditions on-site. In both cases, the chemical sterilization system is not truly self-contained as its operation still requires transportation of biocidal chemicals or heavy-duty equipment, which often creates logistical challenges for austere environments or "in theatre" use. For example, the production of chloride dioxide gas requires sodium hypochlorite and chlorine gas, if using the dry process; and sodium hypochlorite and hydrochloric acid, if using the wet process (Lowe, Gibbs, Iwen, Smith, & Hewlett, 2013; Rastogi et al., 2010). Aerosolized hydrogen peroxide relies on a source of 5% hydrogen peroxide to generate the aerosol (Andersen et al., 2006), while carbon dioxide gas requires a tank of carbon dioxide (Zhang et al., 2006).

[007] Attempt has been made to design a portable chemical sterilizer that uses only a small amount (approximately 0.25 lb.) of safe, dry, chemical reagents. Patent No. 8,133,450 to Doona et al. describes a portable chemical sterilizer adapted to controllably generate sterilization conditions inside the apparatus using a chemical combination. Mixtures of specially formulated iron-activated magnesium Mg (Fe) containing salt, sodium chlorite (NaClO2), sodium sulfite (Na2SO3), and sodium hydrogen ascorbate (C6H7O6Na), which are packed in the sterilizer, react in water and produce of biocidal chlorine dioxide, heat, and relative humidity required for the sterilization. Although, this chemical based sterilizer is light-weight and self-contained, the chemical combination used for sterilization needs to be frequently replaced, which still pose logistic problems relating to the

transportation and storage of these chemicals. Furthermore, after sterilization, gaseous chemical reaction products or excessive heat is released directly into the environment housing the sterilizer, which renders this sterilizer unsuitable for use in a small enclosed area.

[008] Recently, non-thermal plasmas have been shown to be efficient at killing microorganisms on heat sensitive materials, but the supply need of reagent gas continues to create logistical challenges for a field deployable chamber system (Venezia, Orrico, Houston, Yin, & Naumova, 2008; von Keudell et al., 2010).

[009] The utilization of ozone technology for sterilization/disinfection is the focuses of other studies. Some examples of medical equipment sterilization systems that utilize humidified ozone as the sterilization agent are described in patents to Masuda, U.S. Pat. No. 5,120,512 and to Karlson, U.S. Pat. No. 5,069,880; and a plurality of containers and chamber arrangements for use in sterilization processes utilizing ozone as the effluent are shown in patents to Anderson, et al, U.S. Pat. No. 5,118,471; and to Lutz, U.S. Pat. No. 5,087,419. The ozone technology has many advantages over other sterilization methods. It eliminates pre-conditioning, does not require post sterilization dwelling, and allows heat-sensitive devices to be sterilized without compromising materials compatibility, product quality or integrity, while allowing for a rapid turnaround room-temperature sterilization method for medical and dental devices. However, these systems are often bulky and non-portable requiring external ozone or electrical sources. Nor have such earlier systems monitored conditions in the sterilization chamber or deal with the detection, ventilation, destruction and overall management of transient sterilizing agents that may have inadvertently escaped into the environment during sterilization operation.

[0010] Handheld ozone sterilizer has been developed, but mostly for sterilization of food and articles of daily use, such as those described in Chinese Patent No. CN 2142020 and Chinese

Patent No. CN 202945057. However, these devices are not powerful enough to provide the sterilization required for medical setting.

DESCRIPTION OF THE FIGURES

[001] FIG 1. Schematic of the ozone sterilization Chamber System.

[002] FIG 2. Normalized turbidity at O.D. 600nm of *Acinetobacter baumannii* strain BAA 1605. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[003] FIG 3. Normalized turbidity at O.D. 600nm of *Acinetobacter baumannii* strain ATCC 17961. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[004] FIG 4. Normalized turbidity at O.D. 600nm of *Acinetobacter baumannii* strain ATCC 19606. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[005] FIG 5. Normalized turbidity at O.D. 600nm of *Acinetobacter baumannii* strain ATCC 19003. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[006] FIG 6. Normalized turbidity at O.D. 600nm of *Acinetobacter baumannii* strain WRAMC #13. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[007] FIG 7. Normalized turbidity at O.D. 600nm of *Bacillus subdlis* strain JH 642. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[008] FIG 8. Normalized turbidity at O.D. 600nm of *Escherichia coli* strain JM 109. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of

25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[009] FIG 9. Normalized turbidity at O.D. 600nm *of Klebsiella pneumonia* strain BAMC 07-18. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0010] FIG 10. Normalized turbidity at O.D. 600nm of *Klebsiella pneumonia* strain xen-39. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0011] FIG 11. Normalized turbidity at O.D. 600nm of *Klebsiella pneumonia* strain IA-525. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by

absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0012] FIG 12. Normalized turbidity at O.D. 600nm of *Pseudomonas aeruginosa* strain Xcn-41. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent limes. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0013] FIG 13. Normalized turbidity at O.D. 600nm of Pseudomonas aeruginosa strain BAMC 07-4 The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0014] FIG 14. Normalized turbidity at O.D. 600nm of *Pseudomonas aeruginosa* strain PA01. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate

standard deviation of three technical replicates.

[0015] FIG 15. Normalized turbidity at O.D. 600nm *of Staphylococcus aureus* strain IQ0070. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0016] FIG 16. Normalized turbidity at O.D. 600nm *of Staphylococcus aureus* strain Xen-40. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0017] FIG 17. Normalized turbidity at O.D. 600nm of *Staphylococcus aureus* strain ATCC 33591. The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0018] FIG 18. Normalized turbidity at O.D. 600nm of *Staphylococcus aureus* strain TCH- 1516.

The ozone treated plates of bacteria were exposed to ozone for 5, 15, 30, or 60 minutes at an ozone output of 25%, 50%, 75%, or 100%. Control plates of bacteria were left untreated for equivalent times. Post-treatment, culture media was added and incubated overnight at 37°C. Turbidity was assayed by absorbance at 600nm for both controls and ozone treated cultures. Ozone treated samples were normalized versus control samples, and averages plotted. Error bars indicate standard deviation of three technical replicates.

[0019] FIG 19. Ozone Sterilization Phase. The diagrams show the primary components of the ozone sterilizer system during Sterilization. Portions shown in grey are active or open, while portions shown in black are inactive or closed. Grey and black dashes indicated portions that are cycled on and off.

[0020] FIG 20. Ozone Destruction Phase. The diagrams show the primary components of the ozone sterilizer system during destruction. Portions shown in grey are active or open, while portions shown in black are inactive or closed.

[0021] FIG 21. User interface during primary state of operation.

[0022] FIG 22. Sterilizer wiring diagram. A simplified wiring diagram shows power distribution to sterilization system components. A rectifier converts 120 VAC into 12VDC when the system is powered externally, and a 12 VDC battery is used when the system is powered internally. A DC-DC converter provides the voltage required by the ozone sensor, and an inverter provides a high voltage AC signal to the ozone generator **1**. Microprocessor-controlled relays energize the ozone generator **1**, pump **6**, and solenoid valves **8**.

[0023] FIG 23. Ozone Sterilizer Configurations. The diagrams show the primary components of the ozone sterilizer in the two configurations that were tested. In the open-loop configuration, air enters the system through the air dryer **5** and exists through the sterilization compartment **4**. In the

closed-loop configuration, air is recirculated through the system, flowing through the active ozone generator 1 during sterilization. During the destruction phase at the end of sterilization, air is redirected through the ozone destruction unit 7 (dashed lines).

[0024] FIG 24. Normalized turbidity of ozone treated bacteria in an open-loop configuration. Bacteria were treated during a 60-minute sterilization cycle. Control plates of bacteria were left untreated for the equivalent time. Post-treatment, sterile media was added and incubated overnight at 37 °C. Turbidity was measured using a spectrometer for both control and treated cultures. Treated samples were normalized to the control samples, and averages plotted. Error bars indicate the standard deviation of ten technical replicates.

[0025] FIG 25. Normalized turbidity of ozone treated bacteria in a closed-loop configuration. Bacteria were treated during a 60-minute sterilization cycle. A) *E. coli*, B) *A. baumannii*, C) *B. subtilis*, D), *K. pneumoniae*, E) *P. aeruginosa*, F) *S. aureus*. Control plates of bacteria were left untreated for equivalent times. Post treatment, sterile media was added and incubated overnight at 37 °C. Turbidity was measured using a spectrometer for both control and treated cultures. Ozone treated samples were normalized to the control samples, and averages plotted. Error bars indicate the standard deviation of three experimental and ten technical replicates.

[0026] FIG 26. Normalized turbidity of ozone treated dental instruments in a closed-loop configuration. Bacteria were treated during a 60-minute sterilization cycle. Control dental instruments were left untreated for equivalent times. Post treatment, dental instruments were placed in sterile media and incubated overnight at 37°C. Turbidity was measured using a spectrometer for both control and treated cultures. Ozone treated samples were normalized to the control samples, and averages plotted. *Note: Dental mirrors results not shown. Error bars indicate the standard deviation of four technical replicates

DETAILED DESCRIPTION OF THE INVENTION

[0027] This application describes the utilization of a portable medical sterilization system employing ozone technology. The technology eliminates pre-conditioning, does not require post sterilization dwelling, and allows heat-sensitive devices to be sterilized without compromising materials compatibility, product quality or integrity. This technology allows for a rapid turnaround room-tempeFIGrature sterilization method for medical and dental devices. The inventive apparatus is a field deployable sterilization chamber system being developed for utilization at the "point-of-care" by front line medics, physicians, and dentists in austere environment.

[0028] This invention allows ozone generation by a battery-powered sterilization chamber system solely reliant on ambient air, thus removing the current needs for external electrical sources, or additional cargo required as in gaseous or liquid sterilizers.

[0029] An embodiment of the inventive ozone sterilizer comprising ozone sterilizer system contained in a ruggedized and portable case with seven primary mechanical components that are monitored and controlled by a microcontroller. The ozone sterilizer assembly comprising: 1) an ozone generator **1**, 2) an ozone sensor **2**, 3) a humidifier **3** 4) a sterilization compartment **4**, 5) an air dryer **5**, 6) a pneumatic pump **6**, 7) an ozone destruction unit **7** (FIGs 19 and 20). The pump **6** circulates air through the system, and solenoid valves **8** direct air flow to different portions of the system depending on the status of the sterilization cycle. In the Sterilization Phase, the ozone generator **1** is active, and the pump **6** circulates ozonated air into the sterilization compartment **4**, while recycling air from the sterilization compartment **4** back to the generator **1**. An ozone sensor **2** monitors the generator output, and when the ozone concentration reaches a target level, the sterilization timer begins. The humidifier **3** switches on and off to add moisture to the sterilization compartment **4**. After the sterilization cycle is complete, the ozone generator **1** turns off, and

solenoid valves 8 redirect air to the ozone destruction unit 7 to convert any remaining ozone back into oxygen. The air dryer 5 is used to remove moisture from incoming air entering the system to ensure proper functions and extend life of the pump 6, ozone generator 1 , and ozone sensor 2.

System Components

[0030] Ozone Generator 1 - Ozone is powerful oxidizing agent that is unstable at high concentrations, which quickly decaying into oxygen. For a sterilizer to be practical for field-use, ozone must be generated by the system (Guzel-Seydim, 2003). Ozone can be produced from either pure oxygen, or ambient air, via either UV light or a corona discharge, and has been shown to be effective at killing bacteria, including spores (Kowalski, Bahnfleth, & Whittam, 1998; Nagayoshi et al., 2004; Young & Setlow, 2004). In an embodiment of this inventive system, the corona discharge method is employed to generate ozone. Corona discharge is a cost-effective, small form-factor method for producing ozone from ambient air, which can result in high ozone output with relatively low energy costs compared to other ozone-generating techniques (Summerfelt, 1997). The ozone generator 1 produces heat as a byproduct, which reduces the efficiency of the generator 1 , so heat removal was an important design consideration for the system (Carlins, 1982). The corona discharge generator selected for this invention features a tube design with heat sinks running the full length of the tube. Cooling fans may also be incorporated into the system to remove excess heat and maximize generator efficiency.

[0031] Ozone Sensor 2- An ultraviolet (UV) system ozone sensor 2 may be used to measure ozone concentration entering the sterilization compartment 4 by weight. The sensor 2 compensates for temperature and pressure fluctuation, and uses two detectors, one to measure ozone concentration, and the other to adjust for changes in the UV light intensity, allowing the sensor 2 to operate for extended periods without calibration or maintenance. The ozone sensor 2 provides feedback to

the system's microcontroller or processor to ensure the sterilization compartment 4 receives the target ozone concentration during the sterilization cycle and to verify that all ozone is removed during the destruction phase. The sensor 2 selected for the prototype system contains a microprocessor and can communicate with other devices using the RS232 serial communication protocol.

[0032] Humidifier 3- Previous tests have shown that gaseous ozone in the presence of moisture is more effective at killing bacteria than dry ozonated air. Therefore, a bubble humidifier 3 was added at the inlet of the sterilization compartment 4 to add moisture to the air entering the chamber (Sharma, 2008). A second input to the sterilization compartment 4 bypasses the humidifier 3, allowing humidity to be controlled by switching the humidifier 3 branch on and off. In an embodiment, the humidifier 3 compartment is made of polycarbonate and filled with water. A crushed glass stem circulates ozonated air into the water, producing small bubbles, allowing moisture to be picked up by the dry, ozonated air.

[0033] Sterilization Compartment 4 - A sterilization compartment 4 was designed to hold small medical instruments. The prototype sterilization compartment 4 measures 15" x 6" x 6". The compartment 4 has a hinged top with an airtight seal to prevent ozone from escaping during the sterilization cycle. The compartment 4 is constructed of ozone-compatible polycarbonate siding with a silicone seal. Medical instruments are placed on a stainless steel mesh, resting approximately 1 inch above the base of the compartment, to maximize surface contact between instruments and the ozonated air.

[0034] Air Dryer 5- Several system components, including the pump 6, ozone generator 1, and ozone sensor 2, require dry air to operate efficiently. Therefore, an air dryer 5 is placed at the input of the sterilization system to remove any moisture added by the humidifier 3 (Carlins, 1982).

In one embodiment, the air dryer **5** is made of stainless steel, and uses a rechargeable silica desiccant. A moisture indicator placed downstream of the air dryer **5** indicates when the desiccant is saturated.

[0035] Pump **6** – A diaphragm pump is selected that features all ozone-compatible wetted materials, including a Teflon-coated aluminum head, and a Teflon diaphragm. The pump **6** is driven by a brushless DC motor, which pushes air through the system at a rate of 5 liters per minute, resulting in a full air exchange in the sterilization compartment **4** approximately once every minute.

[0036] Ozone Destruction unit **7**- A high flow, high concentration ozone destruction unit **7** destroys the ozone at the end of the sterilization cycle is also included in the inventive system to ensure the device operator in enclosed environment is not exposed to ozone when the sterilization compartment **4** is opened. During sterilization phase, the destruction unit **7** is bypassed, but once the cycle is complete, all air in the sterilization system is directed to the ozone destruction unit **7**. The ozone destruction unit **7** may be made of stainless steel and uses a low-temperature oxidation catalyst to eliminate remaining ozone by converting it into oxygen.

[0037] The sterilization system is controlled by a microprocessor or microcontroller, such as Arduino Mega microprocessor. The microprocessor receives user input, provides status information to the operator, communicates with the ozone sensor **2**, controls the ozone generator **1** and pump **6**, and actuates solenoid valves **8** that distribute air flow to different portions of the system. Any common interfaces for the microprocessor/microcontroller may be used to display information during operation and accepts inputs. The interface used for the prototype is a 3.2" LCD touchscreen panel that allows user input via virtual buttons.

[0038] The portable medical sterilization system this invention may operate in either open-loop

configuration or closed-loop configuration (FIG 23). In the open-loop configuration, ambient air enters the system through an air dryer **5** and is pumped through the ozone generator **1**, humidifier, and into the sterilization compartment **4**. The air dryer **5** removes moisture from air entering the system, increasing the output of the ozone generator **1** and extending the life of the pump **6**, ozone generator **1**, and ozone sensor **2**. The humidifier **3** adds moisture to the air prior to entering the sterilization compartment **4**, which has been shown to facilitate the ozone sterilization process (Sharma, 2008). Ozonated air exits the sterilization chamber and vents into an enclosure, such as a fume hood. In the open-loop configuration, average ozone concentrations range between 4.2 and 6.3 g/Nm3. Peak concentrations are generated at the beginning of the cycle and fall slightly to a steady-state value at around 20 minutes. Humidity levels range between 65% and 75%, and the temperature gradually rises during the process, beginning at 29 °C and reaching 33 °C at the end of the cycle.

[0039] The closed-loop configuration expands on the original design and affords a greater level of efficiency and control over environmental parameters during the sterilization process. The process is split into two distinct phases: sterilization and destruction. During sterilization, the ozone generator **1** is active and the pump **6** circulates ambient air through the closed-loop system. Once the sterilization cycle is complete, the system enters a destruct cycle to eliminate remaining ozone. The air circulating through the sterilizer is diverted to an ozone destruction unit **7**, which contains an oxidation catalyst that converts the remaining ozone into oxygen. The recirculating air passes through the destruction unit **7** multiple times to ensure the ozone is removed before the sterilization compartment **4** is opened (Dory, 2014). Average ozone concentrations in the closed-loop configuration range between 5.4 and 6.8 g/Nm3, again peaking at the beginning of the cycle. Humidity and temperature level are consistent with the open-loop configuration, 65-75% and 29 - 33°C, respectively.

[0040] There are five primary states of operation of this inventive sterilizer controlled by inputs

from the user via a microcontroller or processor: Idle, Setup, Maintenance, Sterilization, and Destruction (FIG 21). When the system is turned on, the device first enters the Idle State, in which the ozone generator **1** is off, the pump **6** is off, the air vent is open, the ozone destruction unit **7** is bypassed, the humidifier **3** is bypassed, and the ozone sensor **2** is energized. For the prototype system, the ozone sensor **2** requires a 15-minute warm-up period; therefore, as soon as the ozone sensor **2** is energized, a 15-minute internal timer begins.

[0041] From the Idle State, the user can enter the Setup state. The Setup window allows sterilization cycle parameters to be defined and saved. The user can set the sterilization runtime, destruction runtime, and minimum threshold ozone concentration for the sterilization cycle. During the sterilization cycle, the sterilization runtime timer begins only once the minimum threshold concentration is reached. From the Setup window, the user can also define the duty cycle of the humidifier **3** and the ozone generator **1**. For instance, the ozone generator **1** can be configured to turn off once an upper threshold is reached and turn back on once the ozone concentration fall below a lower threshold, allowing the system to conserve energy. When the user presses the "Save and Exit" button, the values are stored in the microprocessor's memory, and processor returns to the Idle State.

[0042] The Maintenance state allows the user to actuate different components of the system independently to facilitate troubleshooting and maintenance. The ozone generator **1**, pump **6**, and actuating valves **8** can be independently toggled on and off. When the user exits the Maintenance window, the valves **8**, pump **6**, and generator **1** are all returned to Idle State values.

[0043] When the "Start" button is pressed on the Idle State window, the sterilization state begins. The processor first checks the status of the ozone sensor **2** warm-up timer. After the required 15-minute warm-up period, the processor initializes the ozone sensor **2**, and checks the ozone

sensor status to ensure there are no faults. If an error occurs, a window indicates the fault to the user, and the system returns to the Idle State. If no errors occur, the sterilization cycle begins. The ozone generator 1 and pump 6 turn on, the vent closes, and the ozone destruction unit 7 is bypassed. The processor monitors ozone concentration, and, the sterilization timer begins once the concentration reaches the user defined threshold. If ozone concentration falls below threshold at any time during sterilization, the sterilization timer pauses. If the "Stop"' button is pressed while the system is in the Sterilization State, the processor will advance to the Destruction State. Otherwise, once the sterilization timer reaches the defined sterilization runtime, the processor automatically enters the Destruction State.

[0044] In the Destruction State, the ozone generator 1 is turned off, and solenoid valves 8 direct air through the ozone destruction unit 7. When the ozone concentration reaches zero percent, the destruction timer begins. The system continues to operate in the Destruction State until the destruction time reaches the defined destruction runtime. If the "E-Stop" button is pressed while the device is in the Destruction State, the processor will return to the Idle State; however, there may still be ozone circulating in the system, so stopping the device using this method is not recommended. Otherwise, the processor indicates to the user when the sterilization is complete, and the system returns to the Idle State.

[0045] The ozone sterilizer can be operated using battery power or standard 120 VAC power. A mechanical switch selects the type of power used during operation. The battery pack used for the system may features a high discharge current and a long lifecycle, such as a 12 VDC, rechargeable, lithium iron phosphate batter. This battery pack provides approximately 6 hours of continuous runtime, and an indicator on the user interface displays the status of the battery. When operated using 120 VAC, a rectifier converts the 120 VAC into 12 VDC. The 12 VDC power, whether

generated by the battery or the rectifier, is regulated to the voltage required by individual system components. In the prototype system, all system components are controlled by TTL level digital signals generated by the Arduino microprocessor. The TTL level signals drive a bank of opto-isolated solid-state relays, which apply or remove power to the ozone generator **1**, pump **6**, and air distribution valves **8** depending on the state of the controller (FIG 22).

[0046] It will be understood that many additional changes in the details, materialsand arrangement of parts, which have been herein described and illustrated in order to explain the nature of the invention, may be made by those skilled in the art within the principles and scope of the invention as expressed in the appended claims.

Example1: Sterilization Tests Using Ozone

[0047] A series of sterilization tests were conducted to evaluate of the prototype, ozone generating deployable chamber and its ability to kill a series of military medicine relevant microorganisms, including: *Methicillin-resistant Staphylococcus aureus* (MRSA, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Bacillus subtilis* and *Acinetobacter baumannii*).

Method and Materials

Bacterial Strains

[0048] Seventeen strains of bacterial cultures representing Gram positive and Gram negative species of various morphological types (rods, cocci, or coccobacillary forms) were tested for their survival after exposure to ozone. Bacteria included strains of *Acinetobacter baumannii, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis* (Table 1). Bacterial stock cultures were generated by growing cultures on 100mm plates containing nutrient agar at 37°C overnight. A single bacterial colony was picked using a sterile toothpick and added to 3 ml of sterile trypticase soy broth or nutrient broth for *A. baumannii* in a 14 ml sterile polypropylene tube overnight at

37°C without shaking. Overnight grown cultures were mixed with sterile glycerol to generate a 50% glycerol stock and aliquoted into cryovials at 100µl each and stored at -80°C.

Table 1. Bacterial Strains

No.	Name	Marking	Number (from military)
1	*Acinetobacter baumannii*	AB-1 or St-1	ATTC BAA-1605
2	*Acinetobacter baumannii*	AB-2 or St-2	ATTC 17961
3	*Acinetobacter baumannii*	AB-3 or St-3	ATCC 19606
4	*Acinetobacter baumannii*	AB-4 or St-4	ATTC 19003
5	*Acinetobacter baumannii*	AB-6 or St-6	WRAMC #13
6	*Klebsiella pneumoniae*	KP-8 or St-8	BAMC 07-18
7	*Klebsiella pneumoniae*	KP- 9 or St-9	Xen- 39
8	*Klebsiella pneumoniae*	KP -10 or St-10	IA- 525
9	*Escherichia coli*	EC- 11 or St-11	JM 109
10	*Pseudomonas aeruginosa*	PA-14 or St-14	Xen-41
11	*Pseudomonas aeruginosa*	PA – 15 or St-15	BAMC 07- 4
12	*Pseudomonas aeruginosa*	PA- 16 or St-16	PA 01
13	*Staphylococcus aureus*	SA- 17 or St-17	IQ 0070
14	*Staphylococcus aureus*	SA – 18 or St-18	Xen 40
15	*Staphylococcus aureus*	SA – 19 or St-19	ATCC 33591
16	*Staphylococcus aureus*	SA – 22 or St-22	TCH 1516
17	*Bacillus subtilis*	BS-23 or St-23	JH 642

Ozone Sterilization Chamber System Prototype

[0049] The ozone generator (Model MP 8000 from A2Z Ozone Inc., Hudson, NH) was connected to an

ozone monitor (Model 454, Teledyne Instrumentations, San Diego, CA) via tygon tubing (outer diameter 6mm, inner diameter 4mm), and subsequently to the sample case (Pelican 1500, Pelican Products, Torrance, CA) with dimensions 19" x 14.5" x 6.5" (l x w x h) (depicted in FIG 1) (Dory, 2014). A small hole was drilled in the back of the briefcase with an inner diameter of 7mm through which the tubing was run through. The ozone chamber was placed in a certified chemical safety fume hood for all studies.

Ozone concentration detection

[0050] The ozone concentration was determined by UV using the Teledyne model 454 ozone monitor (Teledyne Instrumentations, San Diego, CA), which can detect ozone from 0-50g/Nm with an accuracy of ±1%. Ozone concentrations were continuously monitored at each ozone output % for the entire experimental test period ranging from 5 minutes to 1 hour.

Ozone treatment of bacteria

[0051] Except for *Acinetobacter baumannii,* bacterial strains were inoculated at a 1:100 dilution using the frozen culture stock into 3.0 ml of sterile trypticase soy broth in a 14 ml sterile, polypropylene tube and grown at 37°C without shaking. *Acinetobacter baumannii* was grown in nutrient broth. Ten microliter aliquots of the overnight cultures were plated in duplicate in 12-well plates (Corning Inc., Corning, NY) for both the ozone exposure and control plates. Control plates were incubated in a laminar air-flow purifier biological safety lab chamber, for equivalent time periods consistent with ozone treated cultures. Bacteria were exposed to ozone outputs of 25%, 50%, 75%, and 100% for 5, 15, 30, or 60 minutes. Percent killing was assayed by a standard spread plate or turbidity assay.

Spread plate assay

[0052] After ozone exposure, 200 µl of sterile trypticase soy broth was added to each well of the ozone treated and untreated plates and samples were transferred to sterile 100mm plates containing nutrient agar. Bacteria were spread onto the plates using a LAZY-L-SPREADER™ (Sigma, White Plains, NY) and

allowed to grow overnight at 37°C without shaking. Colony forming units were manually counted and results were recorded as:'+++', >300 colonies; *++', 100-300 colonies;'+', <10 colonies, and '—'; no growth. All samples were analyzed as technical duplicates.

Turbidity assay

[0053] After ozone exposure, 2.0 ml of tryptic soy broth (or nutrient broth *for A. baumannii)* was added to each well of the ozone treated and untreated plates and incubated overnight at 37 °C without shaking. Following overnight growth, plates were sealed with PCR sealing film (VWR catalog # 82018-846) and absorbance was read at 600 nm on the BIOTEK SYNERGY™ HI Hybrid plate reader (Biotek, Seattle, WA) using GEN5™ software version 2.01.Turbidity was normalized by dividing the average turbidity of the ozone exposure wells by the average turbidity of control wells. The average turbidity of the control wells was set to 1.0.

Bacterial counting by serial dilution

[0054] Bacterial counting for each strain was performed by generating 10-fold serial from 10^{-1} to 10^{-8}. Bacteria were spread onto nutrient agar plates in duplicate by adding 100 µl of each dilution to each plate and grown overnight at 37°C without shaking. Dilution plates showing between 30-300 colonies were counted and the colony count was averaged from the duplicate plates and expressed in colony-forming units/ml (CFU/ml).

RESULTS

Ozone Output of Prototype Sterilization System

[0055] The prototype ozone generator utilized for sterilization was rated at a maximum ozone output of 8 g/Nm^3 when utilizing a pure gaseous oxygen source. However, given the desire for utilization of ambient air as the source for ozone generation, it was imperative to determine the

ozone output under ambient air conditions. The ozone output was measured under four manufacturer settings, 25%, 50%, 75%, or 100%, at time periods ranging from 5 minutes to 60 minutes. The ozone output utilizing ambient air ranged from an average ozone concentration of 1.6 g/Nm^3 at 25% output to as high as 4.7 g/Nm^3 at 100% output, far below the manufacturer's reporting of 8 g/Nm^3 at 100% utilizing pure gaseous oxygen (Table 2).

Table 2. Percent Ozone Generated in Sterilization Chamber System at 25%, 50%, 75%, and 100% Output

Ozone Generated (g/Nm^3)				
Time (minutes)	25%	50%	75%	100%
5	1.6 g/Nm^3 (1.4-1.8)	2.3 g/Nm^3 (2.0-2.6)	2.8 g/Nm^3 (2.4-3.1)	4.7 g/Nm^3 (4.4-5.1)
15	1.9 g/Nm^3 (1.8-2.0)	2.5 g/Nm^3 (2.4-2.7)	3.0 g/Nm^3 (2.8-3.2)	4.2 g/Nm^3 (4.0-4.5)
30	2.1 g/Nm^3 (2.0-2.2)	2.6 g/Nm^3 (2.5-2.7)	3.1 g/Nm^3 (3.0-3.2)	4.0 g/Nm^3 (3.8-4.1)
60	2.2 g/Nm^3 (2.1-2.4)	2.7 g/Nm^3 (2.6-2.8)	3.2 g/Nm^3 (3.0-3.2)	3.9 g/Nm^3 (3.7-4.0)

Note. Ozone generated at 25%, 50%, 75%, and 100% output was measured by UV. Average ozone concentration and range of three experimental trials are reported.

Ozone Killing of Bacteria

[0056] The composite spread plate assay results of all ozone killing studies demonstrate 100% killing of all bacteria species when exposed to ozone for 60 minutes at all four ozone outputs evaluated (Tables 3-6).

Table 3. Spread Plate Assay Results from 25% Ozone Output

25% Ozone Output								
	Control				Ozone-treated			
	Time (minutes)				Time (minutes)			
Strain	5	15	30	60	5	15	30	60
AB-1	+++	+++	+++	+++	+++	---	---	---
AB-2	+++	+++	+++	+++	+++	---	---	---
AB-3	+++	+++	+++	+++	+++	++	---	---
AB-4	+++	+++	+++	+++	+++	++	---	---
AB-6	+++	+++	+++	+++	+++	---	---	---
KP-8	+++	+++	+++	+++	+++	+++	---	---
KP-9	+++	+++	+++	+++	+++	+	---	---
KP-10	+++	+++	+++	+++	+++	+++	---	---
EC-11	+++	+++	+++	+++	+++	+	---	---
PA-14	+++	+++	+++	+++	+++	+++	---	---
PA-15	+++	+++	+++	+++	+++	+++	---	---
PA-16	+++	+++	+++	+++	+++	+++	---	---
SA-17	+++	+++	+++	+++	+++	+++	---	---
SA-18	+++	+++	+++	+++	+++	+	---	---
SA-19	+++	+++	+++	+++	+++	+++	---	---
SA-22	+++	+++	+++	+++	+++	+++	---	---
BS-23	+++	+++	+++	+++	+++	+	---	---

Note. Bacteria were treated with and without ozone for 5, 15, 30, and 60 minutes and then spread on plates. '+++', >300 colonies, '++' 100-300 colonies, '+', <10 colonies, '---', no growth. Plates were assayed in technical duplicate.

Table 4. Spread Plate Assay Results from 50% Ozone Output

50% Ozone Output								
	Control				Ozone-treated			
	Time (minutes)				Time (minutes)			
Strain	5	15	30	60	5	15	30	60
AB-1	+++	+++	+++	+++	+++	---	---	---
AB-2	+++	+++	+++	+++	+++	---	---	---
AB-3	+++	+++	+++	+++	+++	+++	---	---
AB-4	+++	+++	+++	+++	+++	+++	++	---
AB-6	+++	+++	+++	+++	+++	---	---	---
KP-8	+++	+++	+++	+++	+++	++	---	---
KP-9	+++	+++	+++	+++	+++	++	---	---
KP-10	+++	+++	+++	+++	+++	++	---	---
EC-11	+++	+++	+++	+++	+++	---	---	---
PA-14	+++	+++	+++	+++	+++	---	---	---
PA-15	+++	+++	+++	+++	+++	---	---	---
PA-16	+++	+++	+++	+++	+++	---	---	---
SA-17	+++	+++	+++	+++	+++	++	---	---
SA-18	+++	+++	+++	+++	+++	---	---	---
SA-19	+++	+++	+++	+++	+++	+++	---	---
SA-22	+++	+++	+++	+++	+++	+++	---	---
BS-23	+++	+++	+++	+++	+++	---	---	---

Note. Bacteria were treated with and without ozone for 5, 15, 30, and 60 minutes and then spread on plates. '+++', >300 colonies, '++' 100-300 colonies, '+', <10 colonies, '---', no growth. Plates were assayed in technical duplicate.

Table 5. Spread Plate Assay Results from 75% Ozone Output

75% Ozone Output								
	Control				Ozone-treated			
	Time (minutes)				Time (minutes)			
Strain	5	15	30	60	5	15	30	60
AB-1	+++	+++	+++	+++	+++	---	---	---
AB-2	+++	+++	+++	+++	+++	---	---	---
AB-3	+++	+++	+++	+++	+++	---	---	---
AB-4	+++	+++	+++	+++	+++	---	---	---
AB-6	+++	+++	+++	+++	+++	+	---	---
KP-8	+++	+++	+++	+++	+++	+++	---	---
KP-9	+++	+++	+++	+++	+++	+++	---	---
KP-10	+++	+++	+++	+++	+++	+++	---	---
EC-11	+++	+++	+++	+++	+++	---	---	---
PA-14	+++	+++	+++	+++	+++	+	---	---
PA-15	+++	+++	+++	+++	+++	+++	---	---
PA-16	+++	+++	+++	+++	+++	+	---	---
SA-17	+++	+++	+++	+++	+++	+	---	---
SA-18	+++	+++	+++	+++	+++	---	---	---
SA-19	+++	+++	+++	+++	+++	+++	---	---
SA-22	+++	+++	+++	+++	+++	+++	---	---
BS-23	+++	+++	+++	+++	+++	---	---	---

Note. Bacteria were treated with and without ozone for 5, 15, 30, and 60 minutes and then spread on plates. '+++', >300 colonies, '++' 100-300 colonies, '+', ≤10 colonies, '---', no growth. Plates were assayed in technical duplicate.

Table 6. Spread Plate Assay Results from 100% Ozone Output

100% Ozone Output								
	Control				Ozone-treated			
	Time (minutes)				Time (minutes)			
Strain	5	15	30	60	5	15	30	60
AB-1	+++	+++	+++	+++	+++	---	---	---
AB-2	+++	+++	+++	+++	+++	---	---	---
AB-3	+++	+++	+++	+++	+++	---	---	---
AB-4	+++	+++	+++	+++	+++	++	---	---
AB-6	+++	+++	+++	+++	++	---	---	---
KP-8	+++	+++	+++	+++	+++	+++	---	---
KP-9	+++	+++	+++	+++	+++	+++	---	---
KP-10	+++	+++	+++	+++	+++	+++	---	---
EC-11	+++	+++	+++	+++	+++	+	---	---
PA-14	+++	+++	+++	+++	+++	+++	---	---
PA-15	+++	+++	+++	+++	+++	---	---	---
PA-16	+++	+++	+++	+++	+++	---	---	---
SA-17	+++	+++	+++	+++	+++	---	---	---
SA-18	+++	+++	+++	+++	+++	---	---	---
SA-19	+++	+++	+++	+++	+++	---	---	---
SA-22	+++	+++	+++	+++	+++	---	---	---
BS-23	+++	+++	+++	+++	++	---	---	---

Note. Bacteria were treated with and without ozone for 5, 15, 30, and 60 minutes and then spread on plates. '+++', >300 colonies, '++' 100-300 colonies, '+', <10 colonies, '---', no growth. Plates were assayed in technical duplicate.

[0057] In addition to the spread plate assay, the killing effects of ozone on each bacterium species was further validated utilizing a turbidity assay. As was observed in the spread plate assay, the killing effect of ozone on the bacteria strains was impacted by both ozone output, as well as time of exposure, with > 90% loss of absorbance/turbidity observed for all 17 strains of bacteria following 60 minutes of exposure. Results for each experimental exposure condition for each bacteria isolates are shown in FIGs 2-18: *A. baumannii* (FIGs 2-6), *B. subtilis* (FIG 7), *E. coli* (FIG 8), *K. pneumonia* (FIGs 9-11), *P. aeruginosa* (FIGs 12-14), and *S. aureus* (FIGs 15-18).

[0058] The results of this study demonstrate that gaseous ozone is highly effective at killing bacteria at concentrations as high as 10^9 cfu/ml. This killing effect is equivalent or superior to the efficiency of several currently utilized sterilization systems, including heat sterilization, formaldehyde, glutaraldehyde, and hydrogen peroxide. Overall, the data from both the turbidity and spread plate readout reveal that a 60 minute exposure to gaseous ozone at an ozone concentration as low as 1.6 g/Nm^3 (25% ozone output) was sufficient to kill all 17 strains of bacteria tested. Although some discrepancies were seen between the turbidity and spread plate readout, the 60 minute exposure time demonstrated consistent bacterial killing at all ozone concentrations tested under both assay conditions.

Prophetic example 2: Phase II Open and Closed Loop Testing

[0059] In this phase, a diverse group of Gram-positive and Gram-negative bacterial strains, 24 in total, were tested in the inventive sterilizer in both open and closed loop testings. Dental instrument were also tested in closed-loop testing.

Bacterial Strains

[0060] Bacterial species tested included: *S. aureus, P. aeruginosa, K. pneumonia, E. coli, B. subtilis, and A. baumannii* (Table 7).

Table 7. Bacterial Strains for open and closed-loop testing

Name	Strain ID	Military ID
Acinetobacter baumannii	ST-1	ATTC BAA-1605
Acinetobacter baumannii	ST-2	ATTC 11
Acinetobacter baumannii	ST-3	ATTC 19606
Acinetobacter baumannii	ST-4	ATTC 19003
Acinetobacter baumannii	ST-6	WRAMC #13
Acinetobacter baumannii	ST-7	WRAMC #9
Klebsiella pneumoniae	ST-8	BAMC 07-18
Klebsiella pneumoniae	ST-9	XEN-39
Klebsiella pneumoniae	ST-10	IA-525
Escherichia coli	ST-11	JM109
Pseudomonas aeruginosa	ST-14	XEN-41
Pseudomonas aeruginosa	ST-15	BAMC 07-4
Pseudomonas aeruginosa	ST-16	PAO1
Staphylococcus aureus	ST-17	IQ 0070
Staphylococcus aureus	ST-18	XEN-40
*Staphylococcus aureus**	ST-19	ATTC 33591
Staphylococcus aureus	ST-22	TCH 1516
Bacillus subtilis	ST-23	JH 642
Escherichia coli	ST-32	ATTC BAA-2215
Escherichia coli	ST-33	ATTC BAA-2196
Escherichia coli	ST-34	ATTC 51657
Escherichia coli	ST-35	ATTC 35401
Escherichia coli	ST-36	ATTC 51434
Escherichia coli	ST-37	ATTC BAA-184

*Note: Methicillin-resistant *Staphylococcus aureus* (MRSA)

[0061] A single bacterial colony from each strain, grown in 35 mm nutrient agar plates at 37 °C, was inoculated into appropriate sterile media. In accordance with strain American Tissue Culture Collection growth suggestions, Luria Broth was used for *E. coli* (ST36) and *B. subtilis* (ST23), nutrient broth was used for all *A. baumannii* strains and *E. coli* (ST32, ST33, and ST35), and Tryptic Soy Broth was used for all other bacterial strains. All cultures were grown in 50 ml propylene conical tubes (BD #352098 and Thermo Scientific # 339653) overnight at 37 °C with shaking at 200 rpm. All strains were stored at -80 °C in cryovial tubes after aliquoting 500 µl of a 50% sterile glycerol and culture mixture.

Bacterial Sample Preparations

[0062] Bacterial strains from freezer stocks were diluted 1:100 in suitable sterile growth media in 50 ml propylene conical tubes, and grown overnight at 37 °C with shaking at 200 rpm. The following day, the cultures were diluted 1:10 in the appropriate media and aliquoted into the wells of a polystyrene, 12-well flat bottom plate (Costar #3512). The OD600 (Optical Density 600 nm) was read using a BIOTEK® SYNERGY™ HT spectrometer (BIOTEK®, Seattle, WA) with Gen5TM software version 2.01. Bacterial cultures were standardized to 1.5 OD/ml after the OD600 was determined. Serial dilutions were performed to quantify inoculum populations by generating 10-fold serial dilutions from 10-1 to 10-8. For specific quantification, 100 µl per dilution was spread onto nutrient agar plates and placed in a 37 °C incubator overnight.

Open-Loop Configuration Testing

[0063] Ozone Treatment. Ten wells from a 12-well plate were inoculated with 10 µl aliquots of the same bacterial strain in both ozone-treated and control plates. Bacteria were spread within the

well and then allowed to dry inside the laminar air-flow safety cabinet, prior to ozone sterilization. Control plates were closed but remained in the laminar air-flow safety cabinet for 60 minutes. Each bacteria strain was treated once.

[0064] Turbidity and Spread Plate Assays. Following ozone exposure, 2 ml of sterile media was added to each well in the test plate and the control plate to re-suspend the bacteria. After the bacteria were re-suspended, 200 µl from each of the 10 treated wells and 3 control wells were plated without dilution on nutrient agar and placed in a 37 °C incubator overnight. Colony forming units (CFU's) were counted the following day. The 12-well plates with the remaining media were placed in the incubator at 37 °C overnight without shaking. The following day, the plates were removed and sealed with PCR sealing film (VWR catalog #82018-846) and the OD600 was read using a spectrometer. Turbidity was normalized by dividing the average turbidity of the ozone exposure wells by the average turbidity of the control wells. Each of the10 treated wells and 10 control wells were measured independently.

Closed-Loop Configuration Testing

[0065] Ozone Treatment. Twelve-well plates were inoculated with bacteria using the same method described for open-loop testing. Each bacteria strain was treated in triplicate. Additionally, dental instruments, including explorers, probes, hatchet, excavators, cotton pliers, spatulas, and mirrors, were treated in the closed-loop sterilizer configuration. Instruments were placed in 50 ml propylene conical tubes containing bacterial suspensions, with bacterial concentrations averaging 108 CFU/ml. The instruments were allowed to dry lying flat inside the laminar flow safety cabinet. A set of instruments was placed in the ozone sterilization chamber on top of a flat piece of aluminum foil and exposed to ozone for 60 minutes, and the control instruments were left in the laminar flow safety cabinet for the duration of the ozone exposure.

Following exposure, tools were wrapped in the foil to prevent contamination and placed in the laminar flow hood. Each bacteria strain was treated in duplicate.

[0066] Turbidity and Spread Plate Assays. Bacterial growth in the 12-well plates was measured using the same methods described for the open-loop testing. For measuring bacterial growth on the dental instruments, the tools were placed in 50 ml propylene conical tubes containing 10 ml of sterile strain growth media for at least 15 minutes with occasional agitation. For spread plate assays, 100 µl of the media was plated on a 35 mm sterile nutrient agar plate. Ten-fold serial dilutions from 10^{-1} to 10-5 were generated and 100 µl per dilution were plated for control instruments. The agar plates were incubated overnight at 37 °C, and CFU's were counted the following day. Remaining media from control and test instruments was kept in the conical tubes for turbidity testing, and incubated overnight at 37 °C without shaking. The following day, 1 ml from each tube was aliquoted into one well in a 12-well flat bottom plate, the OD600 was read using the spectrometer, and the data were normalized using the method described above.

RESULTS

[0067] Ozone Killing of Bacteria in Open-Loop Configuration

[0068] In order to replicate the results from the "proof-of-concept" experiment (example I), representative strains of *S. aureus (ST19), P. aeruginosa (ST14), K. pneumoniae (ST8), E. coli (ST11), B. subtilis (ST23), and A. baumannii* (ST3) were treated for 60 minutes. Starting concentrations of the bacteria ranged from 106 to 107 CFU/ml as determined by serial dilutions. There was complete elimination of bacterial strains ST3, ST8, ST14 and ST19 in both the turbidity and spread plate assays. *E. coli* ST11 and *B. subtilis* ST23 were eliminated from the spread plate assay (Table 8). There was a 97% and 90% reduction in absorbance from the turbidity measurement, respectively, which was slightly elevated relative to the negative control (Fig. 24).

Table 8. Spread Plate Assay Results from Open-Loop Experiments

Name	Strain ID	Military ID	Control (No Treatment)	Treated (60 Minutes)
A. baumannii	ST-3	ATTC 19606	+++	---
K. pneumoniae	ST-8	BAMC 07-18	+++	---
E. coli	ST-11	JM109	+++	---
P. aeruginosa	ST-14	XEN-41	+++	---
S. aureus	ST-19	ATTC 33591	+++	---
B. subtilis	ST-23	JH 642	+++	---

Note: '+++' indicates >300 colonies; '++' indicates 100-300 colonies; '+' indicates <100 colonies; and '---' indicates no growth

Ozone Killing of Bacteria in Closed-Loop Configuration

[0069] In the closed-loop configuration all *E. coli, A. baumannii,* and *B. subtilis,* and *K. pneumonia* strains were completely eliminated after treatment, as demonstrated by the turbidity and spread plate assays (Fig. 25A-D; Table 9). All *P. aeruginosa* and *S. aureus* strains were eliminated in the spread plate assays. Turbidity measurements for ST16 and ST17 were slightly elevated relative to the negative control (98% and 93% reduction in absorbance, respectively) (Fig. 25E and F; Table 9).

Table 9. Spread Plate Assay Results from Closed-Loop Experiments

Name	Strain ID	Military ID	Control (No Treatment)			Treated (60 Minutes)		
			RUN 1	RUN 2	RUN 3	RUN 1	RUN 2	RUN 3
A. baumannii	ST-1	ATTC BAA-1605	+++	+++	+++	---	---	---
A. baumannii	ST-2	ATTC 11	+++	+++	+++	---	---	---
A. baumannii	ST-3	ATTC 19606	+++	+++	+++	---	---	---
A. baumannii	ST-4	ATTC 19003	+++	+++	+++	---	---	---
A. baumannii	ST-6	WRAMC #13	+++	+++	+++	---	---	---
A. baumannii	ST-7	WRAMC #9	+++	+++	+++	---	---	---
K. pneumoniae	ST-8	BAMC 07-18	+++	+++	+++	---	---	---
K. pneumoniae	ST-9	XEN-39	+++	+++	+++	---	---	---
K. pneumoniae	ST-10	IA-525	+++	+++	+++	---	---	---
E. coli	ST-11	JM109	+++	+++	+++	---	---	---
P. aeruginosa	ST-14	XEN-41	+++	+++	+++	---	---	---
P. aeruginosa	ST-15	BAMC 07-4	+++	+++	+++	---	---	---
P. aeruginosa	ST-16	PAO1	+++	+++	+++	---	---	---
S. aureus	ST-17	IQ 0070	+++	+++	+++	---	---	---
S. aureus	ST-18	XEN-40	+++	+++	+++	---	---	---
S. aureus	ST-19	ATTC 33591	+++	+++	+++	---	---	---
S. aureus	ST-22	TCH 1516	+++	+++	+++	---	---	---
B. subtilis	ST-23	JH 642	+++	+++	+++	---	---	---
E. coli	ST-32	ATTC BAA-2215	+++	+++	+++	---	---	---
E. coli	ST-33	ATTC BAA-2196	+++	+++	+++	---	---	---
E. coli	ST-34	ATTC 51657	+++	+++	+++	---	---	---
E. coli	ST-35	ATTC 35401	+++	+++	+++	---	---	---
E. coli	ST-36	ATTC 51434	+++	+++	+++	---	---	---
E. coli	ST-37	ATTC BAA-184	+++	+++	+++	---	---	---

Note: '+++' indicates >300 colonies; '++' indicates 100-300 colonies; '+' indicates <100 colonies; and '---' indicates no growth

Ozone Killing of Bacteria on Dental Instruments

[0070] Functional testing of the ozone sterilizer was performed by exposing stainless steel dental instruments to representative strains of *S. aureus* (ST19), *P. aeruginosa* (ST14), *K. pneumonia*

(ST8), *E. coli* (ST11), and *A. baumannii* (ST3). *B. subtilis* (ST23) was excluded from these experiments due to repeated failure of the negative controls when inoculated on the dental tools. Turbidity assays and spread plate tests showed no reduction in bacterial load on the dental mirrors, although significant reduction or elimination was seen on the other dental instruments. On the other instruments, no bacterial growth was seen in the spread plate assays, with the exception of ST8 (16 CFU's) and ST19 (1 CFU), and turbidity measurements for ST8 and ST3 remained elevated (<90% reduction in absorbance) (Fig. 26; Table 10).

Table 10. Spread Plate Assay Results from Dental Instrument Experiments

Name	Strain ID	Military ID	Control (No Treatment)		Treated (60 Minutes)*	
			RUN 1	RUN 2	RUN 1	RUN 2
A. baumannii	ST-3	ATCC 19606	+++	+++	---	---
K. pneumoniae	ST-8	BAMC 07-18	+++	+++	+	---
E. coli	ST-11	JM109	+++	+++	---	---
P. aeruginosa	ST-14	XEN-41	+++	+++	---	---
S. aureus	ST-19	ATCC 33591	+++	+++	+	---

Note: '+++' indicates >300 colonies; '++' indicates 100-300 colonies; '+' indicates <100 colonies; and '---' indicates no growth
***Note:** Dental mirror results not shown. All treated mirrors had >300 CFU's.

[0071] During treatment of the dental instruments, signs of oxidation were observed on some of the stainless steel tools. Stainless steel typically resists corrosion and is considered to be ozone compatible, so other sources of oxidation were investigated. The water reservoir used for the humidifier **3**, through which the ozonated air passes, was tested for acidity using litmus paper, and was found to have a pH of ~1. The reservoir water was analyzed using LCMS to determine its constituent parts, and elevated levels of nitrate were detected in the sample, suggesting nitric acid to be a source of the acidity.

[0072] Phase II results in the open-loop configuration replicated those observed in example 1. Consistent with example 1, all bacteria strains were eliminated from the spread plate assays, and turbidity/absorbance measurements were all reduced by >90% after the 60- minute sterilization cycle. In the closed-loop configuration, the sterilizer eliminated both Gram-positive and Gram-negative bacterial strains from polystyrene plates. Again, all bacteria were eliminated from the spread plate assays, and turbidity tests demonstrated a >90% reduction in absorbance.

[0073] No bacterial growth was observed in the spread plate assays, slight elevations in turbidity were observed for some of the strains (ST11, ST16, and ST19) relative to the negative control. The slightly elevated absorbance measurements may simply be caused by debris in the media from the dead bacterial cells.

[0074] Bacterial growth was also significantly reduced on all dental instruments. Remaining growth observed on the dental instrument may also be due to inadequate air exchange with the surface of the dental tools. During tests with the 12-well flat bottom plates, surfaces inoculated with bacteria were completely open and exposed to the sterilization chamber air, whereas during the dental instrument tests, a portion of the inoculated tools, such as the dental mirror was always in contact with the floor of the sterilization compartment 4, which may have obstructed the ozone from reaching all of the bacteria. Elevation of the floor mesh or repeat sterilization of the tools should further reduce the bacteria growth.

REFERENCES

1. Andersen, B. M., Rasch, M., Hochlin, K., Jensen, F. H., Wismar, P., & Fredriksen, J. E. (2006). Decontamination of rooms, medical equipment and ambulances using an aerosol of hydrogen peroxide disinfectant. JHosp Infect, 62(2), 149-155. doi: 10.1016/j.jhin.2005.07.020

2. Aydogan, A., & Gurol, M. D. (2006). Application of gaseous ozone for inactivation of Bacillus subtilis spores. J Air Waste Manag Assoc, 56(2), 179-185.

3. Burdette, A., & Alvarez, R. (2014). *Evaluating the effectiveness of ozone killing bacteria in a portable sterilizer* (NAMRU-SA Report 2014-50).

4. Carlins, J. (1982). Engineering aspects of ozone generation. *Environmental Progress,* 1(2), 113-118.

5. Dory, R. E. (2014). Development of a portable ozone sterilizer (NAMRU-SA Report 2014-51).

6. Guzel-Seydim, Z., Greye, A., & Seydim, A. (2003). Use of ozone in the food industry. *LWT-Food Science and Technology,* 37(4), 453-460.

7. Ishizaki, K., Shinriki, N., & Matsuyama, H. (1986). Inactivation of Bacillus spores by gaseous ozone. JAppl Bacteriol, 60(\), 67-72.

8. Kowalski, W., Bahnfleth, W., & Whittam, T. (1998). Bactericidal effects of high airborne ozone concentrations on Escherichia coli and Staphylococcus aureus.

9. Lowe, J. J., Gibbs, S. G., Iwen, P. C, Smith, P. W., & Hewlett, A. L. (2013). Impact of chlorine dioxide gas sterilization on nosocomial organism viability in a hospital room. IntJ Environ Res Public Health, 10(6), 2596-2605. doi: 10.3390/ijerph 10062596

10. Nagayoshi, M., Fukuizumi, T., Kitamura, C, Yano, J., Terashita, M., & Nishihara, T. (2004). Efficacy of ozone on survival and permeability of oral microorganisms. Oral

Microbiol Immunol, 19(A), 240-246. doi: 10.1111/J.1399-302X.2004.00146.x

11. Rastogi, V. K., Ryan, S. P., Wallace, L., Smith, L. S., Shah, S. S., & Martin, G. B. (2010).

12. Sharma, M., & Hudson, J. (2008). Ozone gas is an effective and practical antibacterial agent. *American Journal of Infection Control,* 36(8), 559-563.

13. Summerfelt, S. (1997). Review of ozone process and applications as an oxidizing agent in aquaculture. *The Progressive Fish-Culturist,* 59(2), 94-105.

14. Systematic evaluation of the efficacy of chlorine dioxide in decontamination of building interior surfaces contaminated with anthrax spores. Appl Environ Microbiol, 76(10), 3343-3351. doi: 10.1128/AEM.02668-09

15. Venezia, R. A., Orrico, M., Houston, E., Yin, S. M., & Naumova, Y. Y. (2008). Lethal activity of nonthermal plasma sterilization against microorganisms. Infect Control Hosp Epidemiol, 29(5), 430-436. doi: 10.1086/588003

16. von Keudell, A., Awakowicz, P., Benedikt, J., Raballand, V., Yanguas-Gil, A., Opretzka, J.,... Deslys, J. P. (2010). Inactivation of Bacteria and Biomolecules by Low-Pressure Plasma Discharges. Plasma Processes and Polymers, 7(3-4), 327-352. doi: 10.1002/ppap.200900121

17. Young, S. B., & Setlow, P. (2004). Mechanisms of Bacillus subtilis spore resistance to and killing by aqueous ozone. J Appl Microbiol, 96(5), 1133-1142. doi: 10.1111/j.1365-2672.2004.02236.x

18. Zhang, J., Davis, T. A., Matthews, M. A., Drews, M. J., LaBerge, M., & An, Y. H. (2006). Sterilization using high-pressure carbon dioxide. The Journal of Supercritical Fluids, 38(3), 354-372. doi: 10.1016/j.supflu.2005.05.005.

19. Burdette, A.J. and Alvarez, R. (2014). Evaluating the effectiveness of ozone in

killing bacteria in a portable sterilizer (NAMRU-SA Report 2014-50).

20. Dory, R.E. (2014). Development of a portable ozone sterilizer (NAMRU-SA Report 2014-51). Harris, G.W., Carter, W.P.L., Winer, A.M., Graham, R.A., and Pitts Jr., J.N. (1982). Studies of trace non-ozone species produced in a corona discharge ozonizer. Journal of the Air Pollution Control Association, 32(3): 274-276.

21. Sharma, M. and Hudson, J.B. (2008). Ozone gas is an effective and practical antibacterial agent. American Journal of Infection Control, 36(8): 559-563.

What is claimed is:

1) An assembly for sterilizing microbiologically contaminated objects, using self-generated ozone, the assembly comprises:
    a. a closable, portable, ruggedized case having an inner chamber;
    b. an air dryer disposed in the inner chamber for receiving ambient air and removing moisture from said air;
    c. an ozone generator disposed in the inner chamber for generating ozone and adding said ozone to the air received from the air dryer;
    d. a humidifier disposed in the inner chamber for receiving said air from said ozone generator and adding moisture to said air;
    e. a closable air sealed sterilization compartment disposed in the inner chamber for accepting one or more microbiologically contaminated objects and for receiving air in at least minimally sufficient conditions of ozone and humidity from said humidifier to effect microbiological sterilization of the objects inside said sterilization compartment;
    f. a pump disposed in the inner chamber for circulating the air through said air dryer, ozone generator, humidifier and sterilization compartment;
    g. a microprocessor or microcontroller for controlling operations of said air dryer, ozone generator, humidifier; and
    h. a power supply for powering the assembly.

2) The assembly of claim 1 further comprises an ozone destruction unit disposed in the inner chamber, powered by the power source and controlled by the microprocessor for

receiving air from said sterilization compartment and converting ozone in said air into oxygen.

3) The assembly of claim 1 further comprises an ozone sensor disposed in the inner chamber, powered by the power source and controlled by the microprocessor for measuring ozone concentration of the air received from said ozone generator.

4) The assembly of claim 1 further comprises a humidifier sensor disposed in the inner chamber, powered by the power source and controlled by the microprocessor for measuring humidity level of the air received from said humidifier.

5) The assembly of claim 2, further comprises at least one valve disposed in the inner chamber fluidly connected to the sterilization compartment, air dryer and the ozone destruction for directing air from air dryer to the ozone destruction unit or the ozone generator.

6) The assembly of claim 1, wherein said power supply is an AV electric source or a DC battery.

7) The assembly of claim 1, wherein said pump is connected to a motor.

8) The assembly of claim 1, wherein said ozone generator is a Corona discharge ozone generator.

9) The assembly of claim 1, wherein said ozone destruction unit uses a low-temperature oxidation catalyst.

10) The assembly of claim 1, wherein said minimally sufficient condition of ozone is set at concentration of approximately 4.2 and 6.3 g/Nm^3.

11) The assembly of claim 1, wherein said minimally sufficient condition of humidity is set a level of approximately 65-75% at approximate 29 - 33°C, respectively.

12) The assembly of claim 1 further comprises user interface.

13) The assembly of claim 1 further comprises a timer connected to the microcontroller and powered by the power source for setting the length of said sterilization.

14) A method for sterilizing microbiologically contaminated objects using the assembly of claim 1, comprising

a) placing microbiologically contaminated objects inside the sterilization compartment;
b) setting sufficient condition of ozone and humidity required to effect microbiological sterilization of said contaminated objects;
c) setting the length of said sterilization;
d) executing said sterilization; and
a. removing said contaminated objects from said sterilization compartment when the sterilization is complete.

15) A method for sterilizing microbiologically contaminated objects using the assembly of claim 5, comprising

a. placing microbiologically contaminated objects inside the sterilization compartment;
b. setting sufficient condition of ozone and humidity required to effect microbiological sterilization of said contaminated objects;

c. setting the length of said sterilization;

d. executing said sterilization;

e. directing air from said air dryer to said ozone destruction unit after the sterilization is complete; and

f. removing said contaminated objects from said sterilization compartment after the ozone is removed from the air.

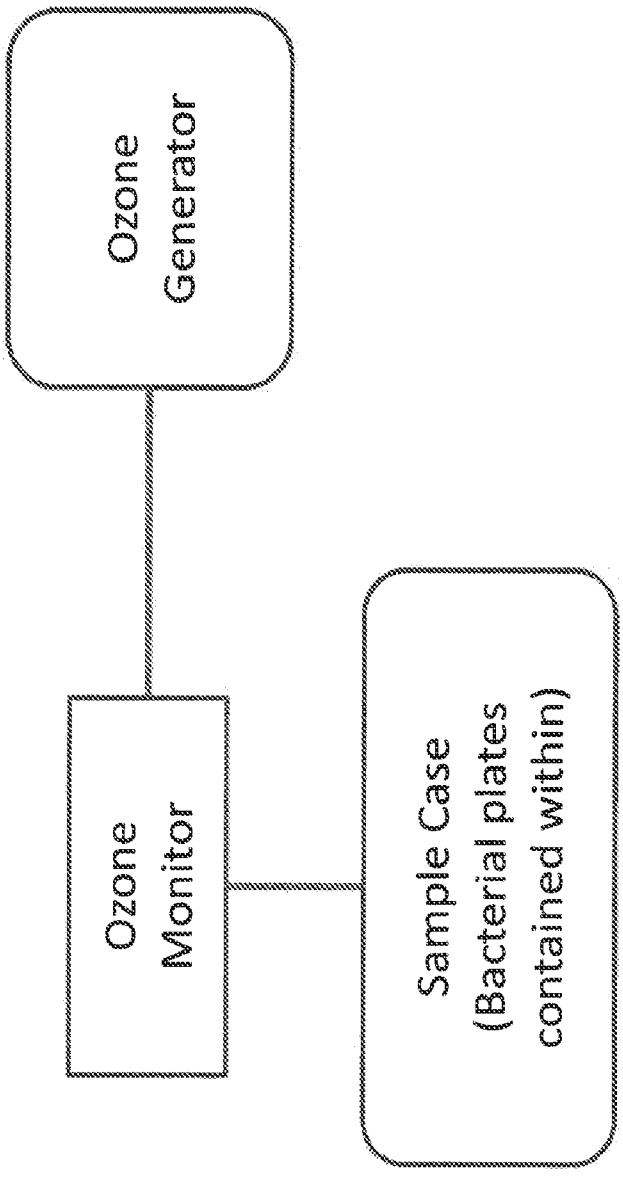
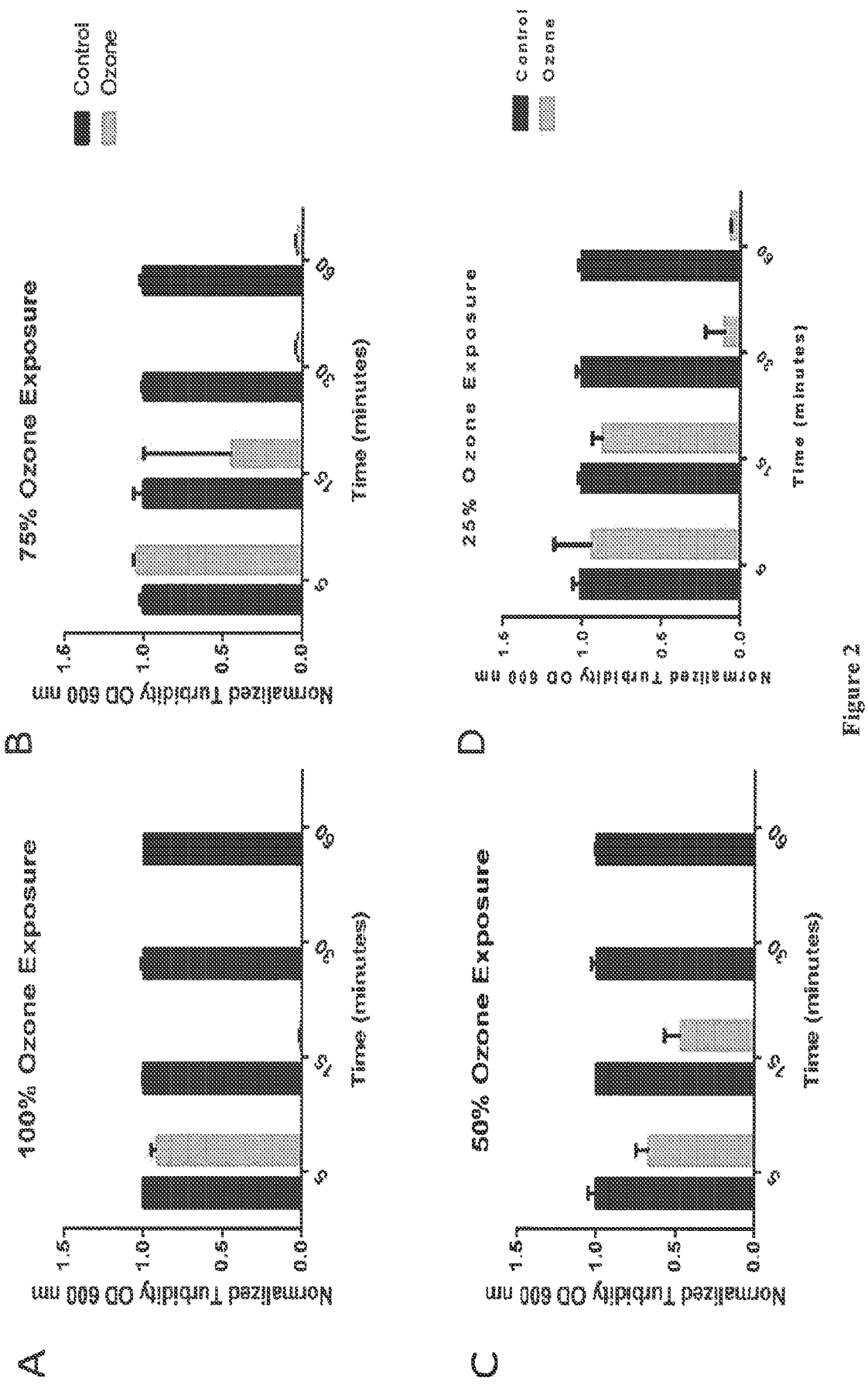

Ozone Generator
Ozone Monitor
Sample Case (Bacterial plates contained within)

FIG 1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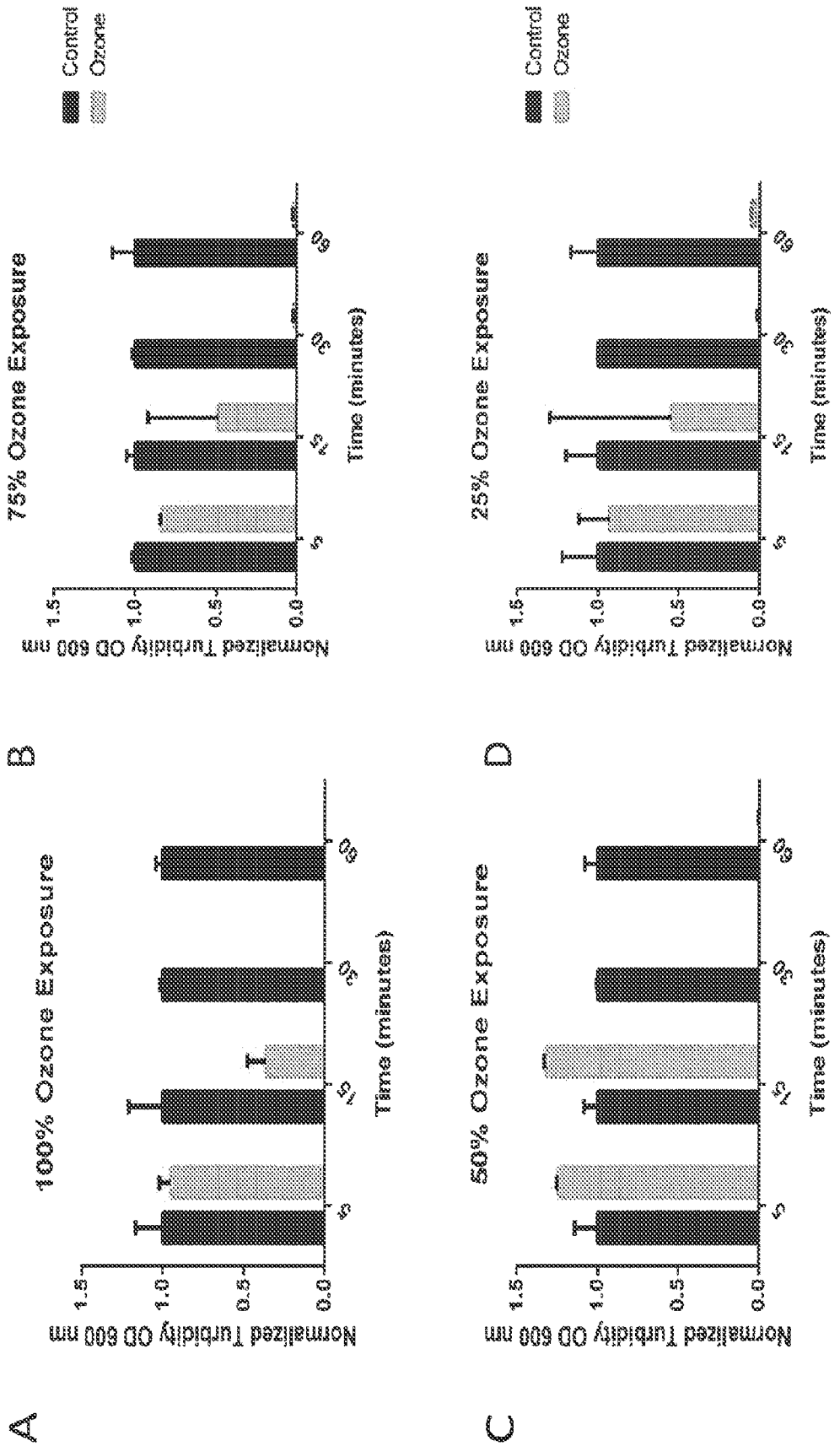
60
Time (minutes)
B
75% Ozone Exposure
Control
Ozone
C
50% Ozone Exposure
D
25% Ozone Exposure

Figure 2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C
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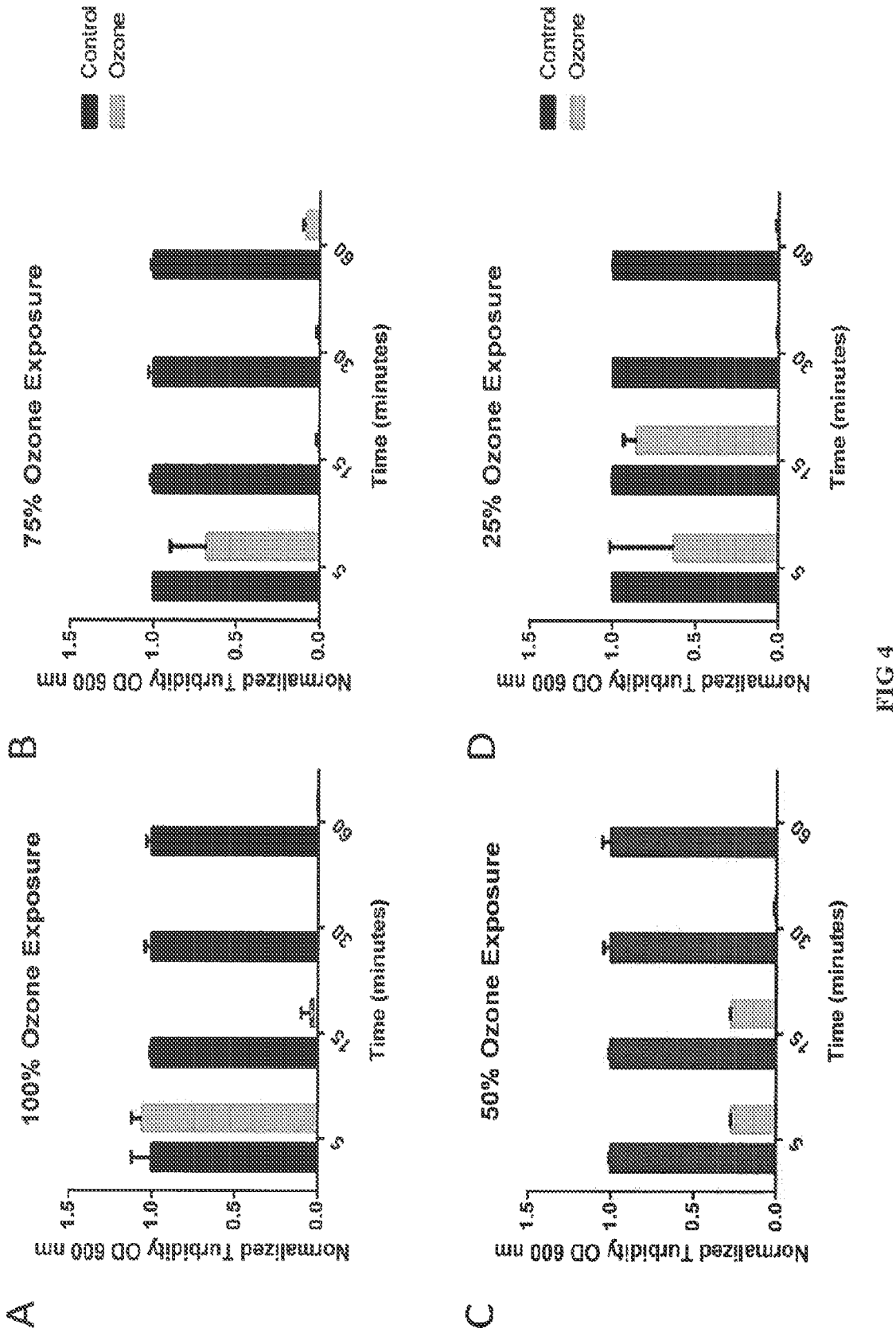
D
25% Ozone Exposure

FIG 3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Time (minutes)
B
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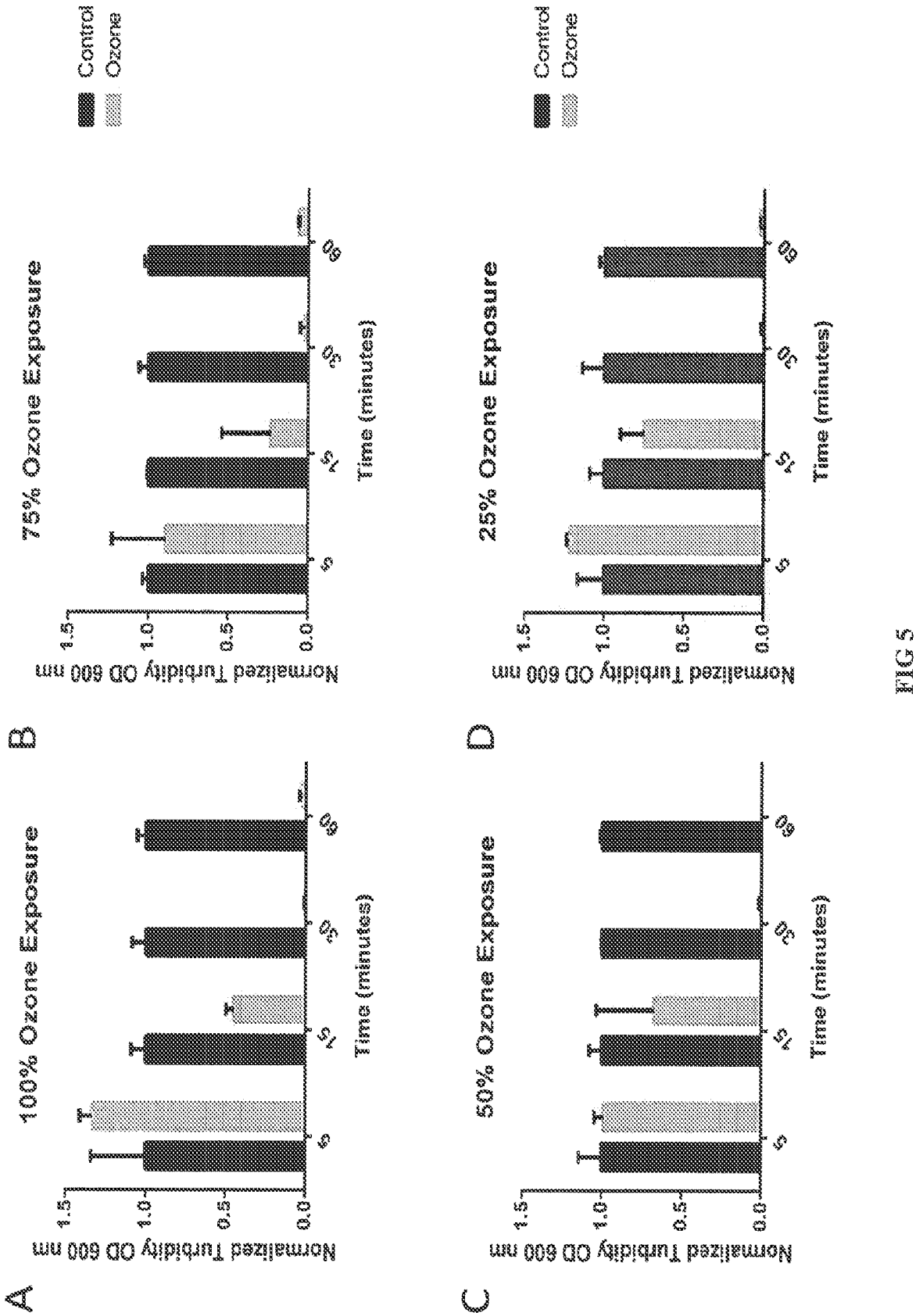
C
50% Ozone Exposure
D
25% Ozone Exposure

FIG 4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C
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25% Ozone Exposure

FIG 5

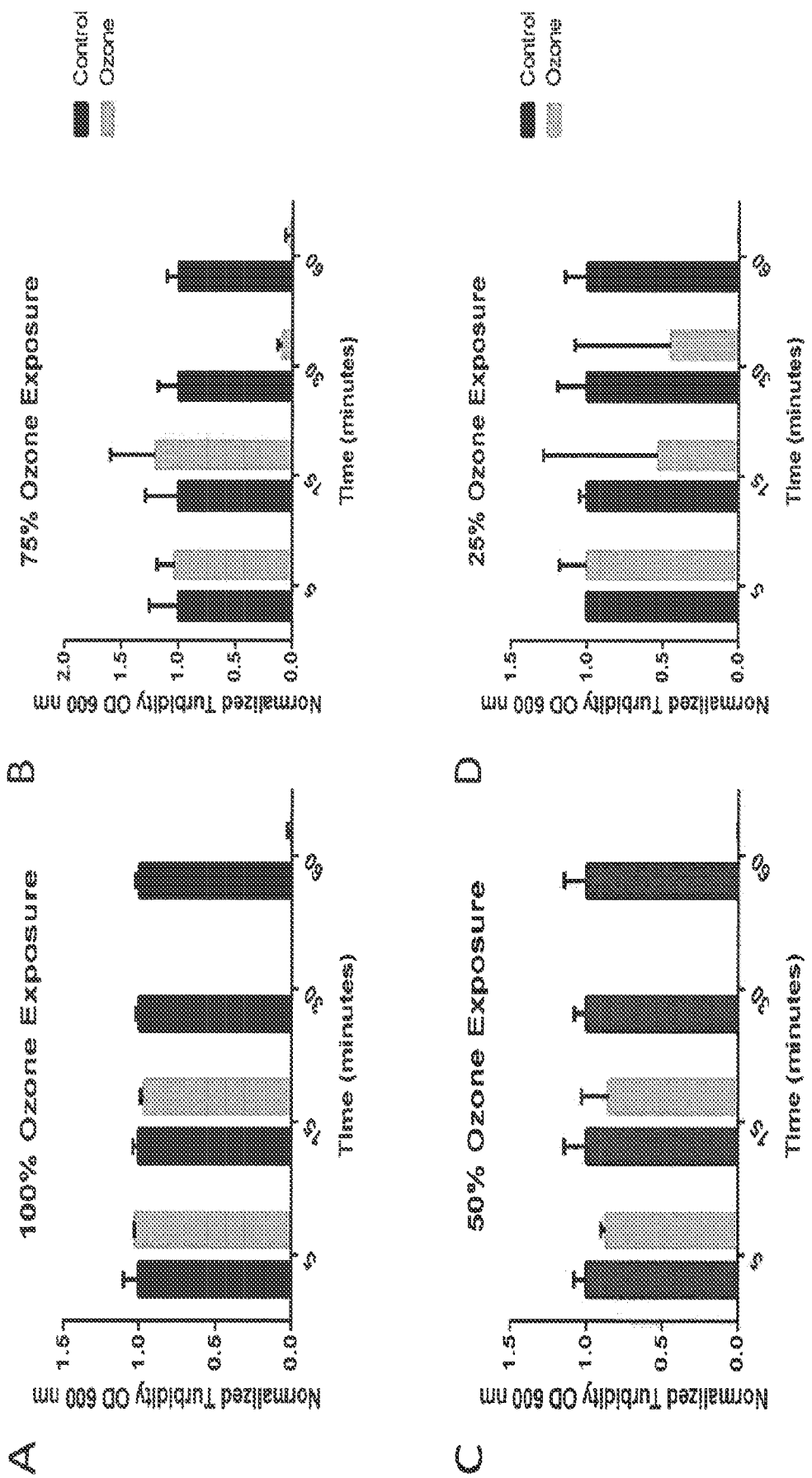
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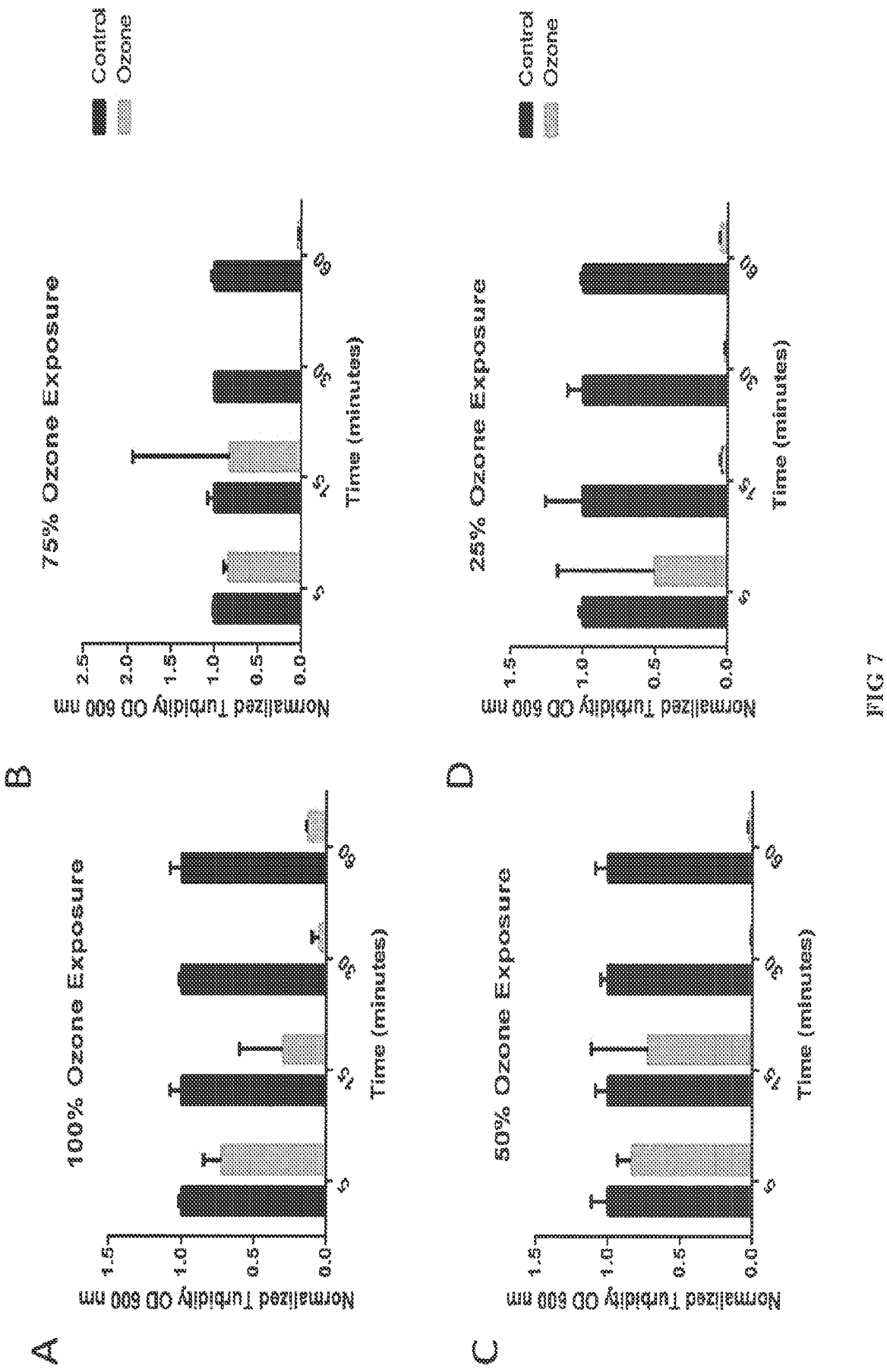
FIG 6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Time (minutes)
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D
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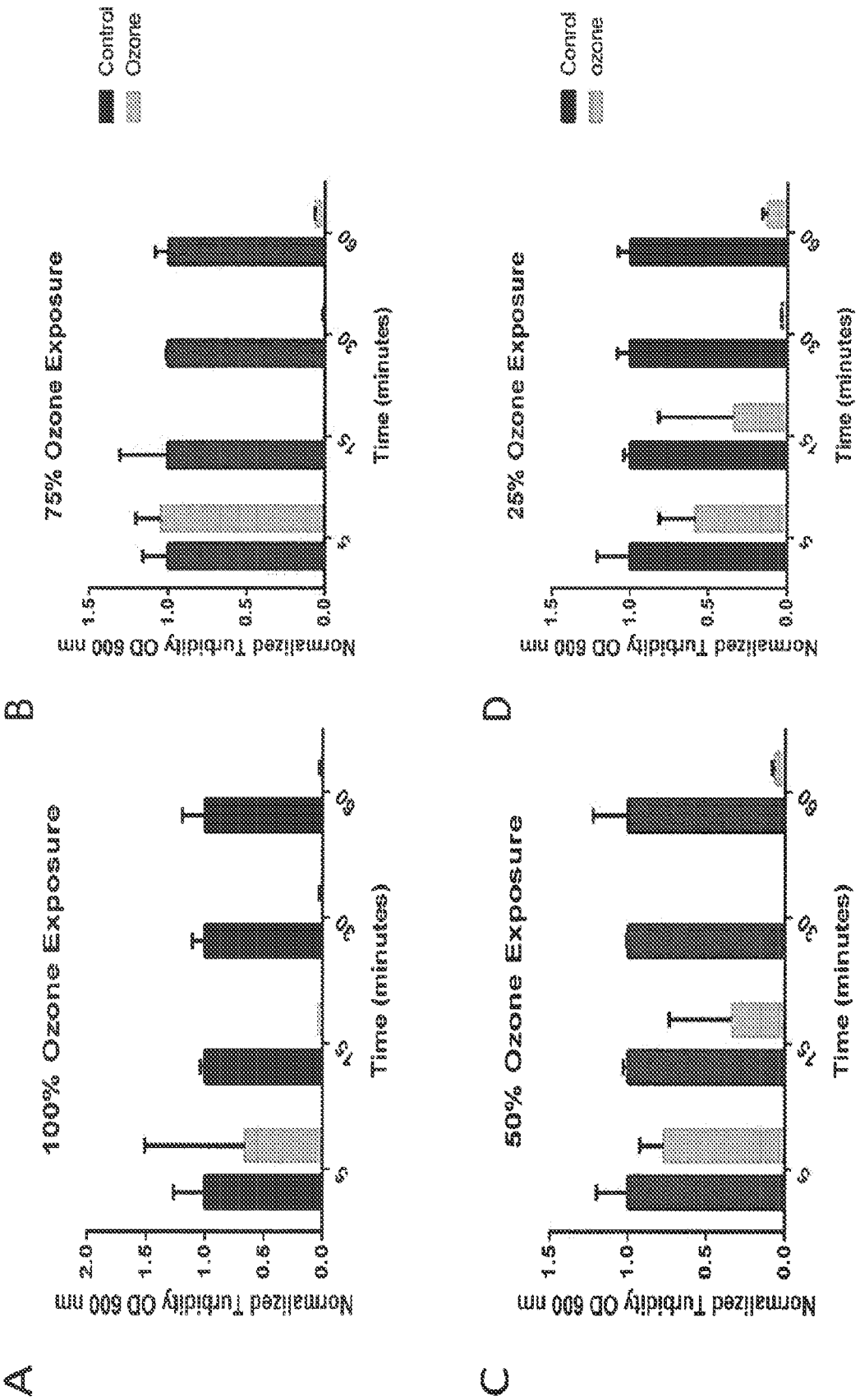
FIG 7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Time (minutes)
B
75% Ozone Exposure
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Normalized Turbidity OD 600 nm
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Normalized Turbidity OD 600 nm
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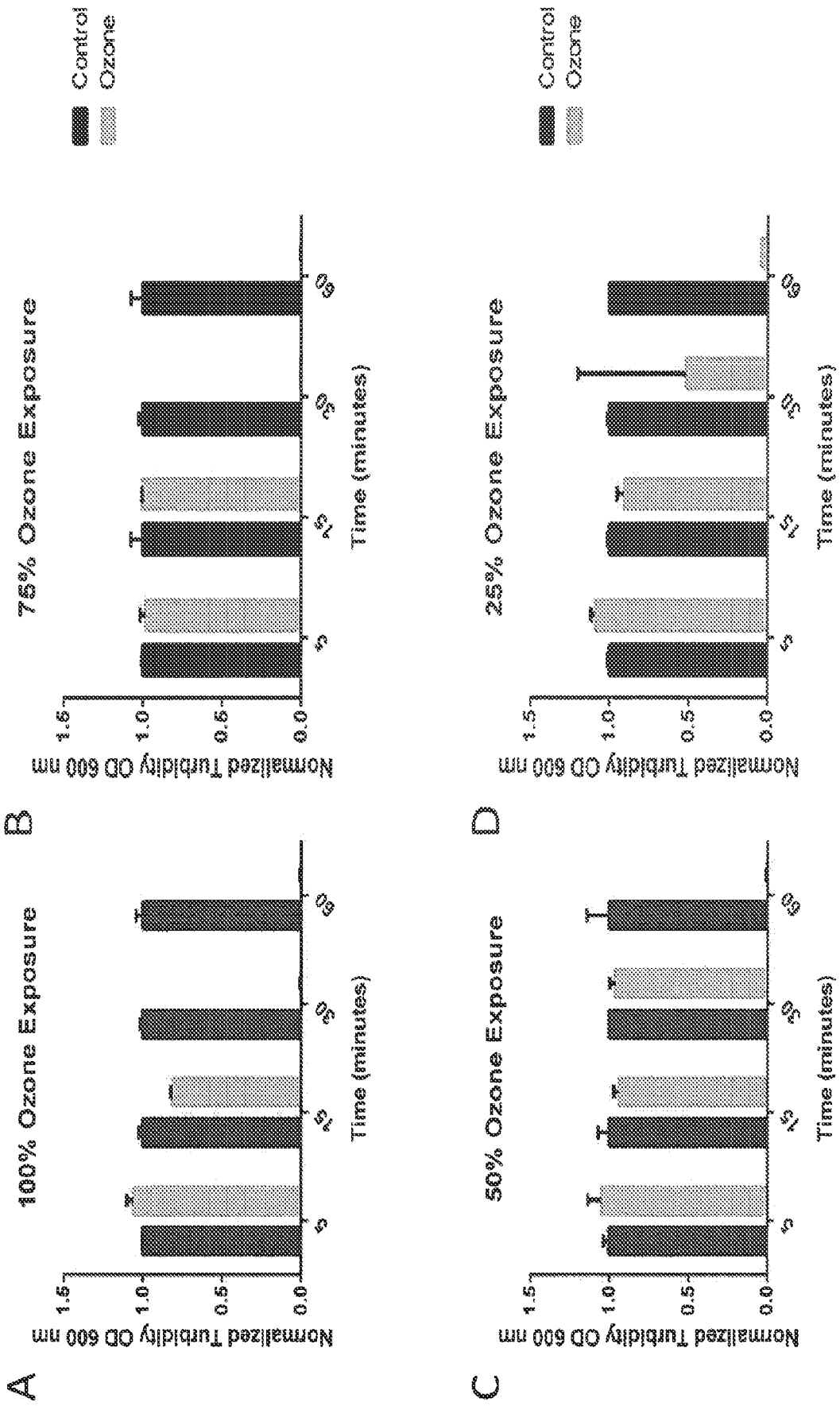
FIG 8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Time (minutes)
B
75% Ozone Exposure
Control
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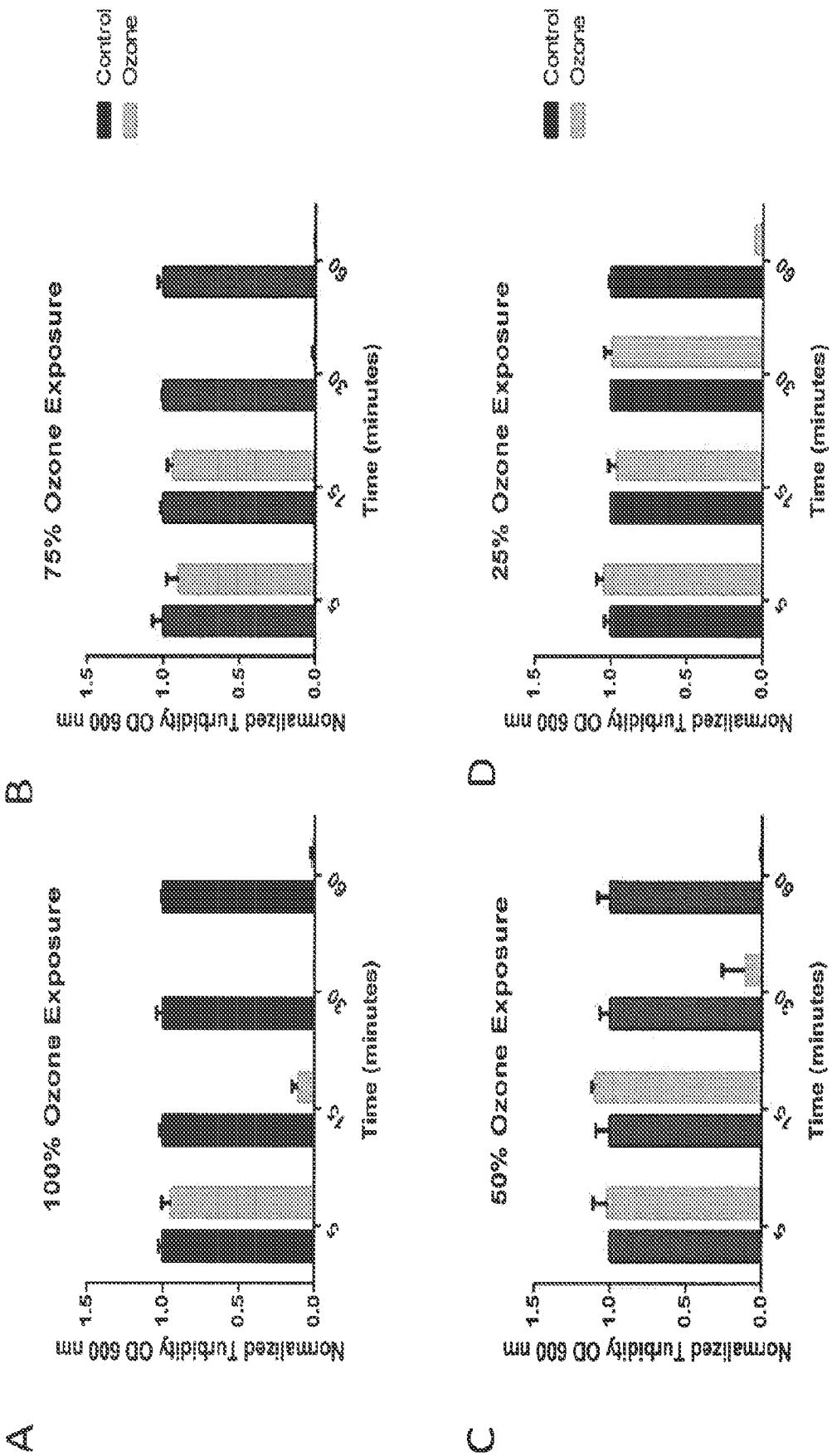
C
50% Ozone Exposure
D
25% Ozone Exposure

FIG 9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Time (minutes)
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C
50% Ozone Exposure
D
25% Ozone Exposure

FIG 10

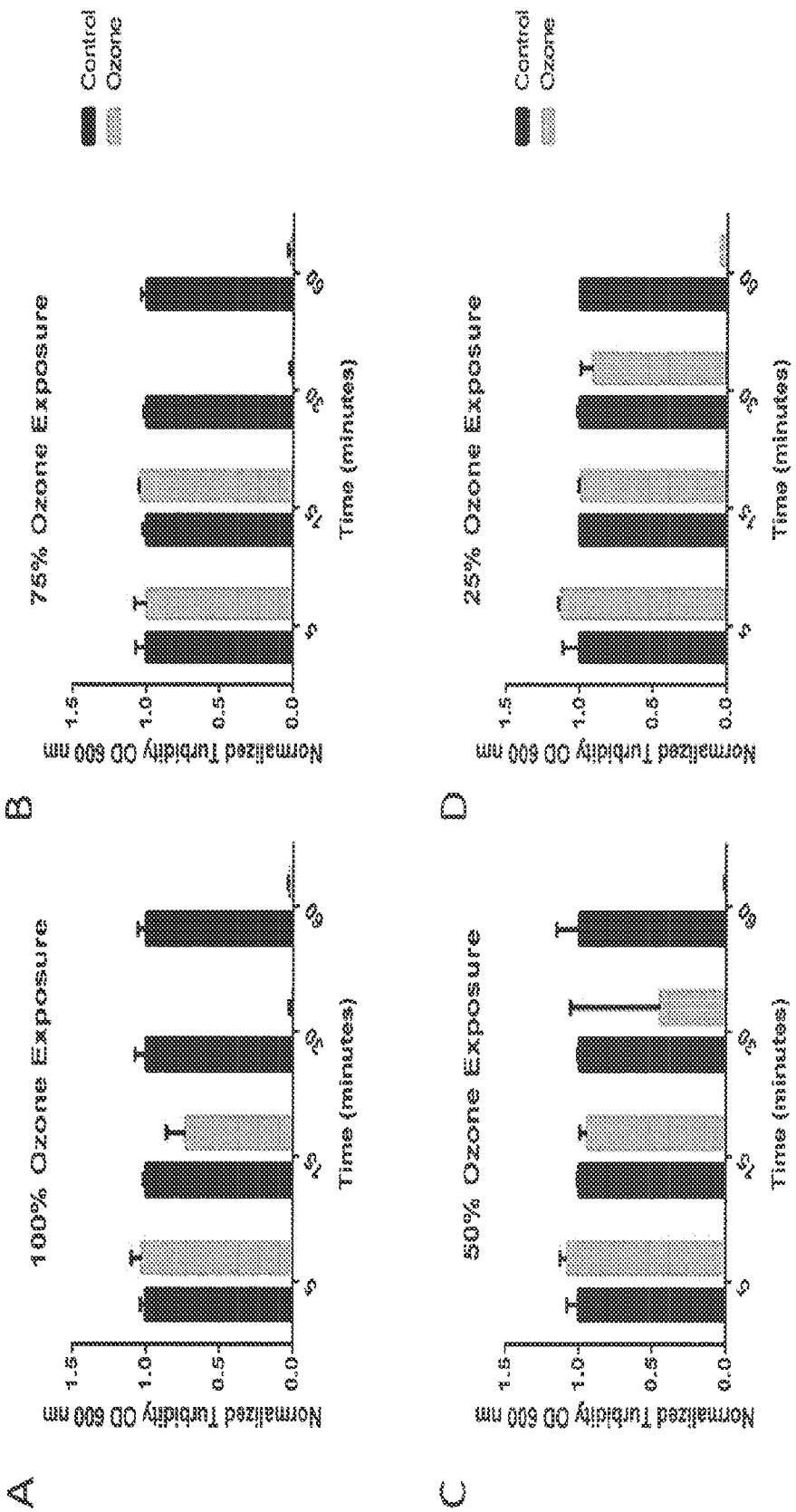
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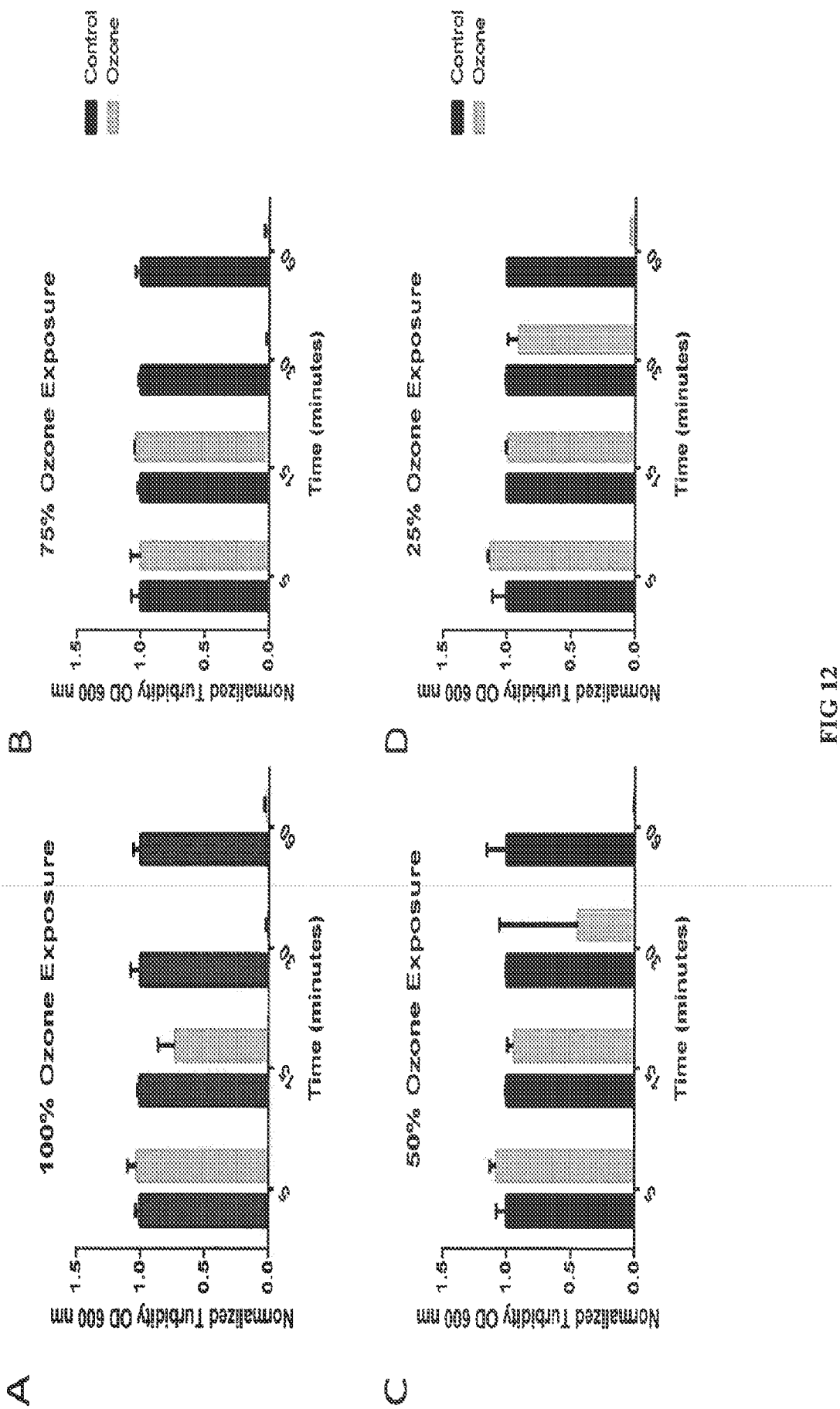
FIG 11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Normalized Turbidity OD 600 nm
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Time (minutes)
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75% Ozone Exposure
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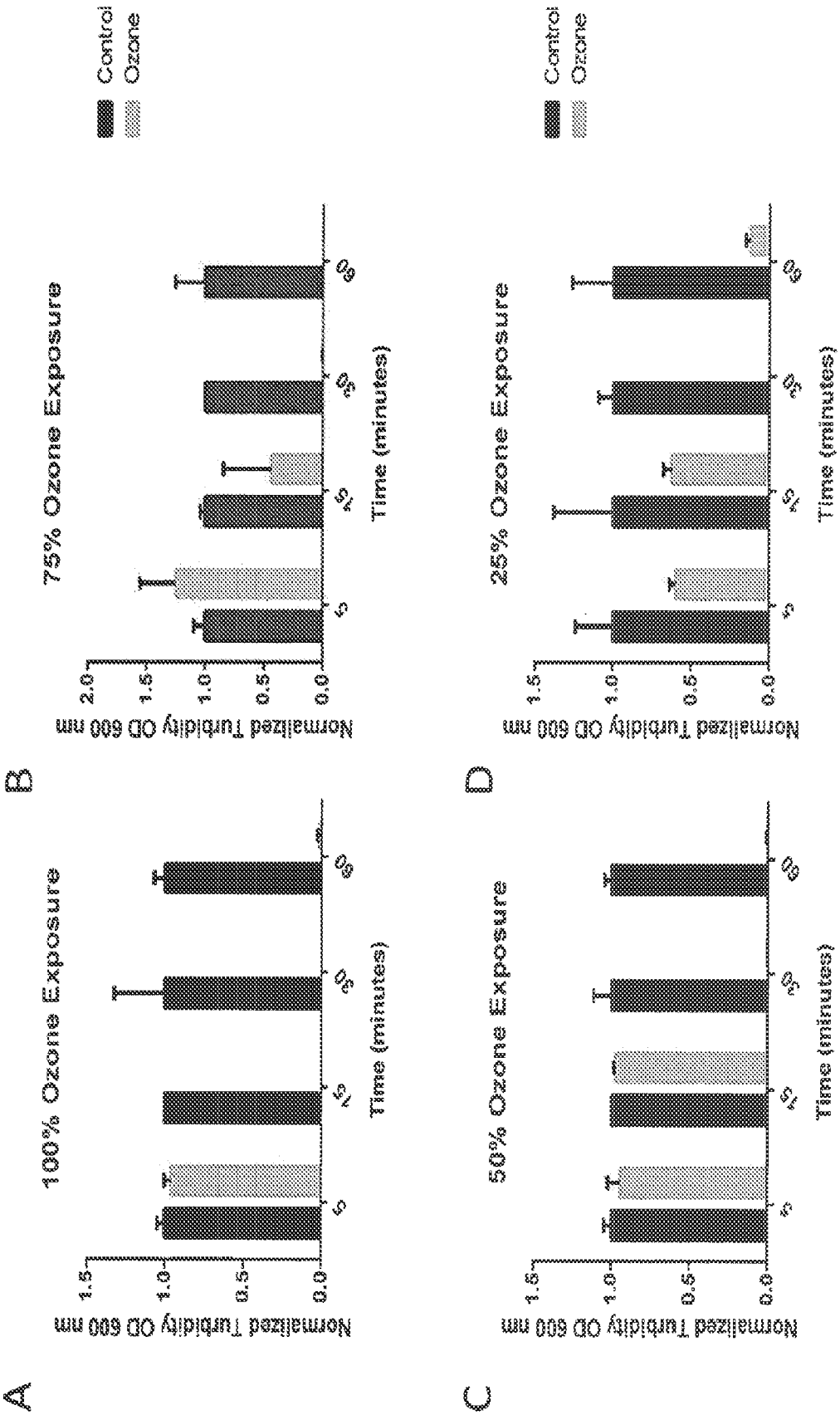
C
50% Ozone Exposure
D
25% Ozone Exposure

FIG 12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Time (minutes)
B
75% Ozone Exposure
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Normalized Turbidity OD 600 nm
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Time (minutes)
C
50% Ozone Exposure
Normalized Turbidity OD 600 nm
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Time (minutes)
D
25% Ozone Exposure
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Ozone
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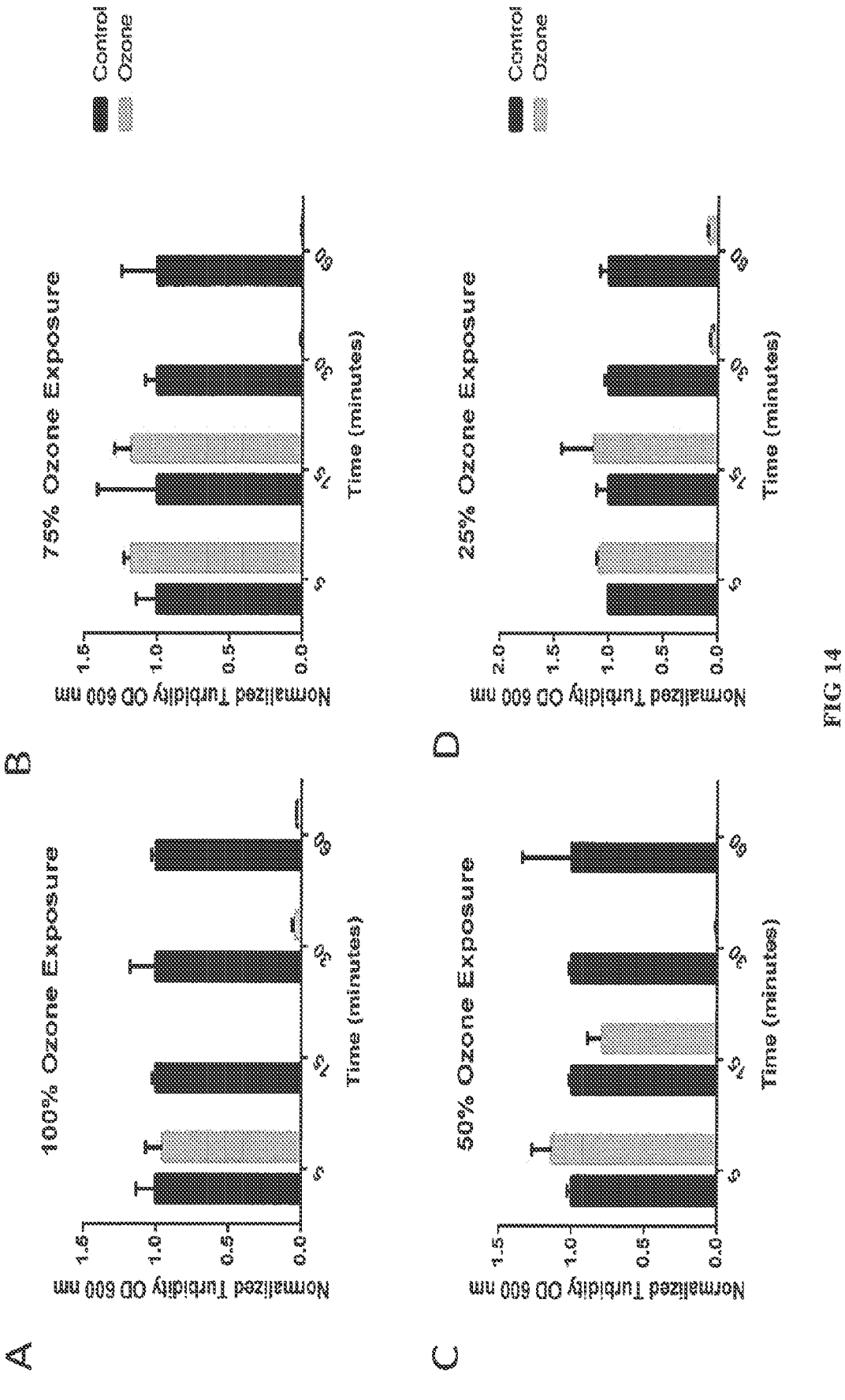
Time (minutes)

FIG 13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25% Ozone Exposure
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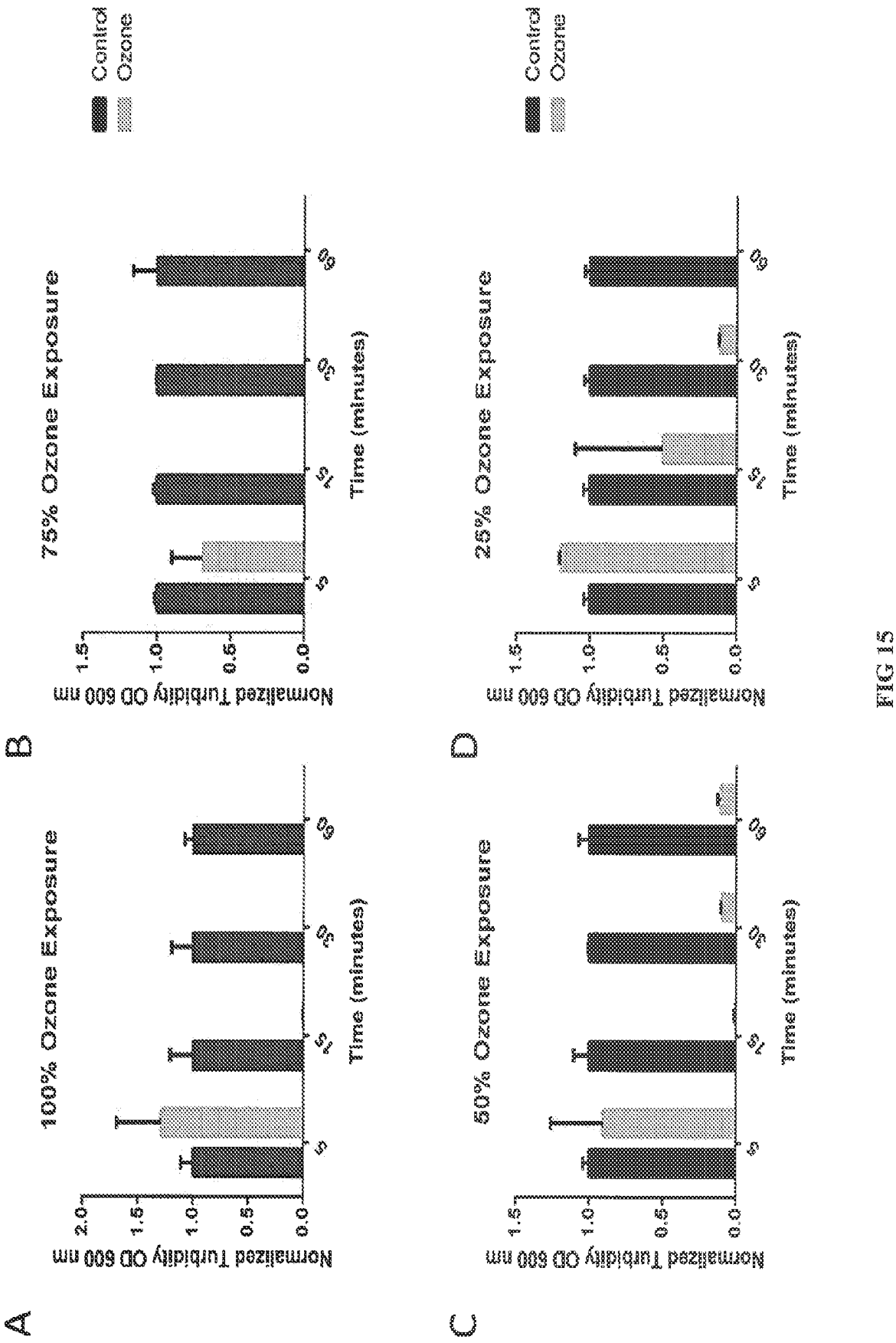
Time (minutes)

FIG 14


A
100% Ozone Exposure
Normalized Turbidity OD 600 nm
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Time (minutes)
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75% Ozone Exposure
Control
Ozone
Normalized Turbidity OD 600 nm
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Time (minutes)
C
50% Ozone Exposure
Normalized Turbidity OD 600 nm
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Time (minutes)
D
25% Ozone Exposure
Control
Ozone
Normalized Turbidity OD 600 nm
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Time (minutes)

FIG 15

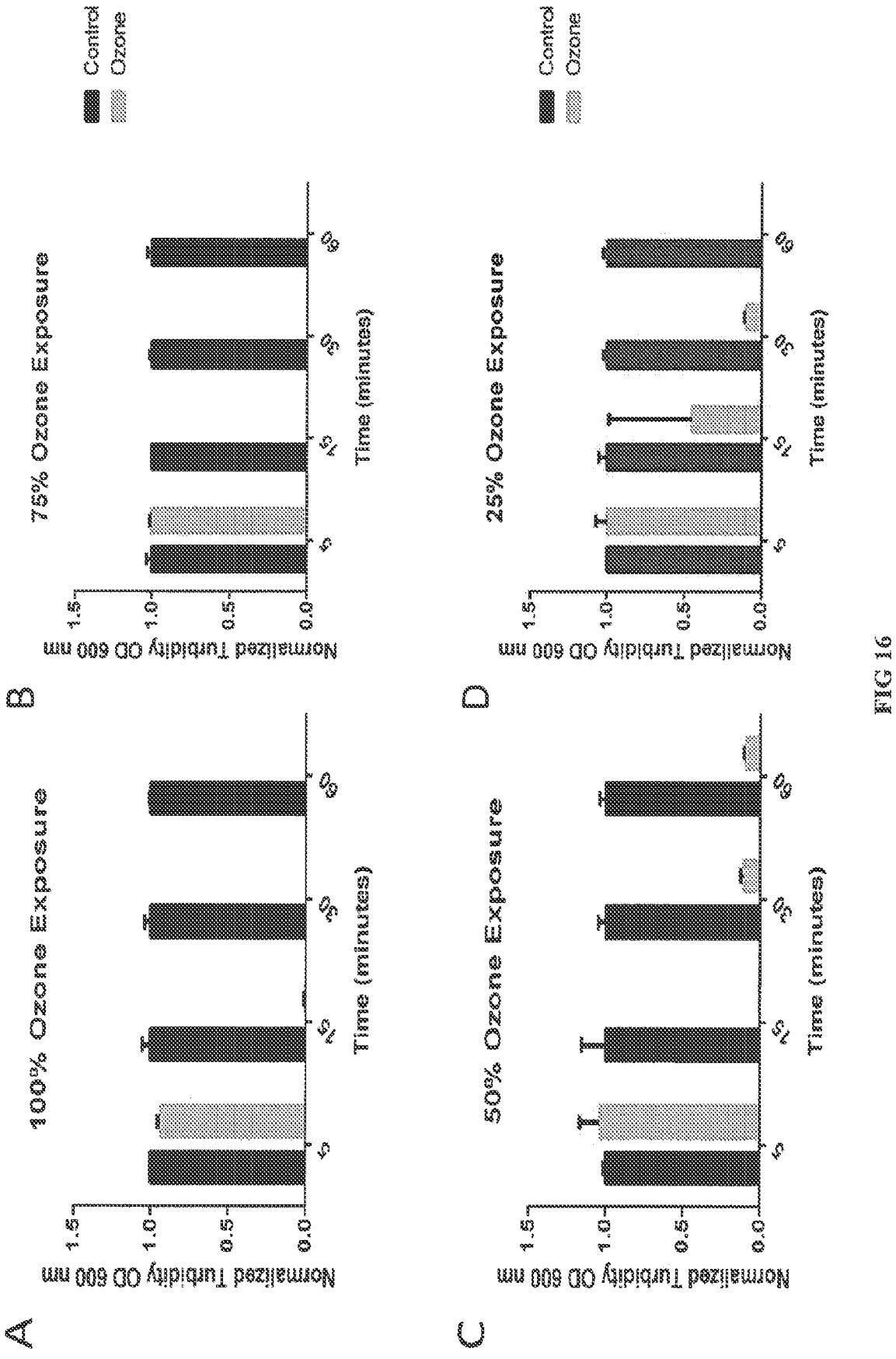

A
100% Ozone Exposure
Normalized Turbidity OD 600 nm
1.5
1.0
0.5
0.0
5
15
30
60
Time (minutes)
B
75% Ozone Exposure
Control
Ozone
C
50% Ozone Exposure
D
25% Ozone Exposure

FIG 16

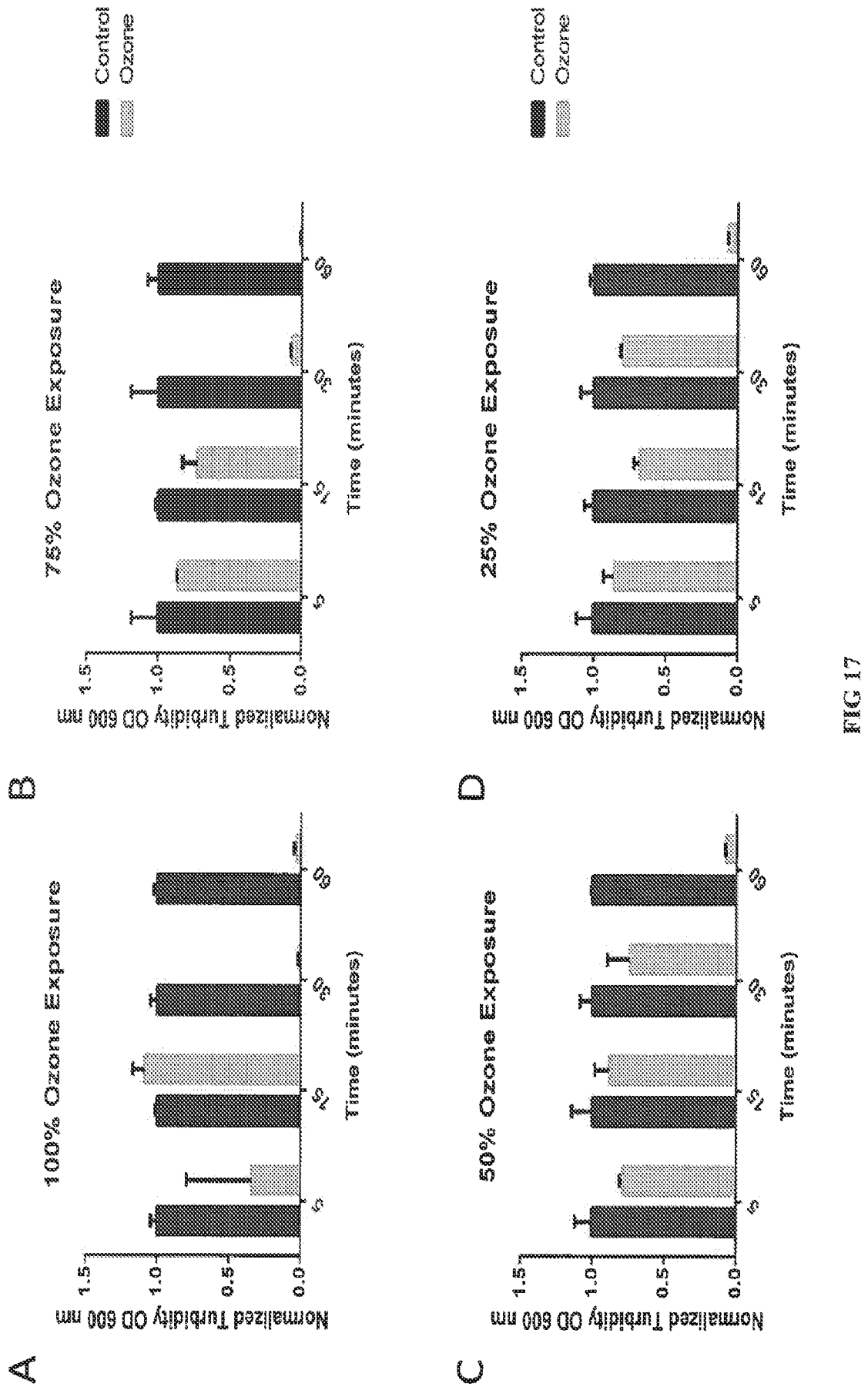

A
100% Ozone Exposure
Normalized Turbidity OD 600 nm
1.5
1.0
0.5
0.0
5
15
30
60
Time (minutes)
B
75% Ozone Exposure
Control
Ozone
C
50% Ozone Exposure
D
25% Ozone Exposure

FIG 17

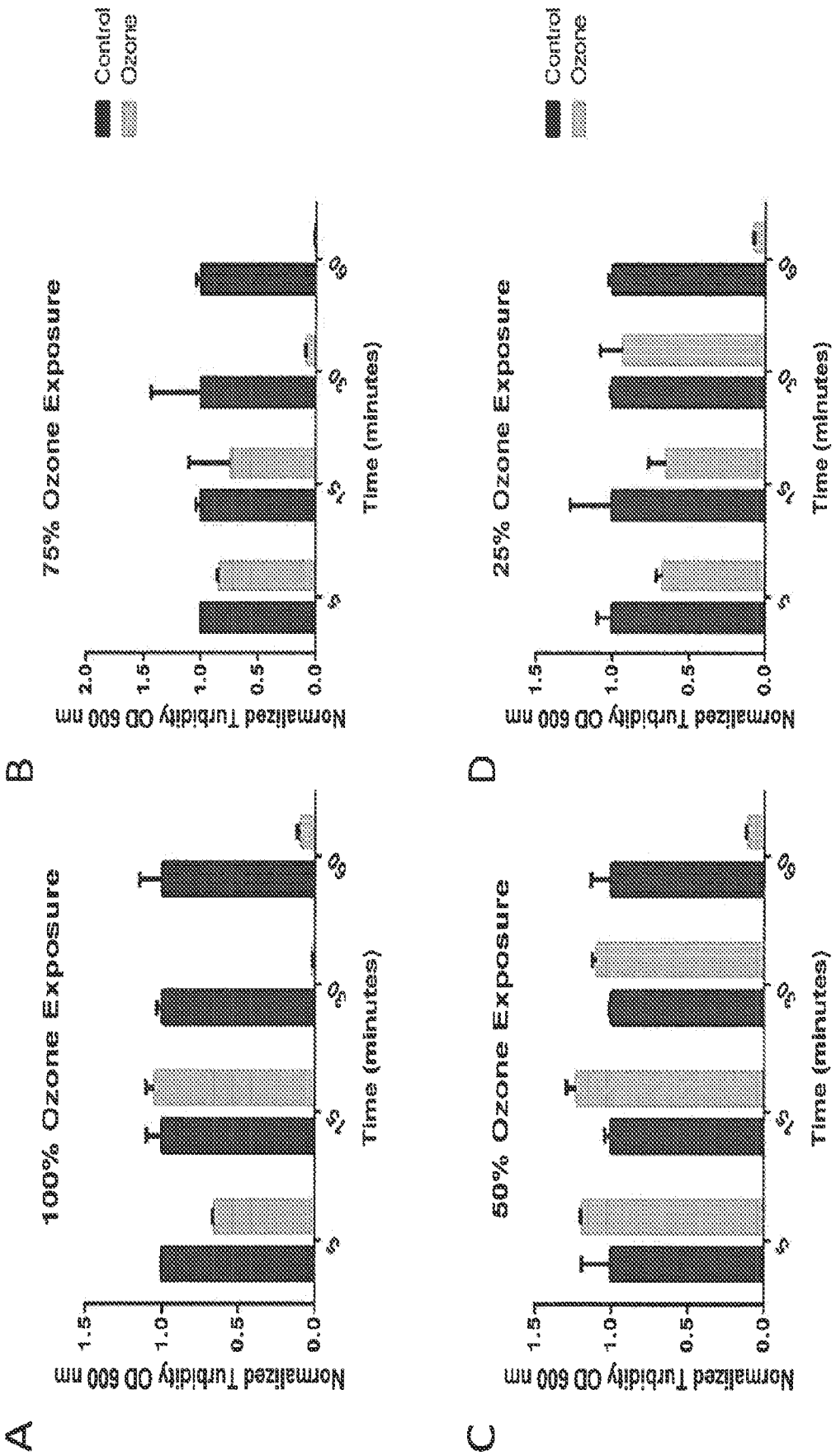

A
100% Ozone Exposure
Normalized Turbidity OD 600 nm
1.5
1.0
0.5
0.0
5
15
30
60
Time (minutes)
B
75% Ozone Exposure
Normalized Turbidity OD 600 nm
2.0
1.5
1.0
0.5
0.0
5
15
30
60
Time (minutes)
Control
Ozone
C
50% Ozone Exposure
Normalized Turbidity OD 600 nm
1.5
1.0
0.5
0.0
5
15
30
60
Time (minutes)
D
25% Ozone Exposure
Normalized Turbidity OD 600 nm
1.5
1.0
0.5
0.0
5
15
30
60
Time (minutes)
Control
Ozone

FIG 18

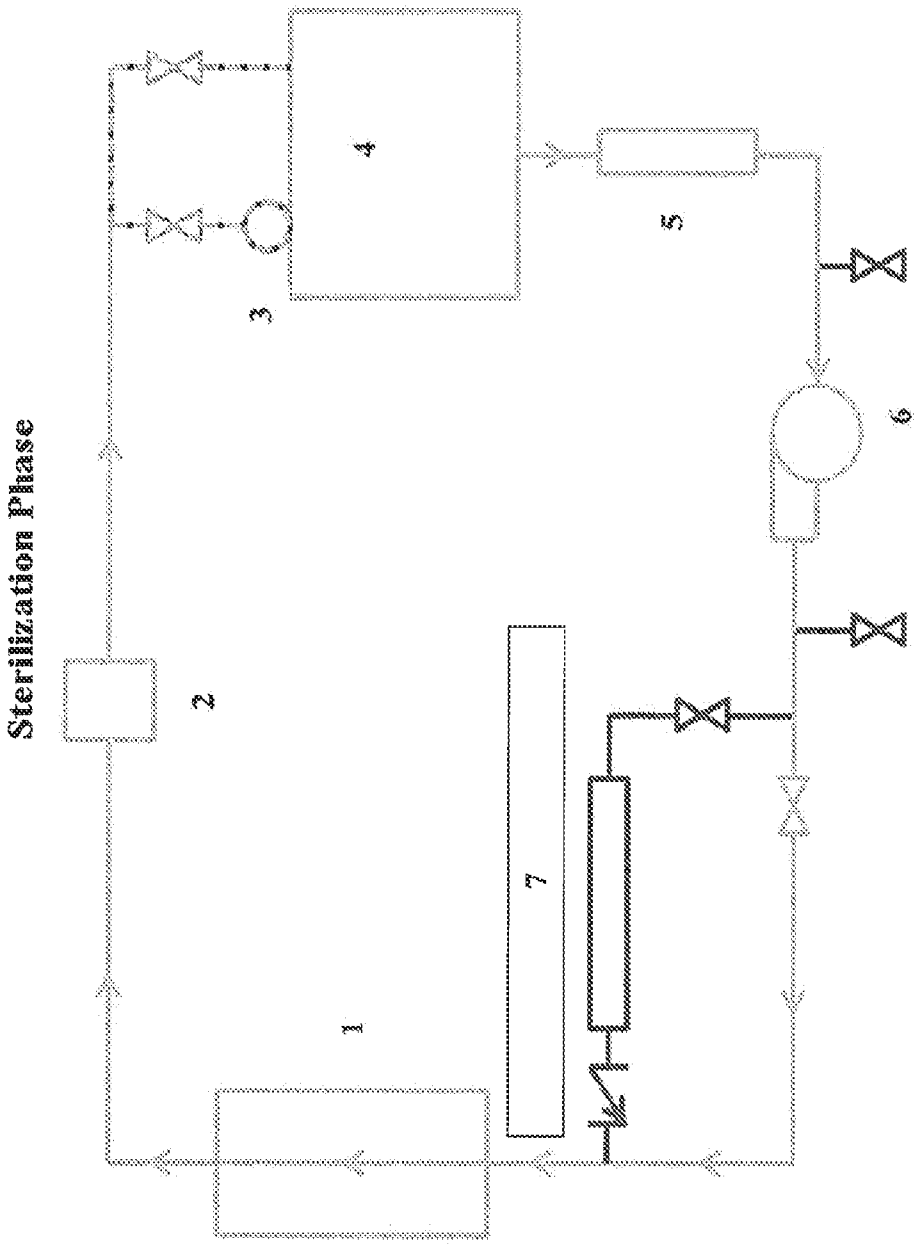


FIG 19

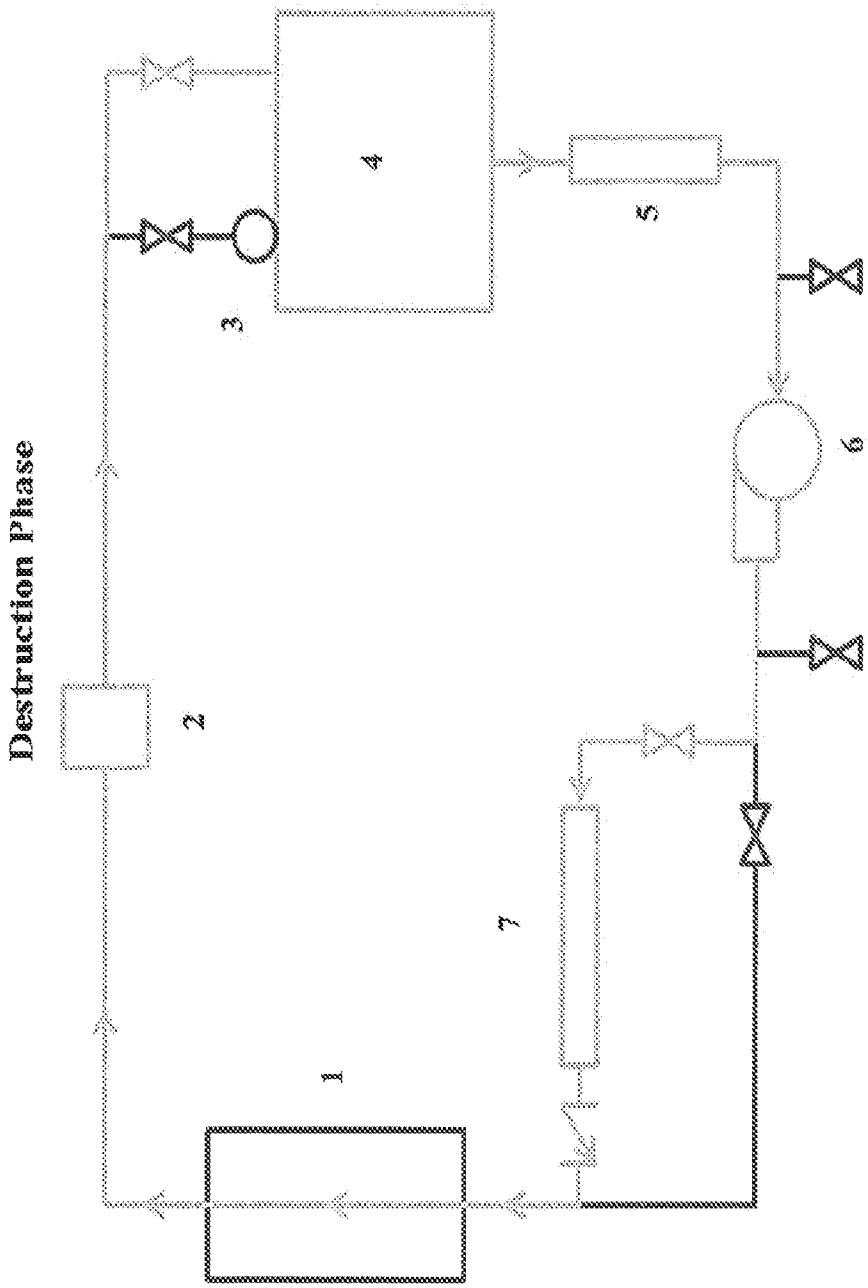


FIG 20

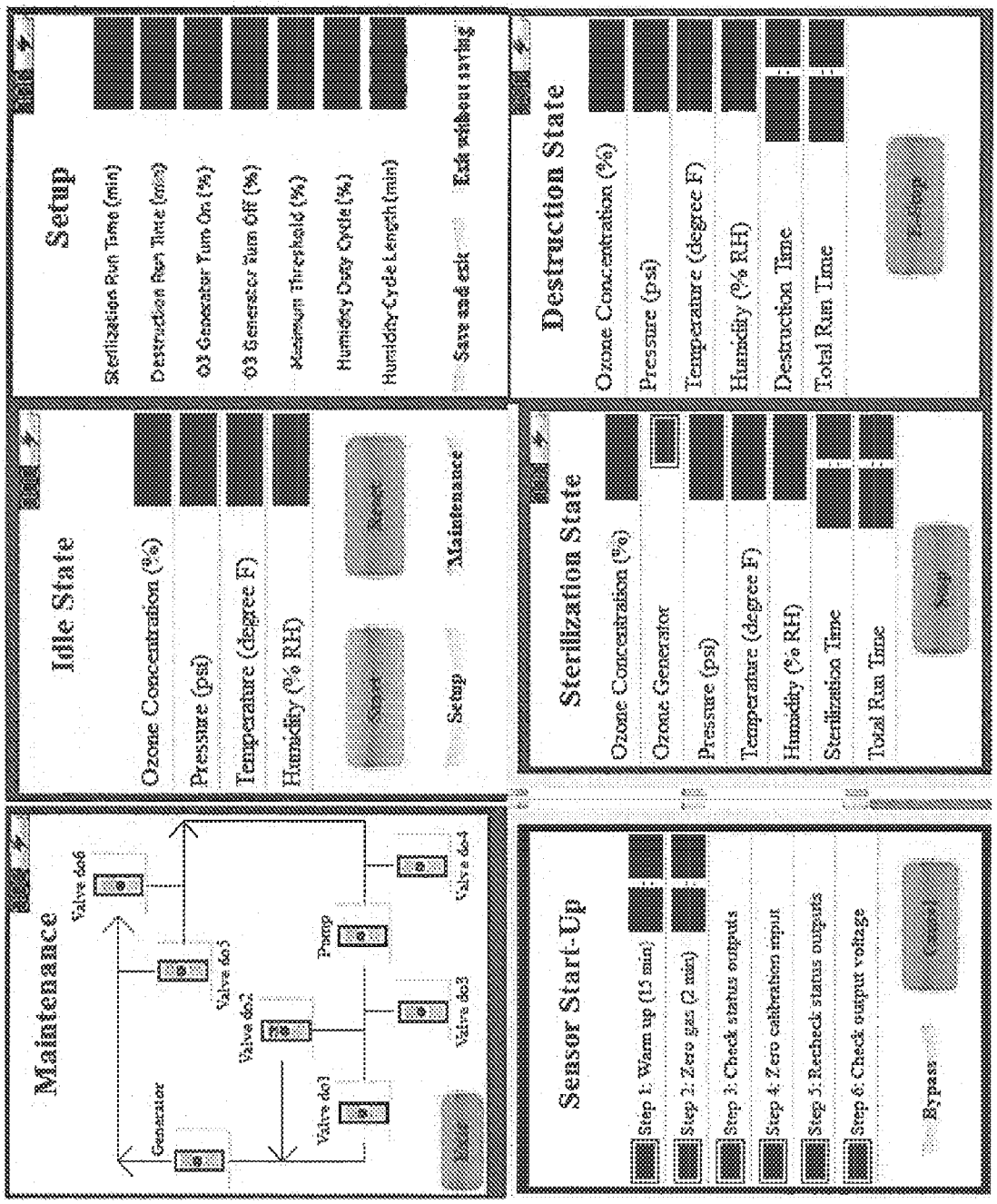

Maintenance
Valve do6
Valve do5
Valve do2
Valve do1
Pump
Valve do3
Valve do4
Generator
Idle State
Ozone Concentration (%)
Pressure (psi)
Temperature (degree F)
Humidity (% RH)
Setup
Maintenance
Setup
Sterilization Run Time (min)
Destruction Run Time (min)
O3 Generator Turn On (%)
O3 Generator Turn Off (%)
Maximum Threshold (%)
Humidity Duty Cycle (%)
Humidity Cycle Length (min)
Save and exit
Exit without saving
Sterilization State
Ozone Concentration (%)
Ozone Generator
Pressure (psi)
Temperature (degree F)
Humidity (% RH)
Sterilization Time
Total Run Time
Destruction State
Ozone Concentration (%)
Pressure (psi)
Temperature (degree F)
Humidity (% RH)
Destruction Time
Total Run Time
Sensor Start-Up
Step 1: Warm up (15 min)
Step 2: Zero gas (2 min)
Step 3: Check status outputs
Step 4: Zero calibration input
Step 5: Recheck status outputs
Step 6: Check output voltage
Bypass

FIG 21

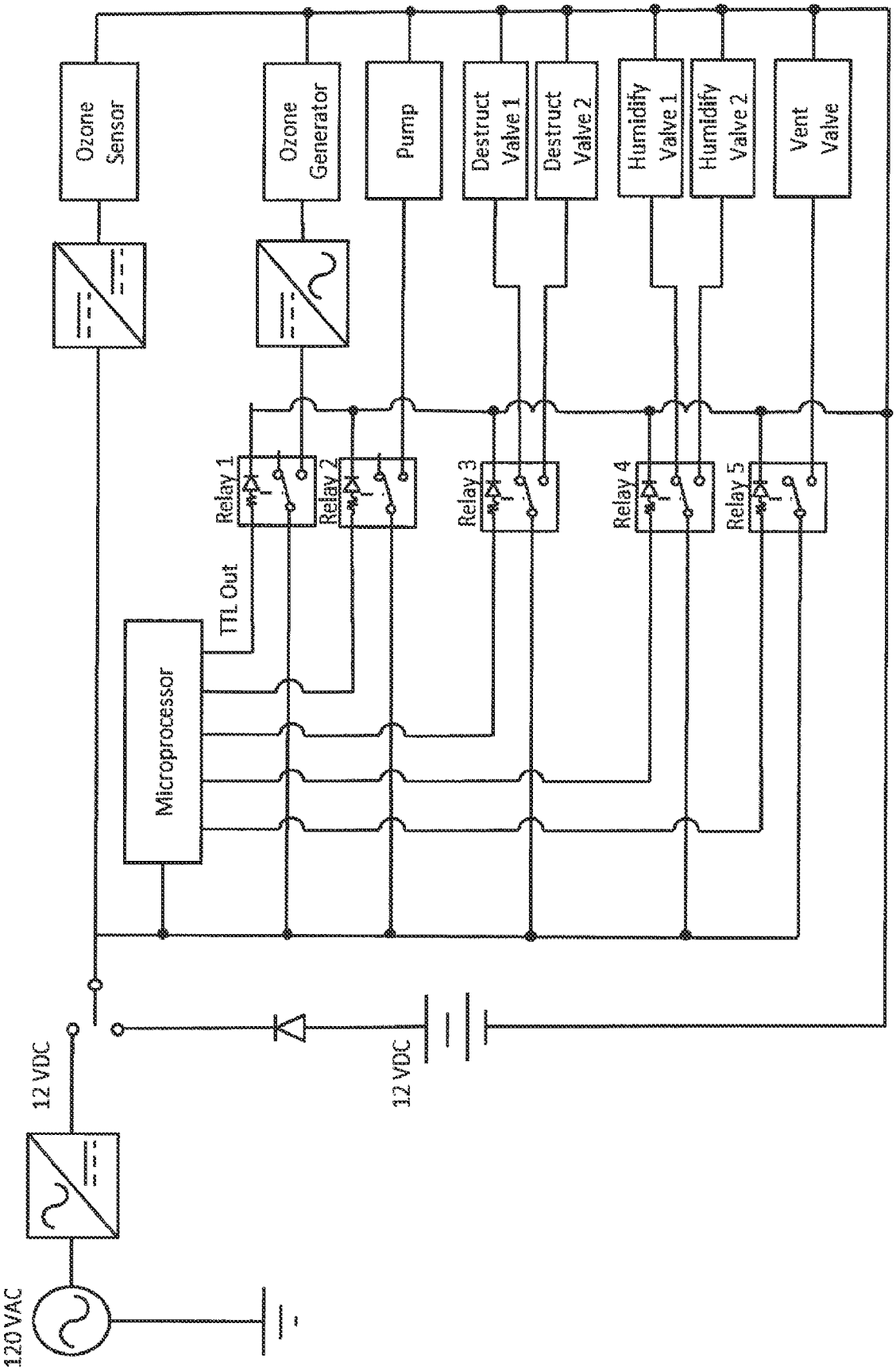

Ozone Sensor
Ozone Generator
Pump
Destruct Valve 1
Destruct Valve 2
Humidify Valve 1
Humidify Valve 2
Vent Valve
Relay 1
Relay 2
Relay 3
Relay 4
Relay 5
TTL Out
Microprocessor
12 VDC
12 VDC
120 VAC

FIG 22

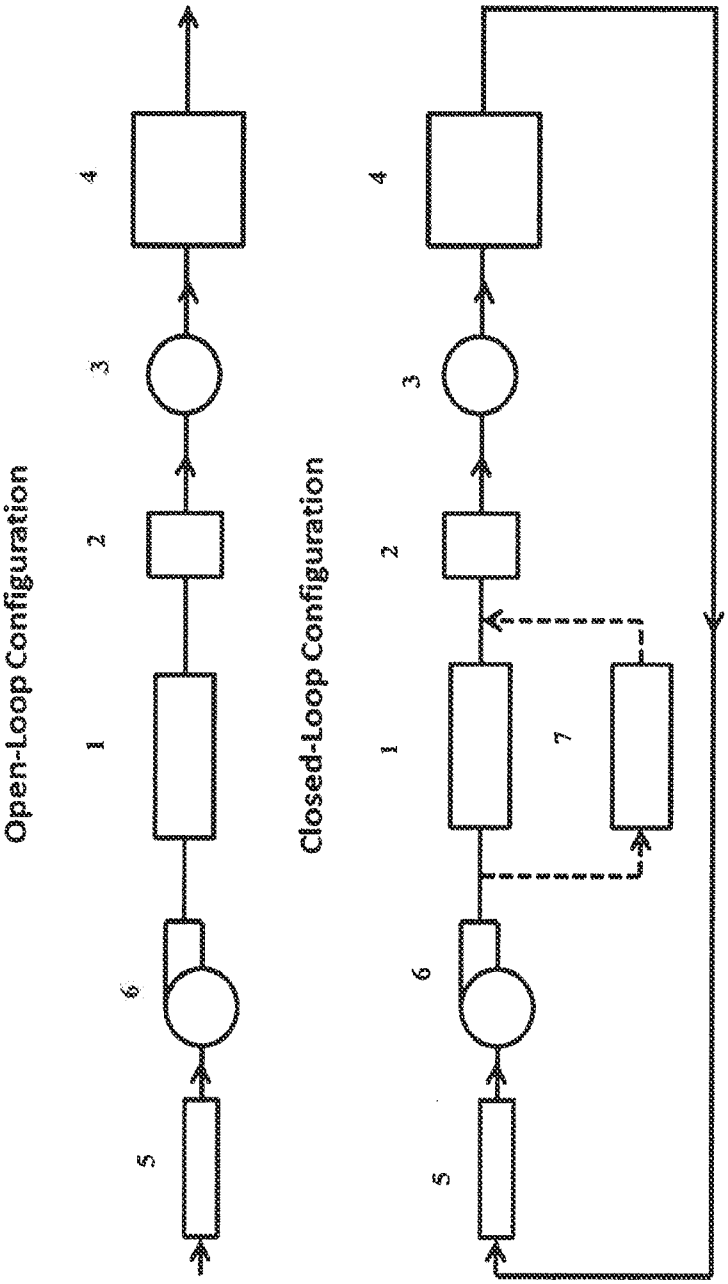

Open-Loop Configuration
1
2
3
4
5
6
Closed-Loop Configuration
1
2
3
4
5
6
7

FIG 23

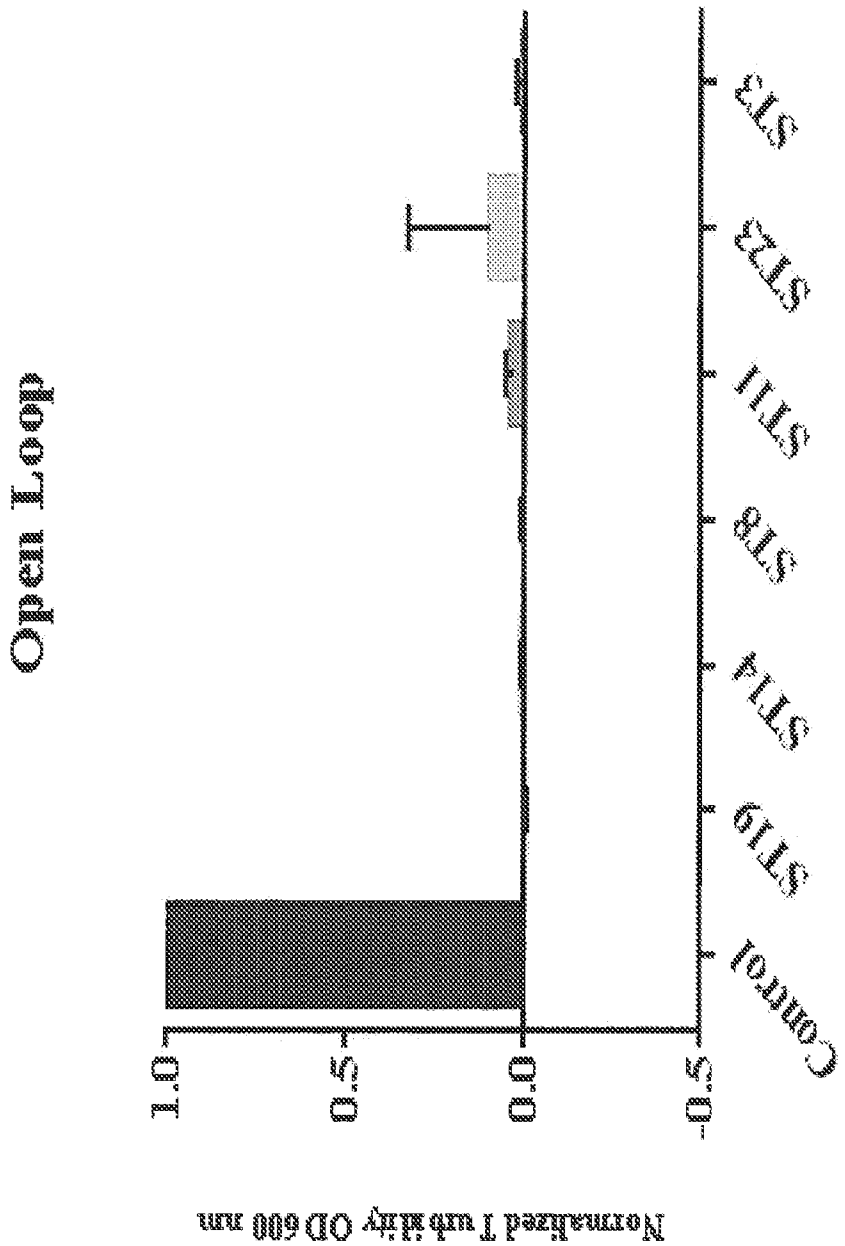
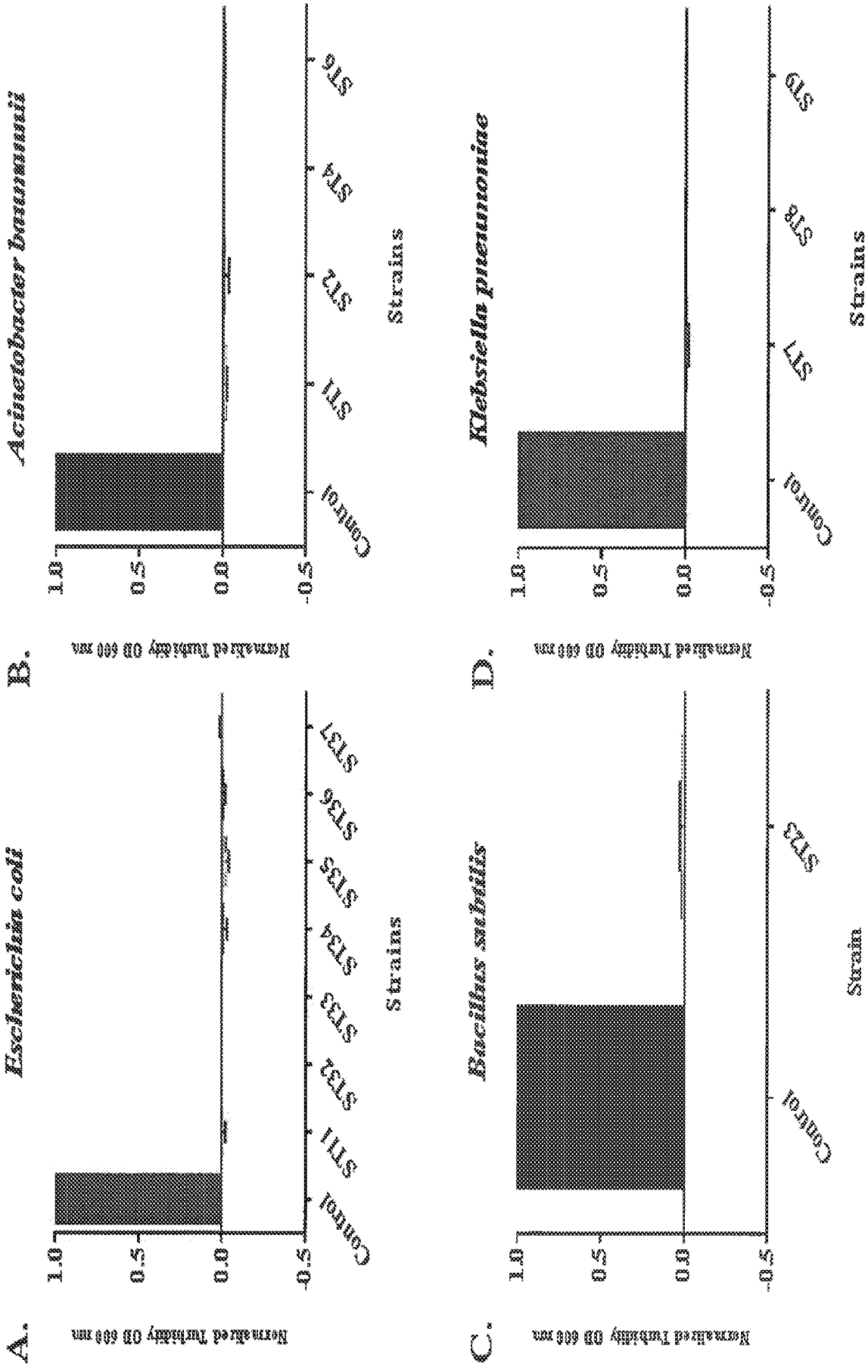

Open Loop
Normalized Turbidity OD 600 nm
1.0
0.5
0.0
-0.5
Control
ST19
ST14
ST8
ST11
ST12
ST3
Strains

FIG 24


A.
Escherichia coli
Normalized Turbidity OD 600 nm
1.0
0.5
0.0
-0.5
Control
ST11
ST32
ST33
ST34
ST35
ST36
ST37
Strains
B.
Acinetobacter baumannii
Normalized Turbidity OD 600 nm
1.0
0.5
0.0
-0.5
Control
ST1
ST2
ST4
ST6
Strains
C.
Bacillus subtilis
Normalized Turbidity OD 600 nm
1.0
0.5
0.0
-0.5
Control
ST23
Strain
D.
Klebsiella pneumoniae
Normalized Turbidity OD 600 nm
1.0
0.5
0.0
-0.5
Control
ST1
ST8
ST9
Strains

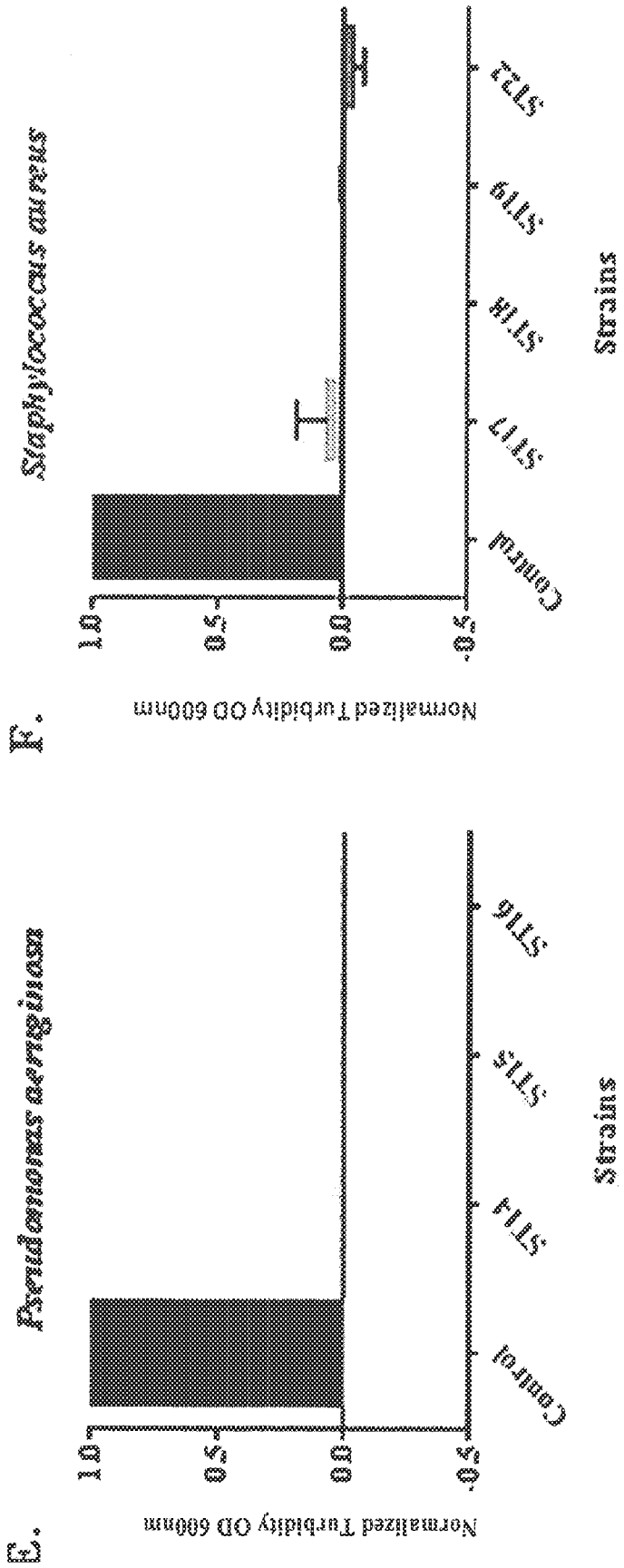

E.
Pseudomonas aeruginosa
Normalized Turbidity OD 600nm
1.0
0.5
0.0
-0.5
Control
ST14
ST15
ST16
Strains
F.
Staphylococcus aureus
Normalized Turbidity OD 600nm
1.0
0.5
0.0
-0.5
Control
ST17
ST18
ST19
ST21
Strains

FIG 25

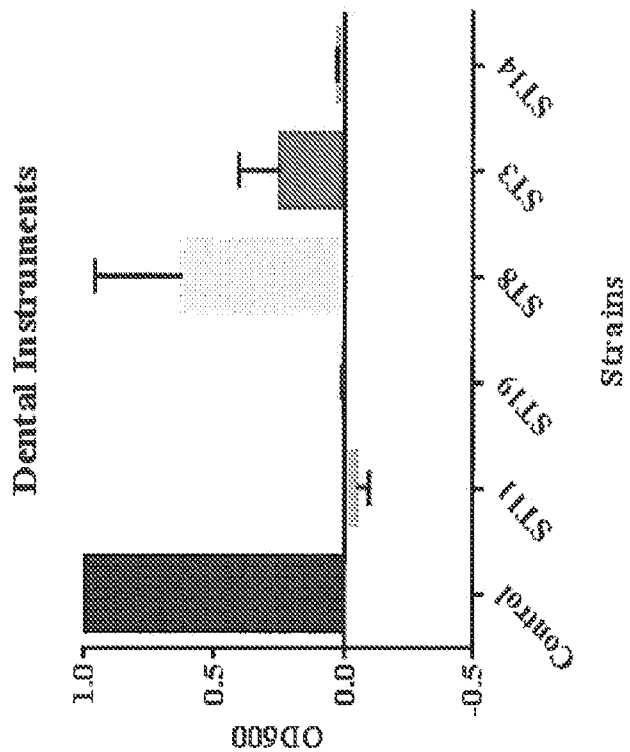

Dental Instruments
OD600
1.0
0.5
0.0
-0.5
Control
ST11
ST19
ST6
ST5
ST3
ST14
Strains

FIG 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/045434

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61L 2/20 (2015.01)
CPC - A61L 2/202 (2015.10)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61L 2/16, 2/20, 2/24 (2015.01)
CPC - A61L 2/202, 2202/16; C02F 1/78 (2015.10)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 422/28, 292; 250/455.11 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Orbit, Google Patents, Google Scholar, Google.
Search terms used: ozone, trioxide, o3, humidifier, moisture, dehumidifier, dry, dehydrate, condensate, seal, air proof, air tight, low temperature, catalyst

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/0105783 A1 (HANTOVER, INC) 17 April 2014 (17.04.2014) entire document	1-15
Y	US 7,976,791 B2 (DOONA et al) 12 July 2011 (12.07.2011) entire document	1-15
Y	WO 01/78793 A1 (PURIZER CORPORATION) 25 October 2001 (25.10.2001) entire document	1-15
Y	US 8,354,057 B2 (HESELTON et al) 15 January 2013 (15.01.2013) entire document	2, 5-7, 13-15
Y	US 2013/0243649 A1 (TSO3 INC) 19 September 2013 (19.09.2013) entire document	5, 15
Y	US 5,221,520 A (CORNWELL) 22 June 1993 (22.06.1993) entire document	9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 2015	12 NOV 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774