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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Development of an online biosensor for in situ monitoring of chlorine dioxide gas disinfection efficacy

Maria del Busto-Ramos · Michael Budzik · Carlos Corvalan · Mark Morgan · Ronald Turco · David Nivens · Bruce Applegate

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Abstract A prototype bioluminescence-based biosensor was designed and constructed to evaluate the antimicrobial efficacy of chlorine dioxide (ClO_2) gas under various treatment conditions. The biosensor consisted of a bioluminescent bioreporter (*Pseudomonas fluorescens* 5RL), an optical transducer (photomultiplier tube), and a light-tight chamber housing, the bioreporter and the transducer. The bioluminescent recombinant *P. fluorescens* 5RL in the biosensor allowed for online monitoring of bioluminescence during ClO_2 gas disinfection. Experiments were performed to evaluate the effects of the two key physical parameters associated with ClO_2 disinfection: relative humidity (40, 60, 80%) and ClO_2 gas concentration (0.5, 1.0, 1.6, 2.1 mg/l) on the bioreporter. Results showed that increasing concentrations of ClO_2 gas corresponded to a faster decrease in luminescence. The rates of luminescence decrease from *P. fluorescens* 5RL, and the log reduction time (LRT, time required to obtain 1-log reduction in luminescence) were calculated for each treatment tested. The LRT values of luminescence were 103, 78, 53, and 35 s for 0.5, 1.0, 1.6, and 2.1 mg/l of ClO_2 gas treatment, respectively, at 78% relative humidity. The gas concentration which caused a tenfold change in LRT (z value) for luminescence of *P. fluorescens* 5RL was 3.4 mg/l of ClO_2. The prototype biosensor showed potential for many applications, such as monitoring real-time microbial inactivation and understanding parameters that influence the efficacy of gaseous decontamination procedures.

Keywords Chlorine dioxide gas · Disinfection efficacy · Online sensor

Introduction

Chlorine dioxide (ClO_2) is a strong oxidizing agent and a highly effective sanitizer for killing vegetative cells and spores of pathogenic and spoilage microorganisms (Beuchat 1998; Beuchat et al. 2004; Han et al. 2003). Aqueous ClO_2 is used for the disinfection of drinking water, processing equipment, and plant sanitation. Over the last two decades, new applications using ClO_2 gas as a disinfectant and sanitizer have been developed. ClO_2 gas is very effective for disinfection of fruits and vegetables (Du et al. 2003; Han et al. 2001a, b), plant equipment sanitation (Han et al. 1999), medical devices and surface sterilization. For example, ClO_2 gas was successfully used to decontaminate the Hart Senate Office building which was contaminated with *Bacillus anthracis* spores in an act of bioterrorism. This was the first time that chlorine dioxide gas was used to decontaminate a large area. However, many challenges were encountered when evaluating the performance and disinfection efficacy due to ClO_2 gas having a rate of spontaneous decay that is influenced by temperature, relative humidity, and light intensity. It was also a challenge to assure that adequate levels of chlorine dioxide reached all areas of the facility that needed to be decontaminated (Betz 2001).

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M. del Busto-Ramos · M. Budzik · C. Corvalan · M. Morgan · D. Nivens · B. Applegate (✉)
Department of Food Science, Purdue University,
745 Agriculture Mall Dr.,
West Lafayette, IN 47907–2009, USA
e-mail: applegate@purdue.edu

R. Turco
Department of Agronomy, Purdue University,
West Lafayette, IN 47907–2009, USA

Traditionally, the effectiveness of a bacterial inactivation process is often evaluated by enumerating surviving organisms (biological indicators or surrogates; US Food and Drug Administration 2000) in colony forming units (CFU) with plate count methodology. Biological indicators containing live Bacillus spores are used to evaluate the effectiveness of sterilization processes. Most of the published studies regarding the biocidal efficacy of chlorine dioxide and other types of sanitizers are reported based on bacterial log reduction during treatment. Based on the Association of Official Agricultural Chemists definition, an efficacious disinfectant is one that can bring about a 5-log reduction of viable organisms within 30 s. However, the current methodology to evaluate the biocidal activity of chemical sanitizers on bacteria or biofilms attached to surfaces is laborious, time-consuming, difficult to standardize, and not amenable to in situ monitoring (Bloomfield et al. 1994). Therefore, alternative methods to monitor the efficacy of disinfectants are needed.

Bioluminescence is an effective alternative method for real-time monitoring of microbial viability. Numerous studies have used bioluminescence to monitor the response to inimical processes. As reported by Duffy et al. (1995), bioluminescence was used to model thermal inactivation of *Salmonella typhimurium*. Another study by Wiles et al. (2005) using bioluminescent reporters evaluated their luminescence response, or decay, when exposed to hydrogen peroxide and heat shock. In a study by Ciarciaglini et al. (2000), *Bacillus subtilis* spores were monitored for bioluminescence upon germination to determine the effect of preservative treatments (such as reduction of pH, use of lactic acid, and pasteurization). In addition, a fluoroluminometric device was used to monitor cell viability and killing of *Escherichia coli* by measuring fluorescence and luminescence on a real-time basis when exposed to increasing ethanol concentrations (Lehtinen et al. 2003). These are examples of how bioluminescence can be used as a tool to evaluate microbial survival after inactivation treatments. However, limited work has been reported utilizing luminescence in an online format.

The purpose of this study was to design a biosensor using bioluminescent bacteria as a potential surrogate to monitor, understand, and optimize chlorine dioxide gas disinfection efficacy in a pilot scale ClO_2 gas treatment process. Bioluminescence is directly related to cell physiology, as the biochemical reactions are dependant on numerous energy-requiring reactions (Heitzer et al. 1998; Meighen 1994). The recombinant bioluminescent reporter *Pseudomonas fluorescens* 5RL (King et al. 1990), which carries the *luxCDABE* genes from *Vibrio fischeri* under the control of the *sal* promoter, is capable of emitting intense levels of luminescence in the presence of salicylate. In this study, the light emitted by the bacteria was detected online using a light measuring sensor, photomultiplier tube (PMT), incorporated in a light-tight chamber and connected to a computer. The data from this biosensor can be visualized online allowing remote monitoring of the gas disinfection process. Changes in bioluminescence during ClO_2 gas exposure were obtained to evaluate the effect of relative humidity and ClO_2 gas concentration on the efficacy of inactivation.

Materials and methods

Bioluminescent sample preparation

P. fluorescens 5RL has the ability to produce high levels of luminescence when grown in the presence of salicylate (King et al. 1990). Bacteria were grown in 250-ml Erlenmeyer flasks containing 100 ml Luria–Bertani medium; 10 g sodium chloride, 10 g tryptone, 5 g yeast extract (Difco), with 14 mg/l of tetracycline and 50 mg/l of salicylate (LBtet/sal) for 48 h at 28°C on a rotatory shaker (100 rpm). A subculture was subsequently grown in LBtet/sal broth at 28°C on a rotatory shaker (100 rpm) until an optical density of OD_{600}=0.5 was obtained. One milliliter of the subculture was then serially diluted in triplicate 1:10 with 9 ml of Luria broth containing salicylate 50 mg/l (LBsal). For cell enumeration, aliquots of 100 µl from each dilution were plated on LBtet/sal plates and incubated at 28°C for 48 h. The remainder of each dilution was subsequently filtered onto a 47-mm, 0.2-µm Supor® 200 membrane filter (Pall Corporation). The membranes were placed on the top of 47 mm LBsal agar plates for bioluminescent assays. Tetracycline was omitted in these assays to eliminate potential adverse effects on the assay. *P. fluorescens* 5RL maintains the reporter plasmid without selection.

Biosensor design

The biosensor consisted of *P. fluorescens* 5RL on Supor® 200 membrane filters on LBsal agar plates and a PMT as an optical transducer. The biosensor was housed in a chamber (Fig. 1) designed to allow gas diffusion but minimize light entry. The housing was constructed using black Delrin, which is inert to ClO_2. The housing consisted of three concentric cylinders with decreasing diameters (95, 74, and 57 mm), 152 mm high with perforations in alternate positions allowing diffusion of gas inside the chamber while preventing ambient light entry. The optical transducer consisted of a Hammamatsu PMT sensor module containing an embedded microcontroller (Hammamatsu Photonics K.K., Toyooka-village, Iwata-gun, Japan) interfaced with a computer through an RS232 port for monitoring luminescence in

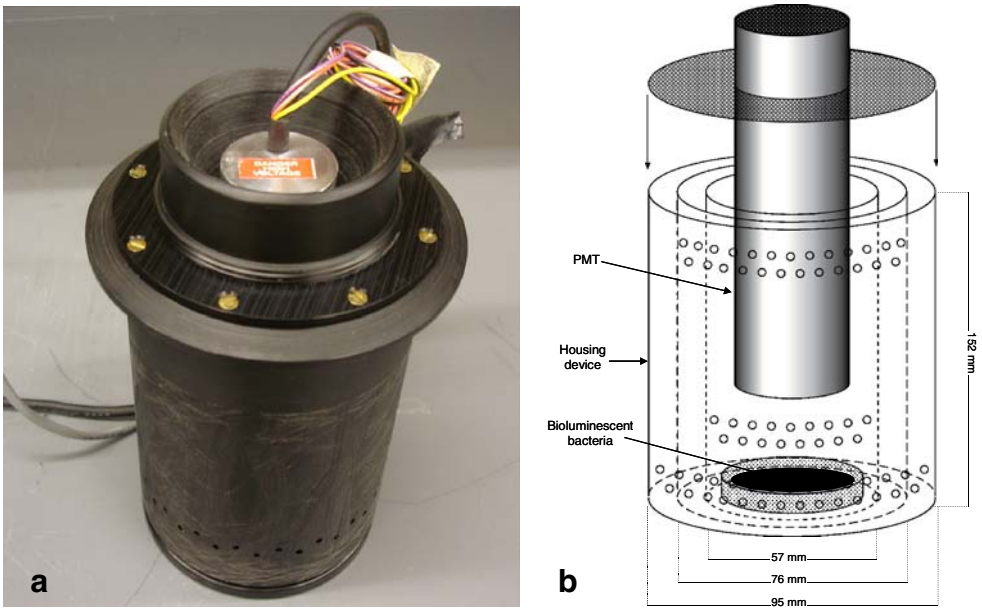

Fig. 1 **a** Biosensor device housing the photomultiplier tube (PMT); **b** schematic representation of the biosensor designed for the online monitoring of chlorine dioxide gas disinfection

real-time. The PMT acquired the luminescence signal by counting the photons as they entered the input window, which had a 21-mm diameter. Light level monitoring was done through the PMT Commander software, which was developed as a Common Gate-way Interface application to interface between a web server and the Hammamatsu PMT sensor module. The PMT Commander application controlled the PMT based on the input provided through the web interface and recorded PMT readings in comma-separated-variable format for easy analysis in Microsoft Excel™ or any statistical analysis package.

Chlorine dioxide gas generation

A pilot scale ClO_2 gas treatment system at the Food Science Department, Purdue University was used for the experiments. This system consists of a ClO_2 generator/monitor unit, ultrasonic humidifier, and a stainless steel treatment chamber (91 cm diameter×122 cm long). Chlorine dioxide gas is generated and monitored by an automated Minidox-M unit (CloriDisys Solutions, Lebanon, NJ, USA), using 0.5–2% chlorine gas in nitrogen (Matheson Gas Products, Joliet, II and BOC Gases, Murry Hill, NJ, USA) that passes through three cartridges containing sodium chlorite. The disinfection process consists of five steps: (1) pre-conditioning, (2) conditioning, (3) charge, (4) exposure, and (5) aeration. The preconditioning and conditioning steps are used to reach the treatment temperature and desired relative humidity. During charge time, the system starts pumping ClO_2 gas into the chamber until it reaches the desired concentration, then exposure time begins. After the end of exposure, the treatment chamber is aerated to remove the gaseous ClO_2. The treatment parameters such as ClO_2 gas concentration, exposure time, % relative humidity (RH), and temperature are set in the generator and monitored by the computer during the treatment.

Luminescence background

Experiments were conducted to evaluate the design of the biosensor and to determine the ambient light detected by the PMT. Luminescence was measured every second by the PMT for 1-h periods at room temperature with the following different configurations: PMT only, LBsal agar plate only, LBsal with dry membrane, dry membrane only, and LBsal plate with membrane wet from LB broth. In addition, the non-luminescent parent strain of *P. fluorescens* 5RL, *P. fluorescens* 5R, was used to identify any background luminescence signal generated by the bacteria. The mean and standard deviation from each configuration was obtained.

Chemiluminescence detection

Experiments were also performed to determine chemiluminescence background during chlorine dioxide gas exposure. The same configurations as outlined above were tested during exposure to 1.2 mg/l of ClO_2 gas for 5 min at 27°C and 80% RH. Luminescence readings were collected every second during the preconditioning, conditioning, charge, 5-min exposure, and aeration steps of chlorine dioxide gas treatment.

Determination of PMT working range

P. fluorescens 5RL culture was grown to an OD_{600} of 0.5 and subsequently serially diluted 1:10 in LBsal broth. Aliquots of 100 μl from each dilution were enumerated in

LBtet/sal plates, incubated at room temperature for 48 h. The remaining solution was filtered on 47-mm 0.2-μm membranes and placed on top of 47-mm LBsal plates. Each plate containing the bioluminescent bacteria on the surface of the membrane was placed inside the biosensor housing containing the PMT. Luminescence was measured in triplicate from each dilution level and reported as average ±1 standard deviation in photons/s.

Chlorine dioxide gas treatments

To compare the effect of chlorine dioxide gas concentrations on the rate of luminescent decrease of *P. fluorescens* 5RL, the biosensor was exposed to 0.5, 1.0, 1.3, 1.6, and 2.1 mg/l ClO_2 at 80% RH and 27°C. The biosensor was placed in the chlorine dioxide gas treatment chamber, and luminescence was monitored online via the web during the chlorine dioxide gas treatment.

Effect of relative humidity

To evaluate the effect of relative humidity during chlorine dioxide gas treatment, the bioluminescent biosensor containing *P. fluorescens* 5RL were treated with 1 mg/l ClO_2 gas for 5 min at 40, 60, and 80% RH. Luminescence was measured as described above. After examining results, further experiments were performed to test the diffusion of humidity into the biosensor housing. Two humidity sensors were used to measure diffusion into the sensor housing. After calibration, humidity was recorded from two sensors, one sensor outside the biosensor housing and one sensor inside the housing, during a humidification-only process.

Results

Background luminescence from ambient light and potential phosphorescence from materials were measured and shown to be nonsignificant (Table 1). Moreover, there was no significant difference between the light signal from the capped PMT which eliminates ambient light (referred to as dark current) and the uncapped PMT incorporated into the biosensor housing ($P<0.01$; data not shown). Light measurements of both the LBsal agar plate and the dry membrane showed minimal dark current but were approximately 30 and 80% higher than the PMT alone. When the membrane was placed on top of the LBsal agar plate, the combination resulted in a threefold increase in luminescence above the PMT alone, and when Lbsal broth was filtered through the membrane and placed on top of LBsal agar plate, the combination increased 1.6-fold over the unfiltered membrane combination. When the non-luminescent strain *P. fluorescens* 5R was included in the filtrate another, approximately twofold increase above the PMT alone was observed. When comparing the signal from the capped PMT dark current to that from the non-luminescent bacterial, there was an eightfold increase.

Table 1 Luminescence background at ambient conditions, no ClO_2 gas exposure, measured every second for 1 h with different configurations and reported as the mean±1SD

Luminescence background	Luminescence photons/second
Dark current (capped PMT)	36.5±8.5
PMT (inside device)	42.8±9.6
LBsal agar plate	57.9±11.8
Dry membrane	78.7±16.7
LBsal/dry membrane	129.9±13.9
LB sal/filtered LB broth membrane	212.4±73.1
LB sal/*P. fluorescens* 5R on membrane	377.9±30.0

To investigate the potential chemiluminescence from chemical oxidation by chlorine dioxide, the aforementioned sensor configurations were exposed in a treatment regime. Luminescence was monitored online during all steps of ClO_2 gas treatment (Fig. 2). The PMT alone showed negligible increase of luminescence during ClO_2 gas exposure (line a). However, the subsequent configurations showed a similar pattern of increased luminescence during exposure, reaching a plateau followed by a decrease upon removal of ClO_2 gas (lines b–f). Maximum luminescence (3,200 photons/s) was observed from the LB sal plate with the filtered LB broth membrane (line f), while the LBsal agar plate with membrane (line d), LBsal agar with *P. fluorescens* 5R filtered on the membrane (line e), and the membrane alone (line c) were approximately 30% lower. The dry membrane showed an increase upon exposure reaching a maximum, but did not show the plateau and began decreasing during the exposure (line c). The LBsal agar plate (line b) was fivefold higher than the PMT alone but sixfold less than the LBsal plate with the filtered membrane. Although significant luminescence (chemiluminescence) was detected, all of these light values were much lower than the light detected from *P. fluorescens* 5RL as described below.

The linear working range of the biosensor design using *P. fluorescens* 5RL as the bioluminescent reporter and the PMT as a transducer of the light signal is shown in Fig. 3. Outside of this linear range, the background noise detected by the PMT was 10^2 photons/s, and the photon counter was saturated above 2.8×10^6 photons/s. Therefore, the linear region or working range of *P. fluorescens* 5RL cells that can be utilized in the biosensor was 10^3 to 10^7 photons/s. The high light emitted per CFU allowed for lower cell detection limits. The working range of luminescence from *P. fluorescens* 5RL from 10^3 to 10^7 photons/s corresponded to only 10^3 to 10^6 CFU/filter.

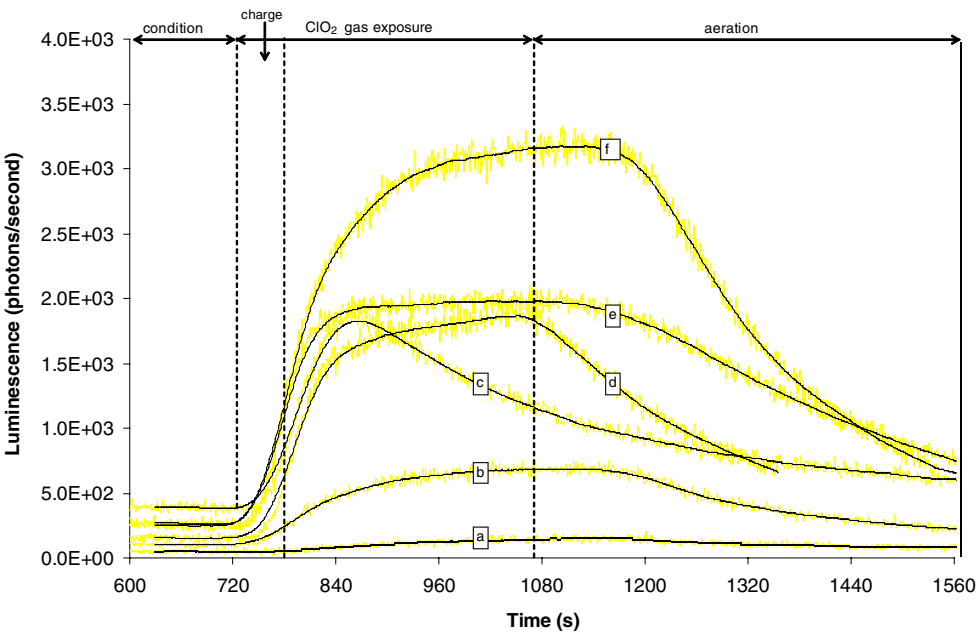

Fig. 2 Luminescence background detected by PMT. Test conditions were 80% RH and 1.2±0.1 mg/l of ClO_2 gas at 27°C. Luminescence background was measured for the following: *a* empty biosensor housing chamber, and lidless, 47 mm Petri plate; *b* plus LB agar with 50 mg/l of salicylate (LB sal); *c* 0.2-μm membrane filter in petri plate; *d* LB sal agar plate with membrane filter; *e* LBsal plate with membrane filter through which 10 ml of *P. fluorescens* 5R culture (OD 0.5) had been filtered; *f* LBsal plate with membrane filter through which 10 ml of LB broth had been filtered

Experiments were performed to determine the effect of increasing ClO_2 gas concentrations on the rates of bioluminescence decrease. The results shown in Fig. 4 demonstrated that the rate of light decrease is directly correlated to increasing ClO_2 gas concentration. Normalized curves in Fig. 4 are expressed as a logarithmic ratio of Ln/Lo vs treatment time where Ln is the luminescence value at time *t* and Lo is the initial luminescence value. All concentrations showed similar reaction kinetics consisting of an initial lag followed by a rapid decrease in luminescence. Linear regression for each treatment concentration was obtained from the linear section of Fig. 4 to compare the different slopes, and the calculated LRT value for each treatment concentration is listed in Table 2. In this study, the LRT represents the time required to decrease bioluminescence by 90%.

The initial cell population of *P. fluorescens* 5RL used for the experiments was 10^6 CFU/filter and after exposure to 0.5, 1.0, 1.6, and 2.1 mg/l of ClO_2 gas survivors numbered 200, 83, 25, and 0 CFU accordingly per filter membrane

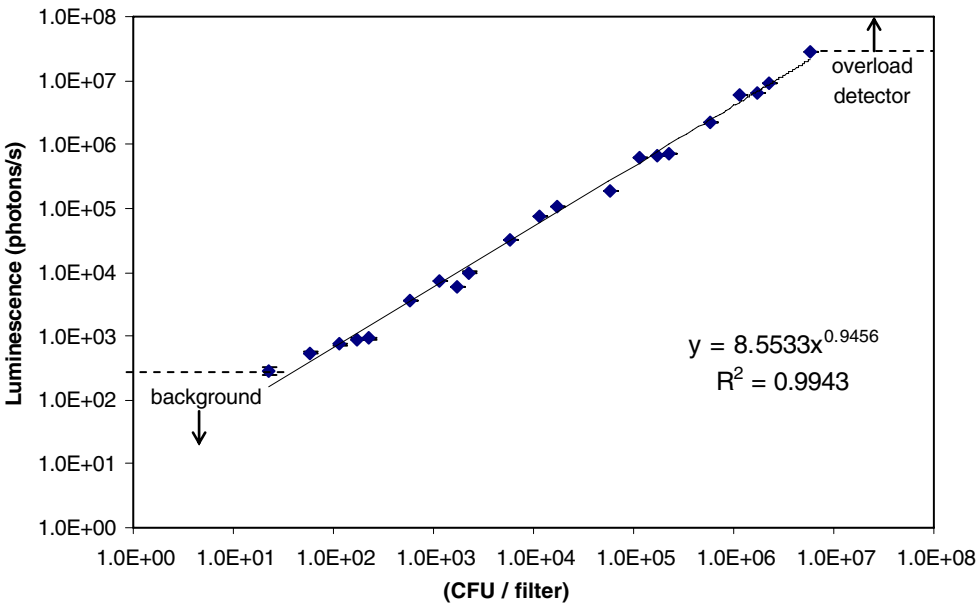

Fig. 3 *P. fluorescens* 5RL working range curve using PMT vs cell count

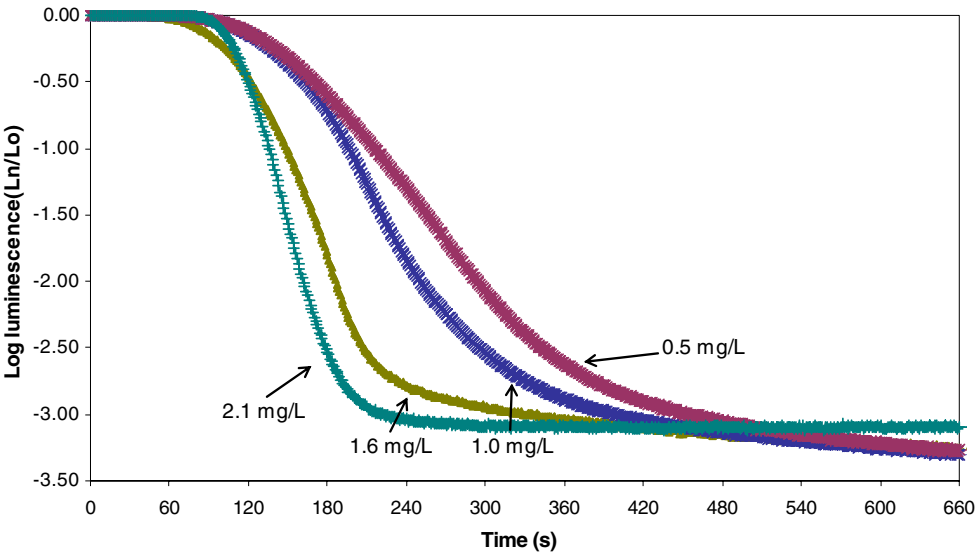

Fig. 4 Luminescence decrease rates of *P. fluorescens* 5RL exposed to 0.5, 1, 1.6 and 2.1 mg/l of chlorine dioxide gas at ×26°C and 78%RH. Normalized light values (*y*-axis) are expressed as Log(Ln/Lo)=Log (light value at time *t*/initial light) versus treatment time (*x*-axis)

obtaining a 4- to 6-log reduction of CFU. The calculated LRT for bioluminescence decrease shows that light decreased faster at higher ClO_2 gas treatment concentrations as shown in Table 2.

Additional experiments were performed to determine the effect of RH on the rates of luminescence decrease during chlorine dioxide gas treatment using a constant gas concentration but different percent of RH (Fig. 5) and replicated three times. A longer lag time was observed at 80% RH compared to 40 and 60% RH, but all curves presented similar rates of light decrease. The treatment with 80% RH showed the greatest lag time, approximately 135 s before the onset of luminescence decrease. This lag time occurs during the charge time which consists of the introduction of ClO_2 gas until it reaches desired concentration. The lag time observed with the treatment at 40 and 60% RH were approximately 65 and 100 s, respectively.

In light of these results, further experiments were performed to determine the rate of diffusion of water vapor into the sensor housing. Signals from two humidity sensors, one outside and one inside the sensor housing, were compared during a humidification-only experiment. In Fig. 6, sensor A (outside the biosensor housing) responded closely to changes in RH when compared to the RH control system sensor. In contrast, sensor B (placed inside the biosensor housing) had no response to the change in RH surrounding the housing. This result indicates that multi-walled sensor housing significantly slowed the diffusion of water vapor into the biosensor, and humidity inside the housing did not change during the three experiments above at 40, 60, and 80% RH.

Table 2 Log reduction time of light decrease for *P. fluorescens* 5RL at various ClO_2 gas concentrations

ClO_2 concentration (mg/l)	LRT (90% light decrease time) (s)	R^2 linear regression
0.5	102.5	0.96
1.0	80	0.96
1.6	52.5	0.96
2.1	35	0.96

Discussion

The biosensor housing device was shown to be light-tight with a low dark current. As even moderate light levels saturate the detector and high light levels can cause irreversible damage to the PMT, a light-tight housing is important to protect the PMT and to measure light attributed solely to the bioluminescent bacteria (less background noise). The increasing luminescence background obtained by including the different materials used for the assay showed some phosphorescence from the materials such as filter membrane, LBsal agar, LB broth, and from the non-luminescent parent strain *P. fluorescencs* 5R. Although a maximum luminescence background (10^2 photons/s) was obtained when all the materials used for the assay were incorporated (LBsal agar plate with filtered LBsal broth on membrane), the background is low when compared to the maximum bioluminescence signal (10^7 photons/s) from *P. fluorescences* 5RL that can be detected by the PMT.

A luminescence increase was also observed in the assay when the materials were exposed ClO_2 gas (Fig. 2). This increase can be attributed to chemiluminescence reactions occurring between ClO_2 gas and some of the materials used

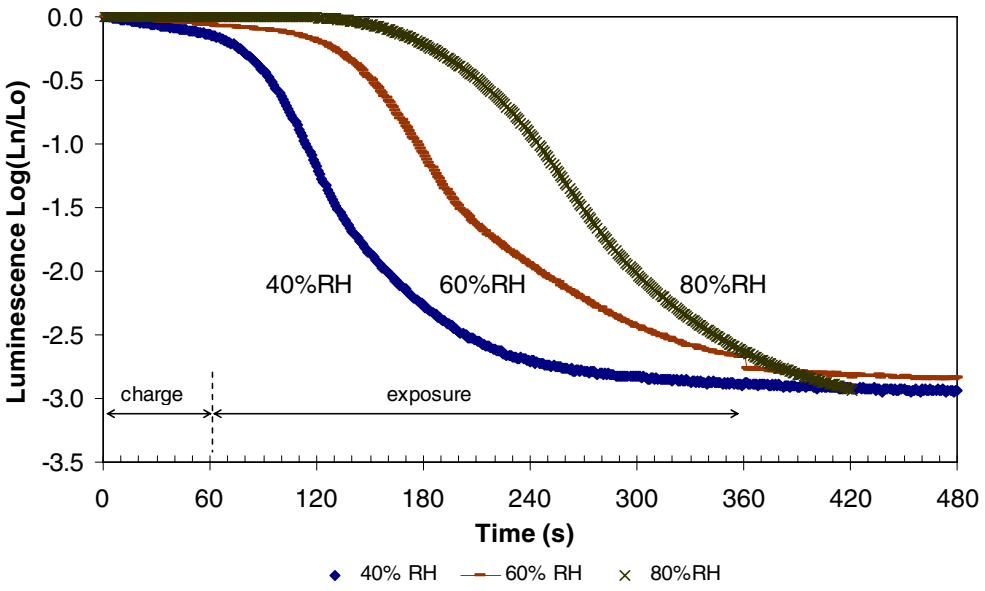

Fig. 5 Effect of relative humidity on luminescence decrease rates during chlorine dioxide (ClO_2) gas treatment of *P. fluorescens* 5RL culture exposed to 1 mg/l of ClO_2 gas at 27°C with 40, 60, and 80% relative humidity. Normalized light values (*y*-axis) are expressed as Log(Ln/Lo)=Log (light value at time *t*/initial light) and treatment time (*x*-axis)

for the experiments such as LB media and the membrane. Chemiluminescence typically increased and then diminished over time when the ClO_2 gas was extracted from the treatment chamber during aeration. The identification of the background light either coming from the environment, materials, or due to chemical reactions was necessary to understand the limitations of the biosensor.

As shown in Fig. 5, higher ClO_2 gas concentrations are directly correlated to the rate of luminescence decrease. The difference in the rates of decrease at different ClO_2 gas concentration clearly shows that a higher gas concentration (2.1 mg/l) generates a more rapid decrease in luminescence as compared to a lower concentration of 0.5 mg/l. The fast signal decrease can be attributed to the toxicity effect of chlorine dioxide gas and accompanied cell death. As suggested by Cronin and Schultz (1998), the true source of a diminished signal is difficult to ascertain, as mechanisms other than toxicity including lack of energy or oxygen can attenuate bioluminescence. However, the effects of lack of oxygen and energy are eliminated in this application due to the cells being utilized in an open air solid media format.

As the results of the water vapor diffusion experiments showed that the relative humidity inside the sensor housing did not change during the tests, i.e., 40-min duration as shown in Fig. 6, the effect of %RH on light reduction when *P. fluorescens* 5RL was exposed to ClO_2 gas was not directly evaluated. However, the fact that changes in %RH outside the sensor housing affected the lag time for light reduction inside the housing (Fig. 5) indicates that some interaction occurred between water vapor and ClO_2 gas.

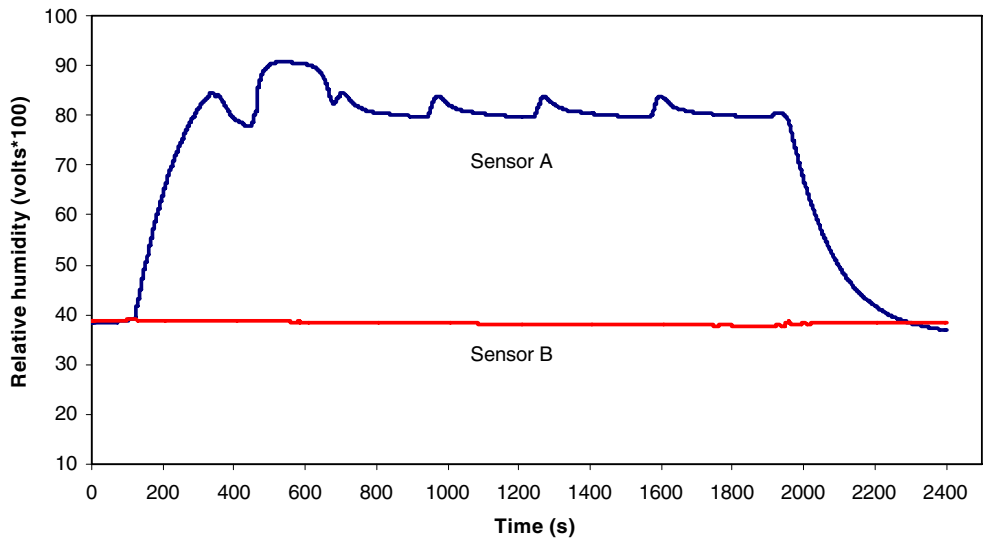

Fig. 6 Humidity inside and outside the biosensor housing. Sensor A was placed outside the biosensor housing and Sensor B was placed inside the biosensor housing. Output signal in %RH for both sensors during exposure to 80% RH is shown

One interpretation of this result is that the higher %RH outside of the sensor housing delayed the diffusion of ClO_2 gas into the housing, as the higher presence of water acts as a sink for ClO_2 gas. In addition, the fact that water vapor did not diffuse into the sensor housing did not hinder the ability of the biosensor to detect the effect of ClO_2 gas on the bacteria. As the *P. fluorescens* 5RL was already fully hydrated inside the biosensor, the presence of high humidity was not necessary to have a lethal effect.

The results of this study demonstrate that the biosensor has potential to be used as a tool to monitor and evaluate chlorine dioxide gas disinfection and other types of gaseous disinfectants. However, the limitation of this biosensor is that only the kinetics of light decrease is obtained, as it is not possible to correlate viable CFU with light decrease due to sampling limitations. However, future work will focus on relating decreases in luminescence using the developed biosensor with inactivation of pathogenic strains of bacteria inoculated on surfaces and treated under the same conditions.

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