Efficiency of an ortho-phthalaldehyde based biocide Against *Pseudomonas fluorescens* adhered to stainless steel surfaces

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Abstract This work reports the study of the action of ortho-phthalaldehyde, OPA, to control Pseudomonas fluorescens adhered to stainless steel surfaces. The action of the biocide was assessed, *in situ*, microscopically using LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit, developed by Molecular Probes. The results show that When OPA concentration is lower than 0.1 % the number of dead cells on the surface are strongly dependent on biocide concentration. For lower biocide concentration, higher dead number of cells can be achieved by increasing the time of exposure to the biocide.
Keywords Adhesion; ortho-phthalaldehyde; *Pseudomonas fluorescens*; biocide; biocide efficiency

Introduction

Disinfectants are widely used in hospitals to control nosocomial infections. In this environment it is necessary to disinfect appropriately all the surfaces that may cause a risk to the health of the patients and of the healthcare personnel. Glutaraldehyde has been traditionally used for low-temperature disinfection of surgical equipment and endoscopes (McDonnell and Russel, 1999). Recently, ortho-phthalaldehyde has been used as a high level desinfectant for endoscopes and other medical devices. Because OPA is a relatively novel disinfectant, few efficacy studies have been performed (Gregory *et al.*, 1999, Alfa, 1994). The mechanism of action of OPA is not fully understood, but a mechanism similar to GTA – reaction with primary amines- and the ability to enter bacterial cell may account for its high lethal action (Walsh et al., 1999).

In most of the situations, the efficacy of the biocides is evaluated through test suspension tests. However, these procedures may not reflect the efficacy of the products against microrganisms adhered to surfaces (Holah *et al*., 1998), since adhered bacterial cells are usually more resistant to the effect of the disinfectants than cells in suspension (Wirtanen *et al.*, 2001). The tests routinely used to assess the efficacy of a disinfectant against adhered cells rely on the removal of cells from the surface after the treatment and enumeration by traditional microbiological techniques. However, the assessment of the viability of the microrganisms *in situ* gives more reliable results.

The aim of this work was to study the action of different concentrations of ortho-phthalaldehyde (OPA) on *P. fluorescens* cells adhered to metal plates. The biocide used was Cidex® OPA solution, Johnson & Johnson medical, Inc. The experimental test were performed using four concentrations of OPA (0.125, 0.1, 0.075, 0.05 % w/v), over a range of exposure times. The action of the biocide was assessed microscopically using LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit, developed by Molecular Probes.

Material and methods

Microorganism

Pseudomonas fluorescens ATCC 13525, a Gram-negative aerobic bacterium, was used through this work.

Biocide

Ortho-phthalaldehyde OPA (Cidex® OPA solution, Johnson & Johnson medical, Inc) The biocide is delivered at 0.55 % (w/v) and, according to the manufacturer it is active till 0.1 % (w/v). The exposure time recommended is 5 minutes.

Micoorganism growth

A continuous pure culture of the *P. fluorescens* was grown in a 2 L glass fermenter, at 27±1ºC, suitably aerated and magnetically agitated. The fermenter was continuously fed with 0.05 L/h of a sterile nutrient solution consisting of 5 g glucose L^{-1}, 2,5 g peptone L^{-1}, 1,25 g yeast extract L^{-1} in phosphate buffer at pH7.

Adhesion assay

A suitable amount of *Pseudomonas fluorescens* culture was removed from the fermenter, centrifuged (5000rpm, 5min,4 ºC) and washed thrice with sterile phosphate buffered saline (PBS, pH7, 1 M). The pellets were ressuspended in PBS and a cell suspension was prepared at 640 nm to an OD of 0.02.

Using aseptic techniques, metal plates (0.9cm x 0.9cm) were placed in the wells (10 mm dia) of a

sterile tissue culture plate (Greiner labortechnik). 2 ml of cell suspension was added to each well. Thereafter, the whole assembly was placed in an incubator for 2 h at 27 ºC with gentle agitation at 90 rev/min. The plates were then washed thrice with sterile PBS.
In order to choose the adhesion time suitable for the experiment the adhesion assay was performed for different incubation times (1, 2, 3, 4 and 5 hours).

Biocide treatment

Six concentrations of OPA Cidex® were tested in the adhesion assay, for 5 minutes exposure time: 0.55 % (as delivered), 0.25 %, 0.125 %, 0.1% (minimum recommended concentration), 0.075% and 0.05 % (w/v).

To evaluate the effect of the exposure time, two concentrations of the biocide (0.075 % and 0.05%) were tested for a contact time of 5, 7 and 10 minutes.

Epifluorescence observations

Bacterial total counts: The total number of bacteria adhered to the surface were obtained using DAPI staining. 500 µl of a 20 µl/ ml DAPI solution was to each well containing the plates and incubated for 30 min. After this time, the plates were rinsed with sterile distilled water and observed under epifluorescence where the cells appeared blue.

Bacligth viability kit

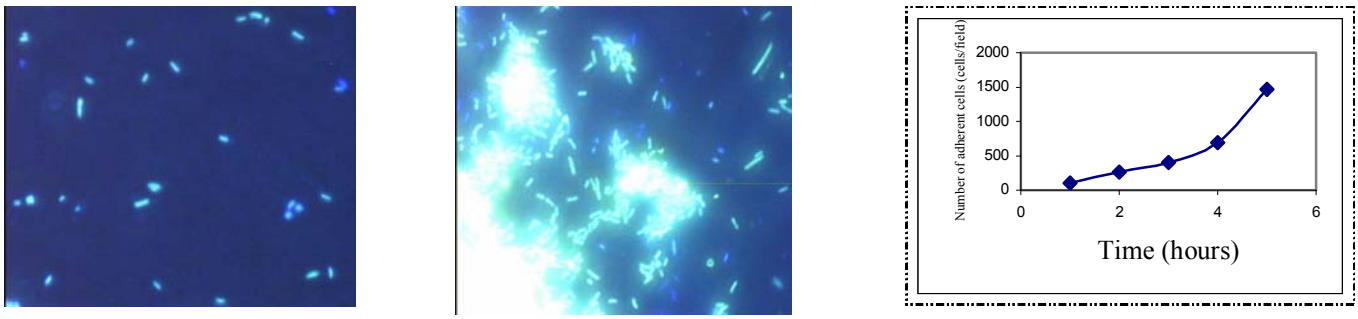
Direct viable and total counts were obtained with *Bac*ligth viability kit. The two *Bac*Ligth stains, SYTO 9 and propidium iodide, dissolved in DMSO, were mixed together (300 µl + 300 µl) and added to sample and incubated for 15 min. After this time, the plates were rinsed with sterile distilled water and observed under epifluorescence (viable cells were fluorescent green, while dead cells were fluorescent red)

Quantification of adherent cells

An image analysis software (Image-Pro Plus, Media Cybernetics) was used for the quantitative estimation of the adherent cells. Thirty fields were counted in each plate at ×630 magnification. As 30 fields were counted for each strip the mean number of cells was expressed as cells per unit square mm. Each image corresponds to 0.05 mm^2. All experiments were repeated on three separate occasions with triplicate determinations on each occasion.
Statistical analysis: Differences in the number of the bacteria remaining on the surface after each particular treatment were assessed statistically by analysis of variance ANOVA, significance is expressed at the 95 % confidence level.

Results

The mean values of the adhesion time for the experiment the adhesion assay are show Figure 1. A 2 hours adhesion time was chosen due to the number of cells obtained after this incubation time.



(a) (b) ©

Figure 1 (a) and (b) Photographs of the surface after the adhesion tests, respectively after 1 and 4 hours; (c) Number of cells/field with adhesion time (bars represent standard deviation)

Table 1 presents the total number of bacteria after biocide treatment and number of dead cells as a function of ortho-phthalaldehyde concentration.

Compared with the control, a highly significant reduction of viability was observed after the exposure to the biocide during 5 minutes. For OPA concentrations higher that 0.1% the percentage of dead cells is not significantly affected by the concentration of biocide. Conversely, for lower concentrations, the number of dead cells is strongly affected by the biocide concentration, as represented in Figue 1.

Table 1 Total number of bacteria after biocide treatment and number of dead cells as a function of ortho-phthalaldehyde concentration.

Biocide concentration (% w/v)	Initial bacterial concentration	Total number of bacteria after biocide treatment (Cells/field)	Number of dead cells (Cells/field)	% Of dead cells
0.55	289.7 ± 27.1	357.3 ± 12.9	352.5 ± 8.1	98.6± 1.4
0.25		348.5 ± 4.8	343.3 ± 7.7	98.5± 1.5
0.125		270.3 ± 0.8	258.1 ± 2.6	95.5± 1.7
0.1		165.5 ± 57.0	156.0 ± 59.1	94.5± 5.5
0.075		144.6 ± 17.2	99.8 ± 21.6	69.0± 1.2
0.05		311.9 ± 8.8	166.6 ± 8.9	53.4± 1.1

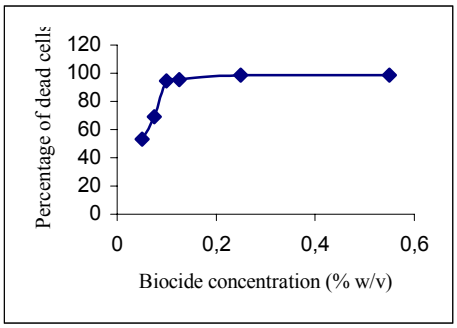


Figure 2 Percentage of dead cells, after 5 minutes, as a function of biocide concentration

Table 2 presents the results concerned with the effect of time of exposure to the biocide, for 0.075 and 0.05 %. The percentage of dead cells as a function of time is represented in Figure 3.

Figure 3 shows that, for OPA oncentrations of 0.075 and 0.05 %, a significant reduction is obtained after 10 minutes exposure to the biocide, achieving values close to the ones obtained after 5 minutes and higher OPA concentrations. However, higher concentrations and a lower exposure time seem to be more effective in killing the bacteria adhered to the surface

Table 2 Total number of bacteria after biocide treatment and number of dead cells as a function of ortho-phthalaldehyde concentration and time

Biocide concentration (% w/v)	Contact time	Initial Bacterial concentration (Cells/field)	Total number of bacteria after biocide treatment (Cells/field)	Number of dead cells (Cells/field)	% Of dead cells
0.075	5 min	289.7 ± 27.1	144.6 ± 17.15	99.8 ± 1.4	69.0± 1.5
	7 min		246.0 ± 22.37	229.0 ± 19.8	93.0± 6.9
	10 min		271.6 ± 11.6	258.2 ± 6.7	95.1± 2.5
0.05	5 min		311.9 ± 8.8	166.6 ± 8.9	53.4± 1.1
	7 min		202.0 ± 23	151.1 ± 23.9	74.8± 11.8
	10 min		147.0 ± 2.4	140.4 ± 1.4	95.5± 1.5

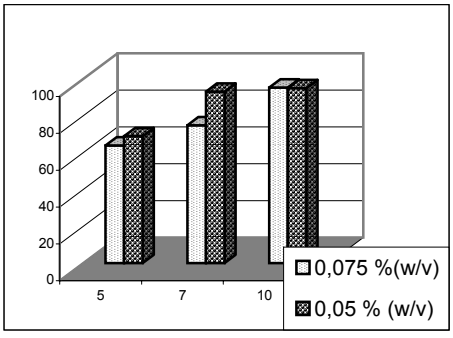


Figure 3 Percentage of dead cells as a function of exposure time, for biocide concentration 0.075 and 0.05%.

Discussion and Conclusions

The present results indicate that a short time exposure of *P.fluorescens* adhered to surfaces to OPA Cidex® reduces their viability when compared with that of the unexposed control. Gregory *et al*., 1999, also found that OPA (0.21% w/v) was very fast in controlling mycobacteria in suspension, nearly six times the speed of glutaraldehyde at its minimum effective concentration.

The results presented in Table 1, also show that nearly 100 % of the bacteria are killed, after 5 minutes, for high OPA concentrations. Concerning tests carried out on the efficacy of OPA to kill adhered cells, Alfa and Slitter (1993) showed that, after exposing endoscopes to 0.5 % OPA (0.55% v/v), no residual bacteria, viruses, parasites and fungi could be detected, obtaining a 5-log $_{10}$ reduction of organisms, after 5 minutes. However, the methodology used (recover microrganisms from the surface and cultivation on selective media) may underestimate the effect of the bacteria, since, after the biocide treatment, some bacteria may continue viable but not culturable. Besides that, the method to remove the bacteria from the surface may be innefective. Table 1 shows that the bacteria continue adhered to the surface after the exposure to the biocide, maintaing a high organic load on the surface. More studies are needed to evaluate the strength of adhesion

When OPA concentration is lower than 0.1 % the number of dead cells on the surface are strongly dependent on biocide concentration. For lower biocide concentration, higher dead number of cells can be achieved by increasing the time of exposure to the biocide.

Acknowledgements

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References

Alfa, M.J., Sitter, D. L. (1993) In-hospital evaluation of orthophthaldehyde as high level desinfectant for flexible endoscopes. *Journal of Hospital Infection* 26, 15-26.

Gregory, A.W, Schaalje, G.B, Smart, J.D, Robinson, R.A. (1999) The mycobactericidal efficacy of ortho-phthaldehyde and comparative resistances of Mycobacterium bovis, Mycobacterium terrae and Mycobacterium chelonae", Infection Control and Hospital Epidemiology, 20 (5), 324-330.

Holah, J. T., Lavaud, A., Peters, W., Dye, K.A., (1998) Future techniques for disinfectant efficacy testing. *International Biodeterioration & Biodegradation* 41, 273-279

Russel A. D. (1994) Glutaraldehyde: its current status and uses. *Infection control and Hospital Epidemiology* 15, 724-733.

Walsh S. E., Maillard J-Y., Simons C. and Russell A. D. (1999) Studies on the mechanisms of the antimicrobial action of ortho-phthaldehyde. *Journal of Applied Microbiology* 87, 702-710.

Wirtanen, G., Salo, S., Helander, I.M., Mattila-Sandhalm (2001). Methods for testing desinfectant on *Pseudomonas* biofilm. *Colloids and Surfaces B: Biointerfaces* 20, 37-50