MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

The effect of Southwell Ltd chlorine dioxide on *Pseudomonas syringae* pv *actinidiae* (PSA) biofilms

Professor Bernd H. A. Rehm

Summary

Biofilms are formed by many pathogenic bacteria. Upon attachment to the host (e.g. plant surface) bacteria differentiate and become non-motile as well as produce polymeric substances which protect them against host defence mechanisms. The biofilm growth mode of *Pseudomonas syringae* pv *actinidiae* (PSA) had been shown to be important for pathogenicity during kiwifruit wine infection. It is well established that the biofilm growth mode of bacteria in general coincides with significantly enhanced resistance to antibacterial treatment. Hence development of new chemicals for treatment of PSA caused plant diseases should target PSA bacteria in biofilms. A method was established to grow PSA in flow chamber cells to form biofilms. Biofilms were treated with 100ppm or 200ppm of ClO_2 (provided by Southwell Ltd) for 1 h and then compared to biofilms treated with a physiological salt solution (saline) as negative control. After treatment of biofilms, live and dead cells were differentially stained and observed by Confocal Laser Scanning Microscopy. Images were analysed with respect to the ratio of live to dead cells. While dead cells naturally occurred at a level of 3-4% of the entire biofilm population, only about 1% of bacteria in biofilms survived treatment with 100ppm ClO_2 and only about 0.1% or less of the bacteria survived treatment with 200 ppm ClO_2. In the ladder case biofilms were observed where complete killing had occurred. These results suggest that PSA biofilms can be efficiently treated with the provided Southwell Ltd ClO_2 i.e. PSA was efficiently killed while embedded in the biofilm matrix.

Introduction

Chlorine dioxide (ClO_2) is widely used as disinfectant for treatment of waste water, fruits and vegetables. Disinfection ability of ClO_2 depends upon its ability to oxidise target compounds or organisms (3). It is commonly used to kill bacteria in planktonic mode and more recently also in biofilm mode. Similar to what has been observed in the case of antibiotics, bacteria are more resistant to ClO_2 in biofilm mode as compared to planktonic mode (1).

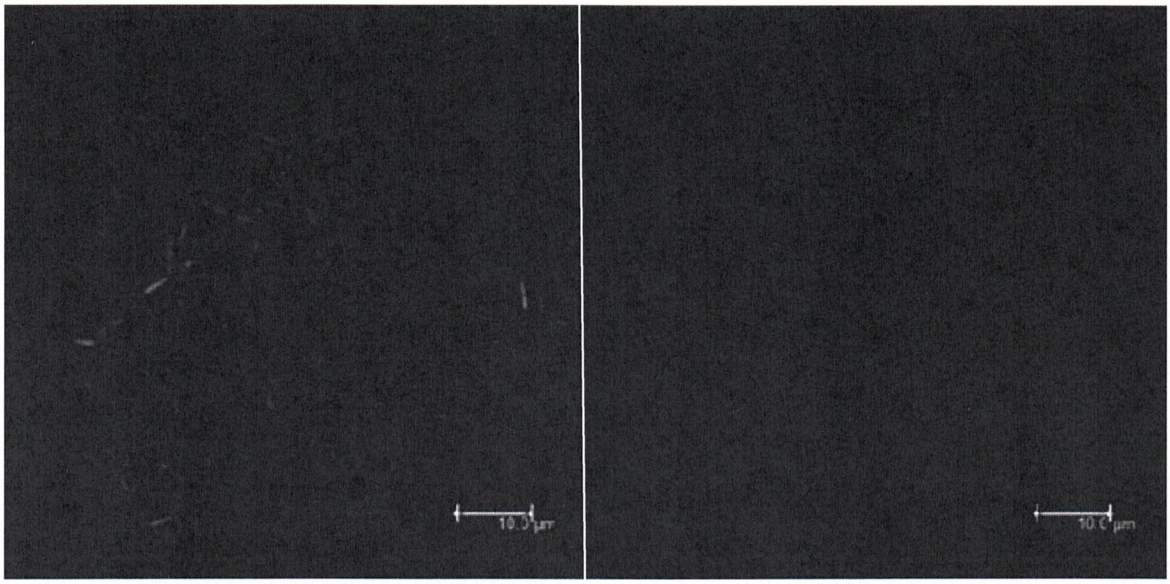
The recent outbreak of *Pseudomonas syringae* pv *actinidiae* in New Zealand has threatened the agriculture based economy of this country (2). The PSA strain isolated in NZ is phylogenetically related to an isolate from China. Symptoms of PSA infection are formation of cankers, production of exudates as well as cane and shoot dieback of kiwifruit wines (2). Previously we have evaluated the ability of ClO_2 to kill PSA grown in planktonic mode. In this study we investigated the killing effect of ClO_2 on PSA grown in biofilm mode, a growth mode relevant to plant pathogenicity as well as for disease prevention/treatment (4).

Material and Methods

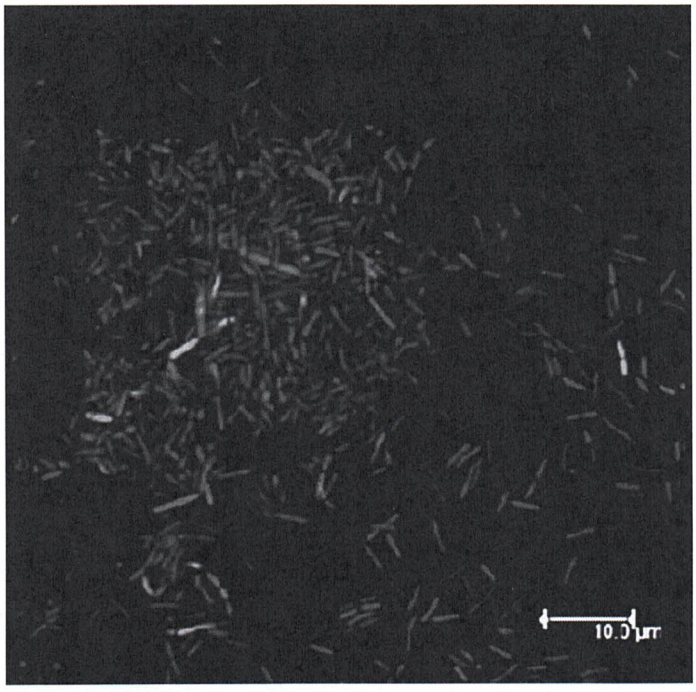
A method was established to grow PSA in biofilms in a defined laboratory environment. For biofilm analysis PSA was firstly inoculated in KB (Kings Broth) media and grown for 18 hrs in planktonic mode at 22^0C. 500µl of overnight culture was used to inoculate flow cells filled with KB media and allowed to attach for 4-5 h. The flow cells with dimensions of 4mm×40mm×1.5mm were used in this study. After the attachment, KB media was allowed to flow through the flow cells at the rate of 0.3 ml/min. The flow cells were incubated at 22^0C for 72 hrs. For treatment with ClO_2, cells were either incubated with normal saline (negative control) or with 100ppm or 200ppm of ClO_2 for 1 h. After treatment with ClO_2, KB media was allowed to flow through the flow cells for 15min for washing. The ClO_2 and normal saline treated biofilms were stained using LIVE/DEAD BacLight bacterial viability kit and visualised using a Confocal Scanning Laser Microscope. Three independent biofilms were assessed. Images were captured using ×100 objective lens (1000x magnification) and analysed by counting live and dead cells (see Appendix for complete set of images).

Results and discussion

It was possible to establish conditions for the growth of PSA in biofilms (Figure 1). Treatment of cells grown in biofilm mode with normal saline (0.9% NaCl) was used as control in order to assess the naturally occurring ratio of dead to live cells. Biofilms were analysed by Confocal Scanning Laser Microscopy (Fig 1). Image analysis indicated that 3-4% of dead cells did naturally occur in the PSA biofilm (Fig. 1C). The green cells in the figure represent the viable cells whereas dead cells are stained red.



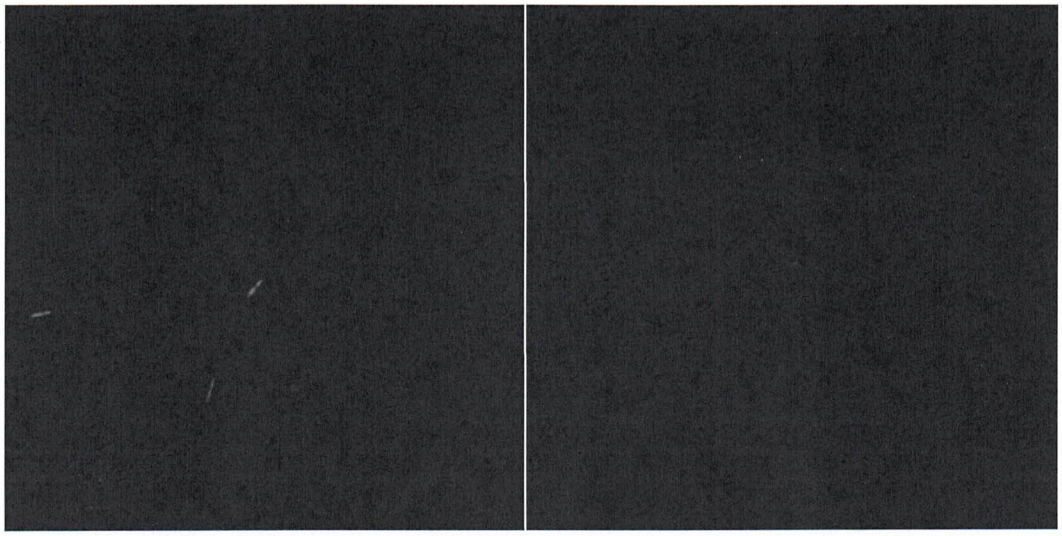
A B



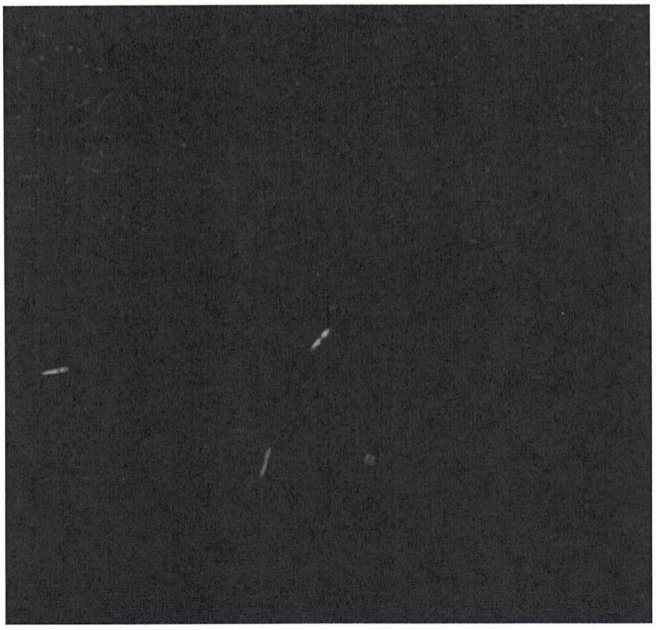
C

Fig. 1: Confocal scanning microscope images of PSA grown for 72 h in the biofilm mode. Cells were treated with normal saline (negative control). (A) Green filter (live cells) (B) Red filter (dead cells) (C) Images A and B superimposed. The viable cells are bright green in colour and dead cells are red in colour.

PSA biofilms were exposed to 100 ppm of the Southwell Ltd chlorine dioxide for 1h and then analysed by Confocal Scanning Laser Microscopy (Fig. 2). This treatment resulted in a significant reduction of live cells to about 1% of the biofilm cell population.



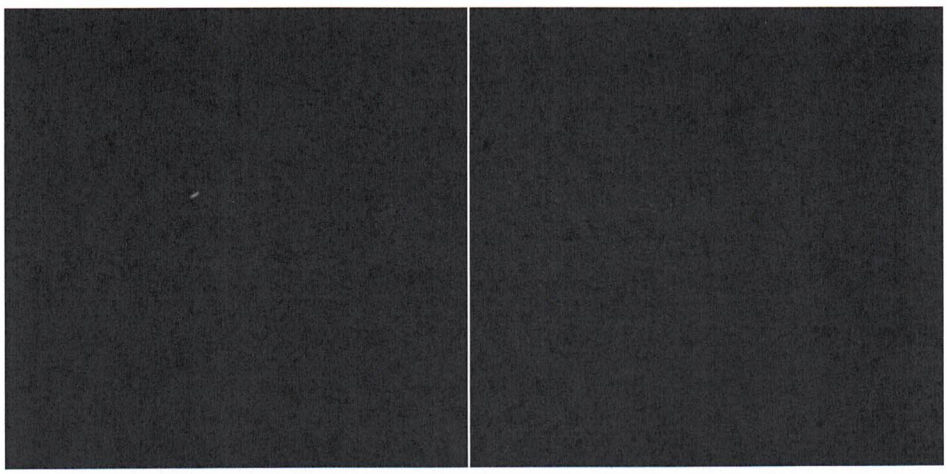
A B



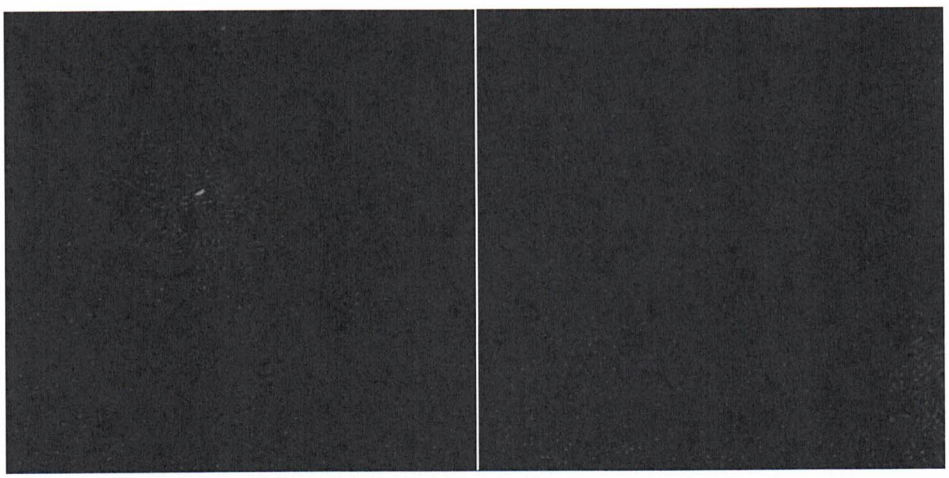
C

Fig. 2: Confocal scanning microscope images of PSA grown for 72 h in the biofilm mode and treated for 1 h with 100 ppm of Southwell Ltd ClO_2. (A) Green filter (live cells) (B) Red filter (dead cells) (C) Images A and B superimposed. The viable cells are bright green in colour and dead cells are red in colour.

To assess whether a further increase in Southwell Ltd ClO_2 concentration would improve killing of PSA in biofilms, the concentration was increased two-fold to 200ppm. Biofilms were analysed by Confocal Scanning Laser Microscopy and a further reduction of live cells to about 0.1% of the biofilm population was found (Fig. 3). In some cases even a total killing was observed (Fig. 3D). Variation in killing efficiency is most likely due to variation in cell density, polymer matrix density and biofilm thickness throughout the biofilm.



A B



C D

Fig. 3: Confocal scanning microscope images of PSA grown for 72 h in the biofilm mode and treated for 1 h with 200 ppm of Southwell Ltd ClO_2. (A) Green filter (live cells) (B) Red filter (dead cells) (C) Images A and B superimposed (D) Superimposed image from independent analysis showing total killing. The viable cells are bright green in colour and dead cells are red in colour.

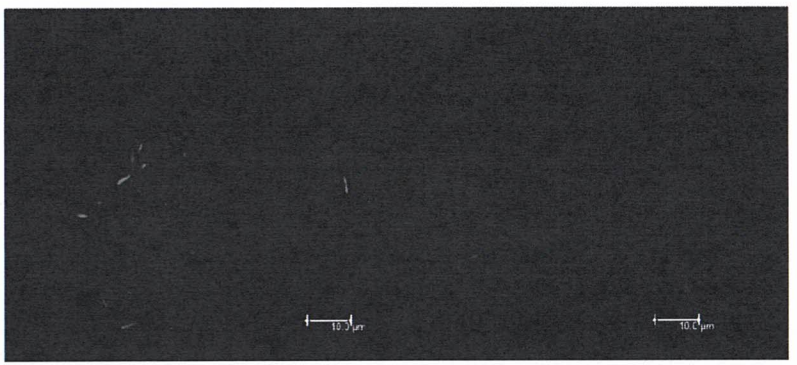
References

1. Behnke, S., and A. K. Camper. 2012. Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells. Biofouling 28:635-47.
2. Chapman, J. R., R. K. Taylor, B. S. Weir, M. K. Romberg, J. L. Vanneste, J. Luck, and B. J. Alexander. 2012. Phylogenetic relationships among global populations of Pseudomonas syringae pv. actinidiae. Phytopathology 102:1034-44.
3. Kozyatnyk, I., J. Swietlik, U. Raczyk-Stanislawiak, A. Dabrowska, N. Klymenko, and J. Nawrocki. 2013. Influence of oxidation on fulvic acids composition and biodegradability. Chemosphere.
4. Renzi M., Copini P., Taddei A.R., Rossetti A., Gallipoli L., Mazzaglia A., Balestra GM. 2012 Bacterial canker on kiwifruit in Italy: anatomical changes in the wood and in the primary infection sites. Phytopathology 102(9):827-40.

Appendix

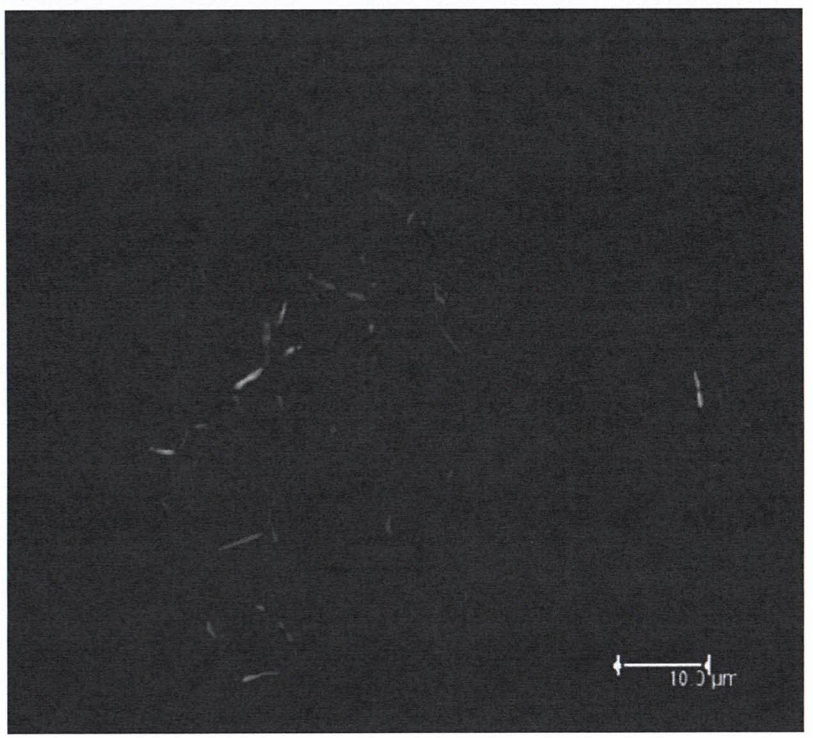
Saline treatment of PSA biofilms: Confocal laser scanning microscope images of biofilm stained for live (green) and dead (red) cells

Green filter



Red filter

Superimposed images

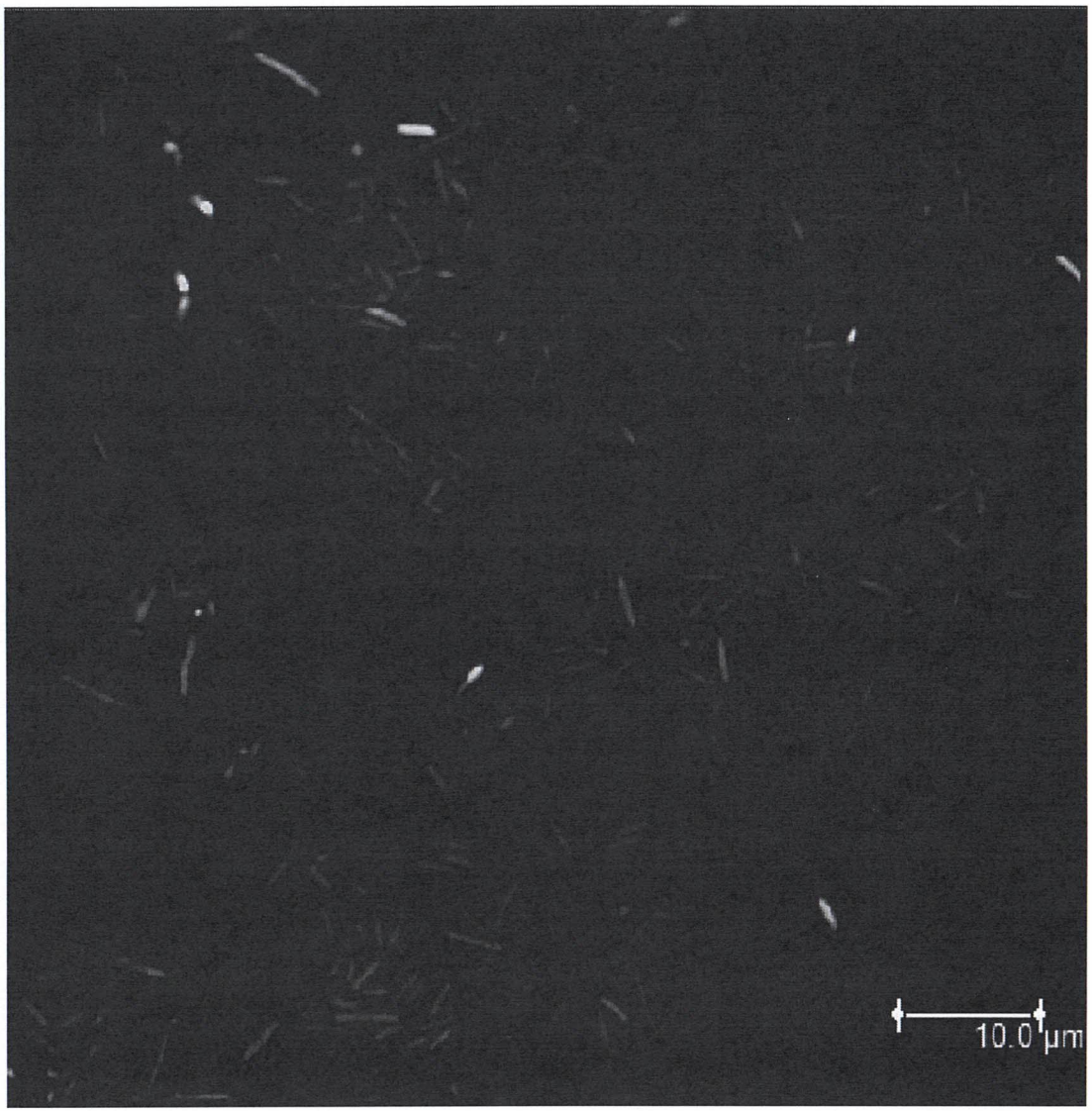


Further superimposed images (saline treatment)

~3-4% dead cells

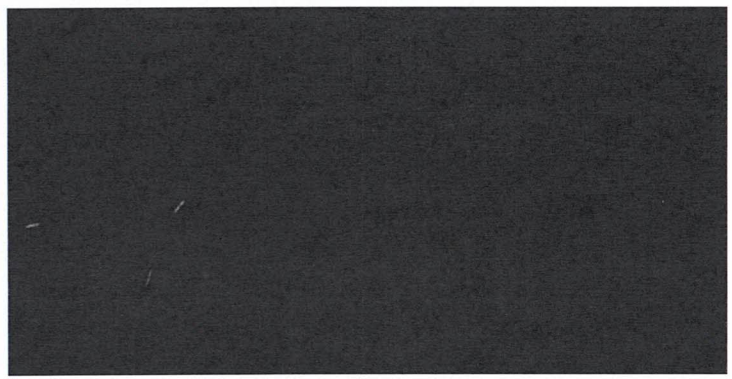
Further superimposed images (saline treatment)

~3-4% dead cells



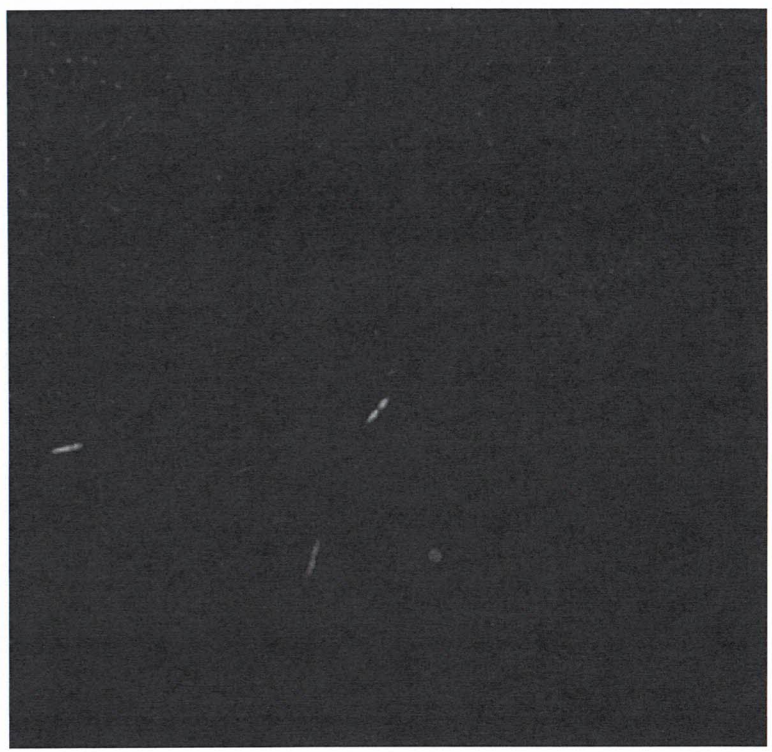
Chlorine dioxide (100ppm) treatment of PSA biofilms: Confocal laser scanning microscope images of biofilm stained for live (green) and dead (red) cells

Green filter



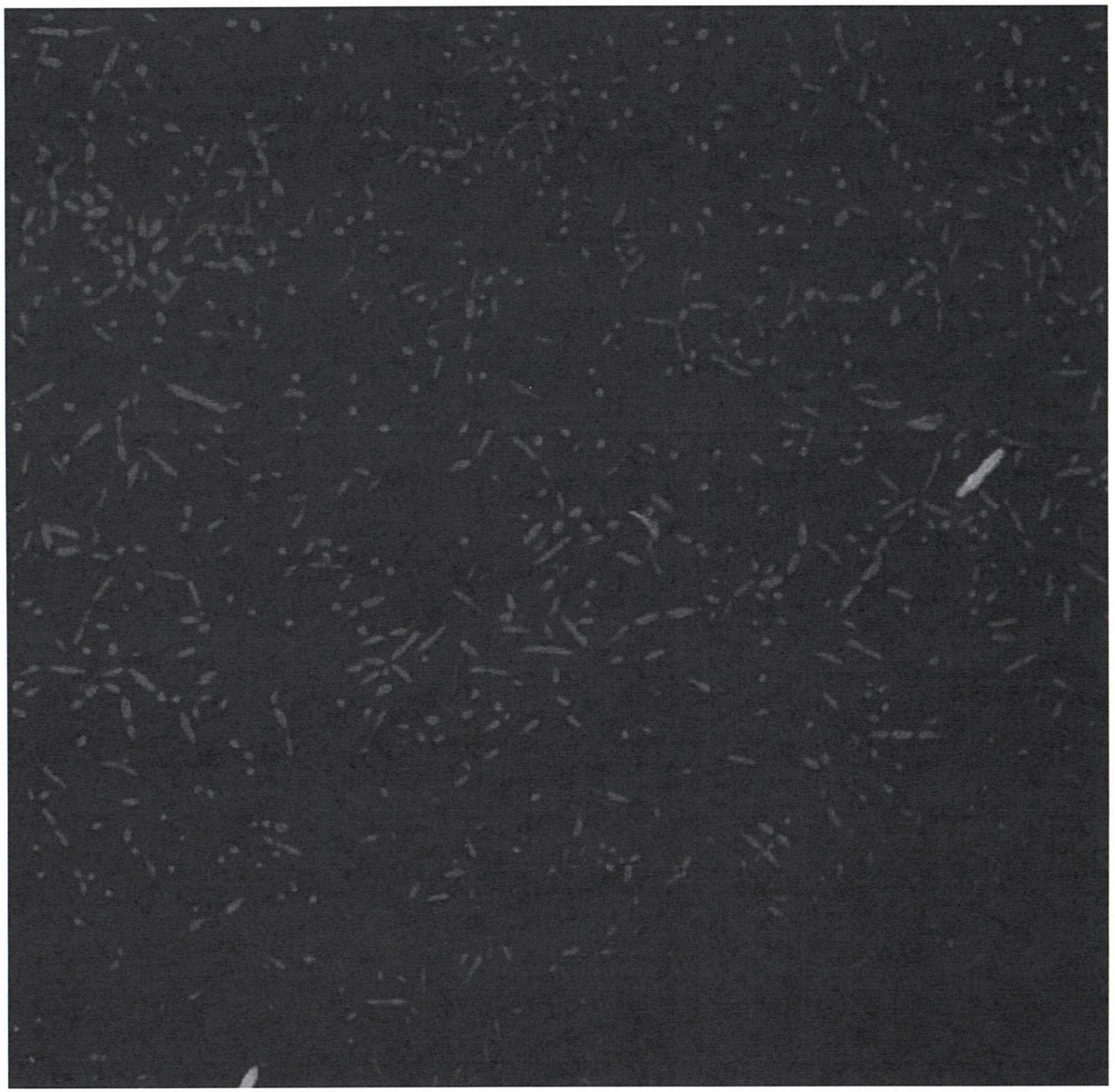
Red filter

Superimposed images



~1-2% live cells

Further superimposed images (Chlorine dioxide 100ppm)

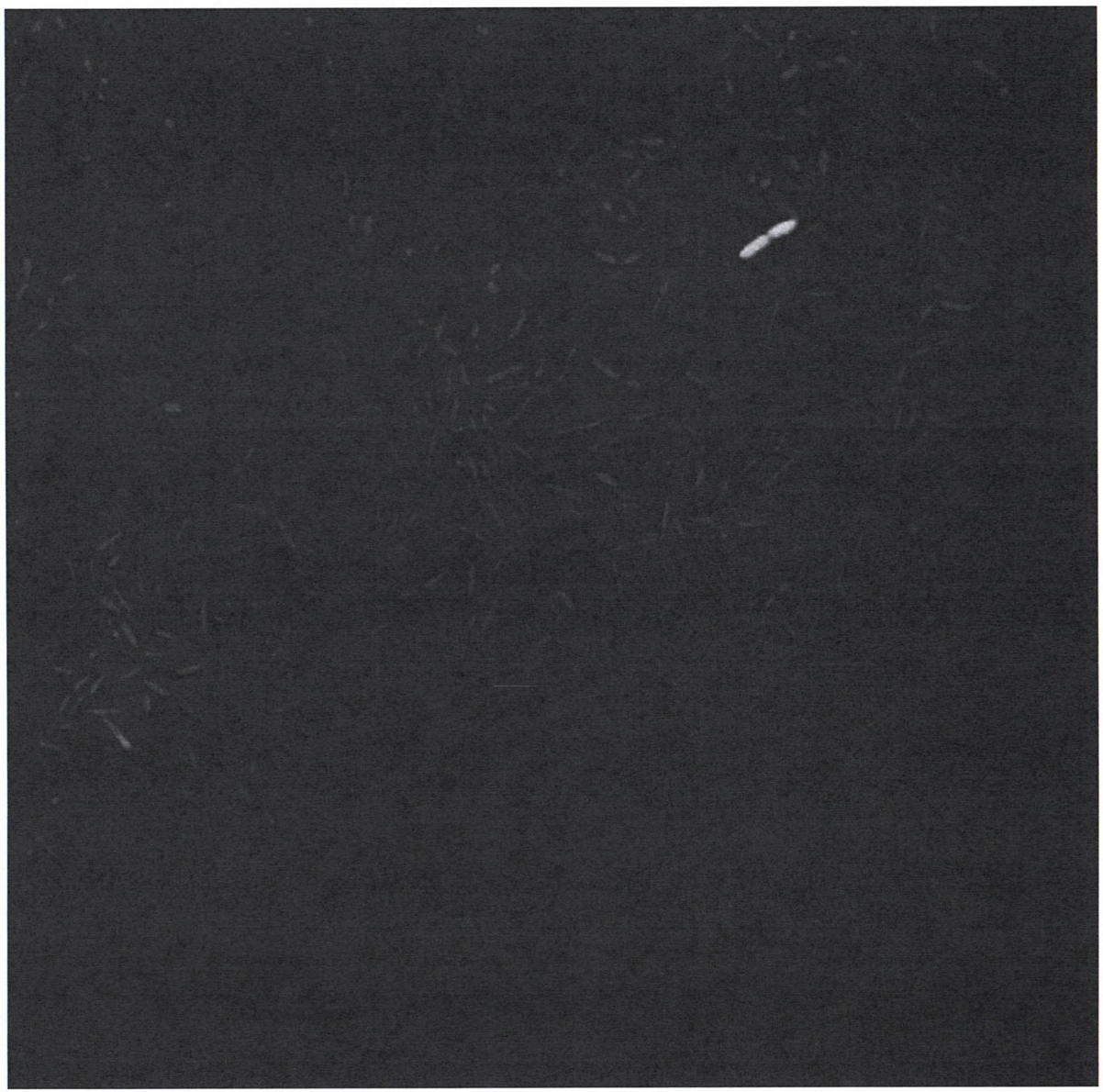


~1% live cells

Further superimposed images (Chlorine dioxide 100ppm)

~1% live cells

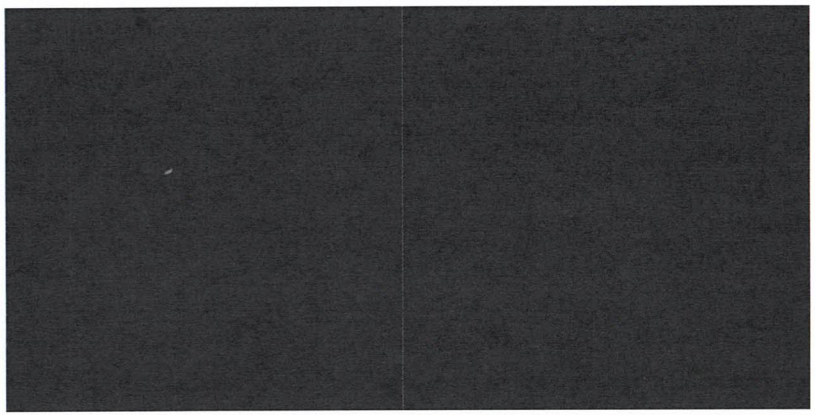
Further superimposed images (Chlorine dioxide 100ppm)



~0.6% live cells

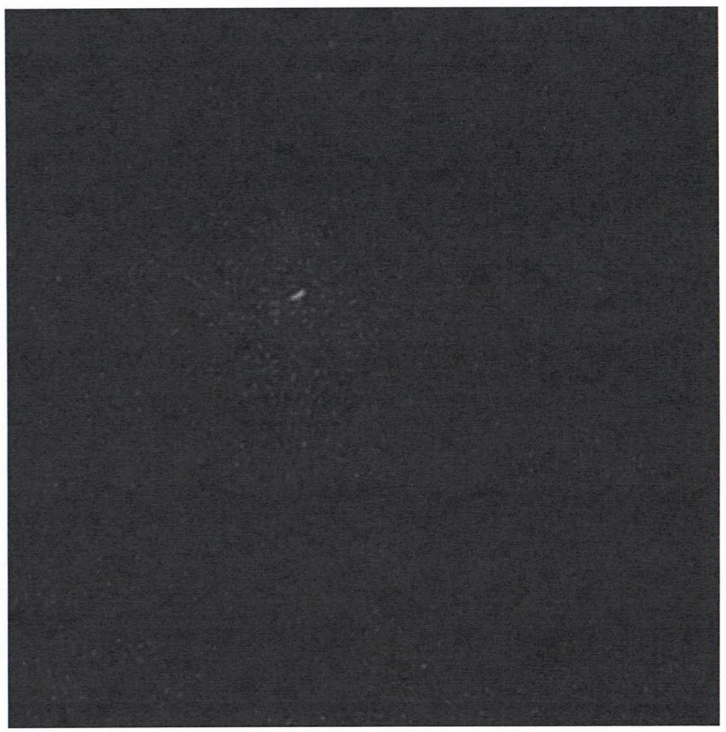
Chlorine dioxide (200ppm) treatment of PSA biofilms: Confocal laser scanning microscope images of biofilm stained for live (green) and dead (red) cells

Green filter



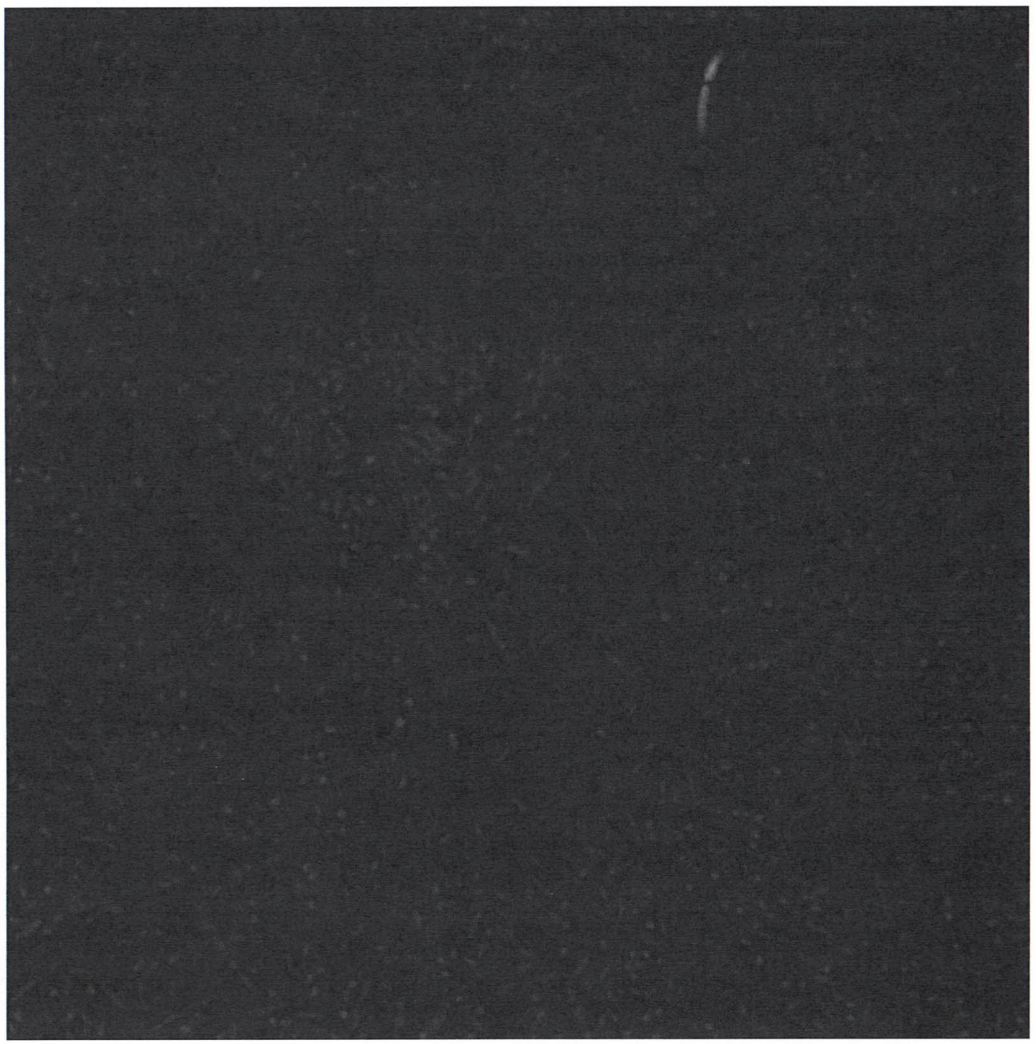
Red filter

Superimposed images



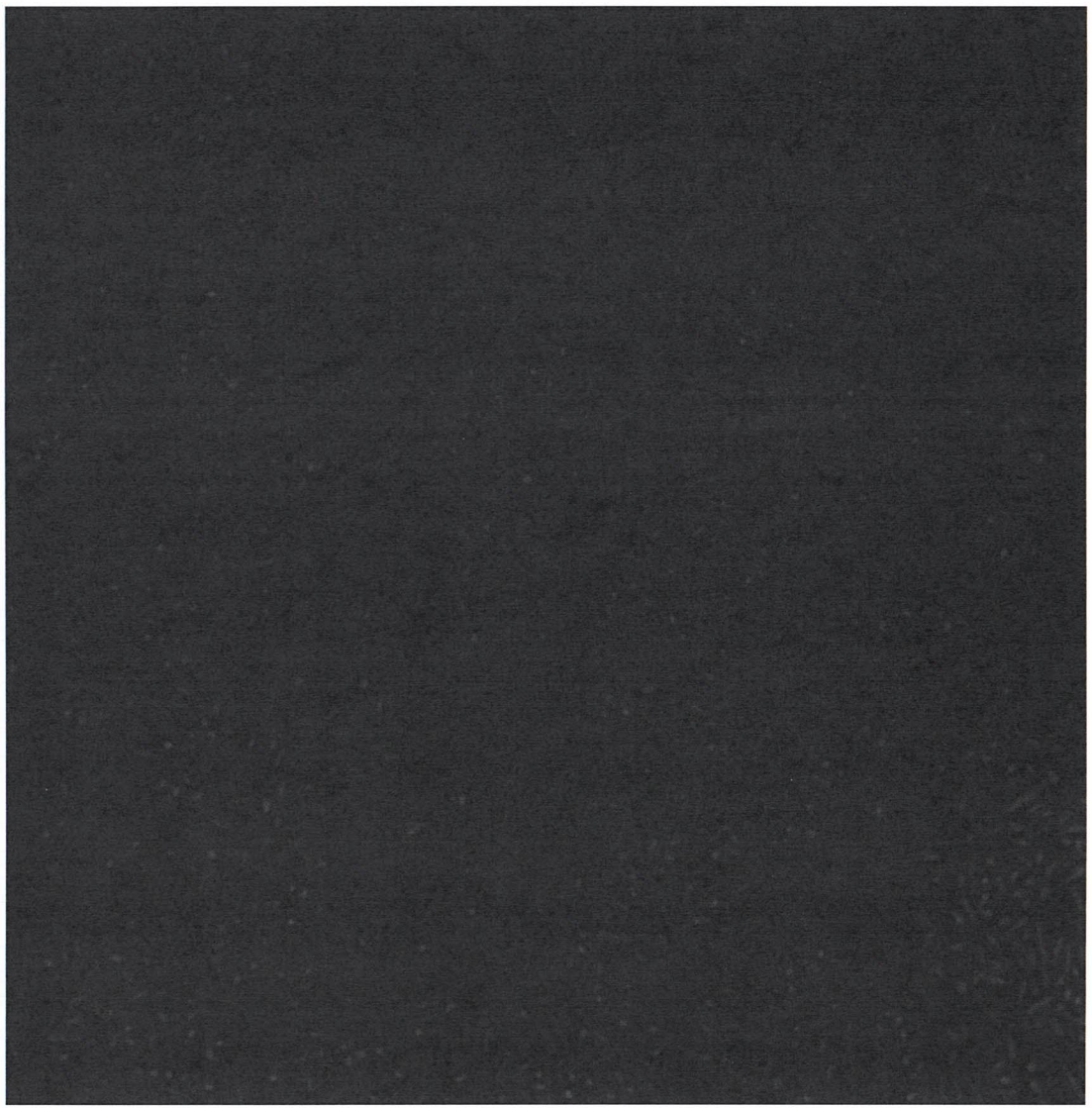
~0.2% live cells

Further superimposed images (Chlorine dioxide 200ppm)



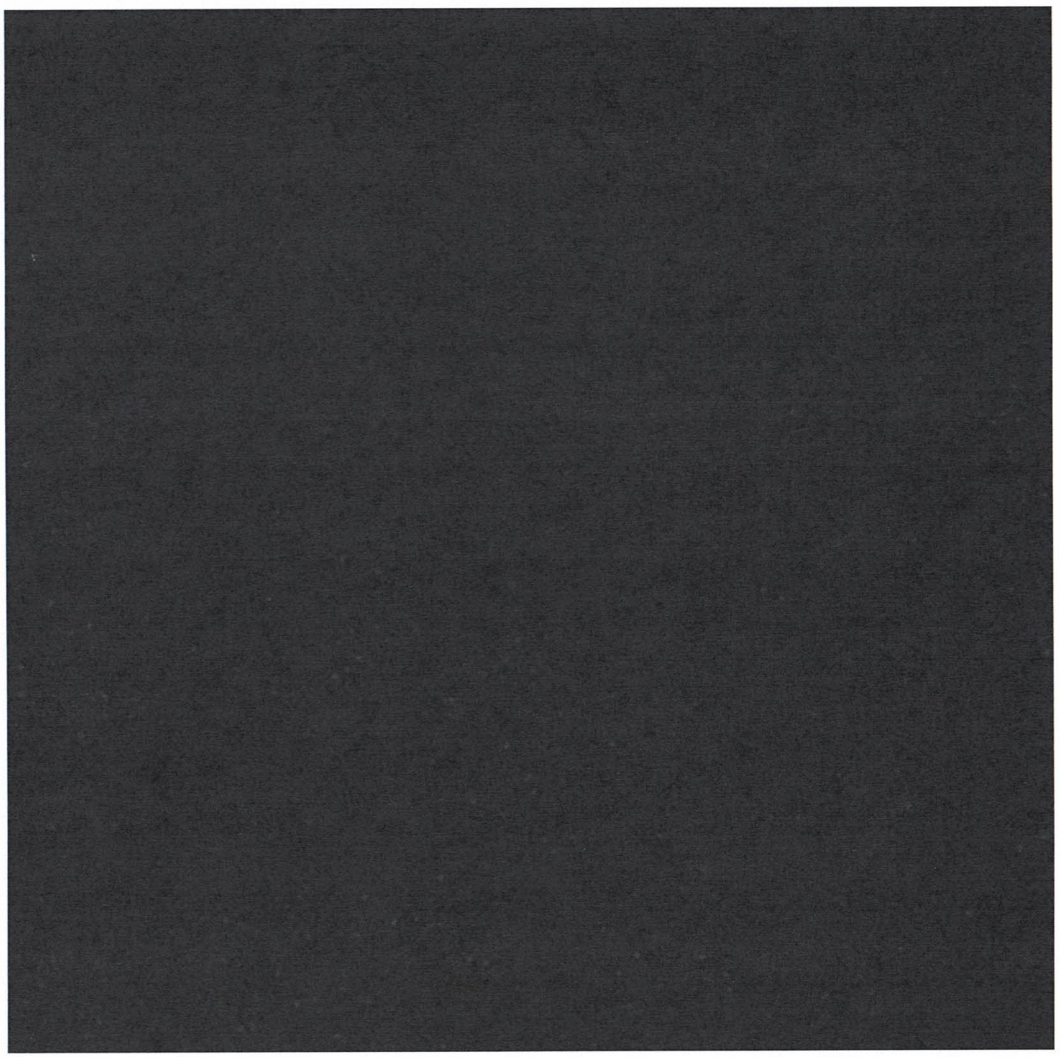
~0.15% live cells

Further superimposed images (Chlorine dioxide 200ppm)



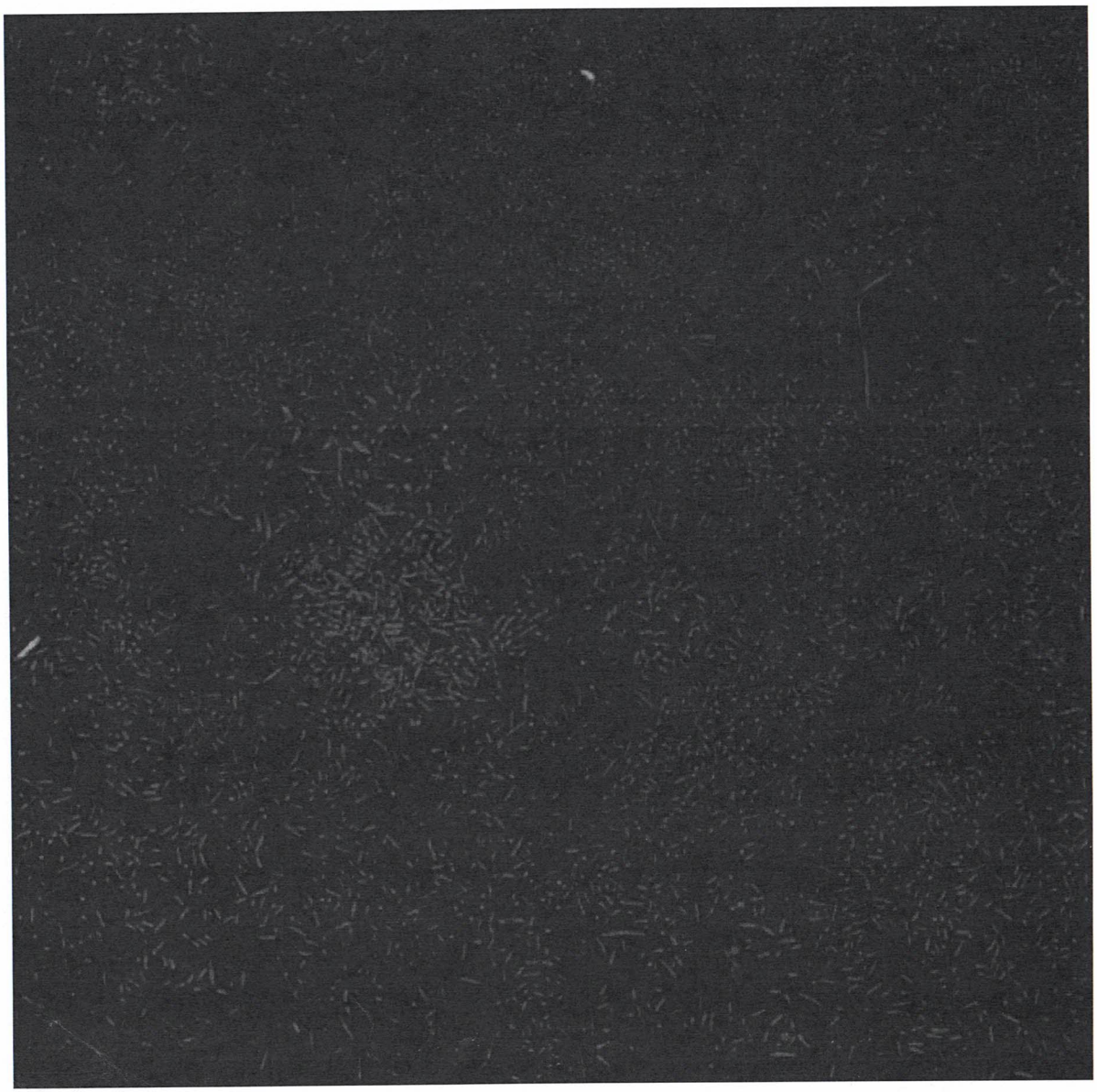
No live cells

Further superimposed images (Chlorine dioxide 200ppm)



<0.1% live cells

Further superimposed images (Chlorine dioxide 200ppm)



~0.1% live cells