The Effect of Chlorine Dioxide on *Pseudomonas syringae* pv *actinidiae* (PSA) Biofilms

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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 vine 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 consider killing of PSA bacteria in biofilms. A method was established to grow PSA in flow chamber cells to form biofilms. Biofilms were treated with 10ppm, 20ppm and 50ppm of ClO2 (provided as 1000ppm stock solution by Tadeusz Krogulec Southwell Products 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 2-3% of the entire biofilm population, only about 0.6% of bacteria in biofilms survived treatment with 50 ppm ClO2. 2.5% and 3.5% of cells in biofilms survived when treated with 20 ppm and 10 ppm of ClO2, respectively. When treated with 50 ppm ClO2 in some cases biofilms were observed where even complete killing could be observed. These results suggest that PSA biofilms can be efficiently treated with the provided Southwell Products Ltd ClO2 i.e. PSA was efficiently killed while embedded in the biofilm matrix.

Introduction

Chlorine dioxide (ClO2) is widely used as disinfectant for treatment of waste water, fruits and vegetables. Disinfection ability of ClO2 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 anti-biotics, bacteria are more resistant to ClO2 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 vines (2). Previously we have evaluated the ability of ClO2 to kill PSA grown in planktonic mode. In this study we investigated the killing effect of ClO2 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 (*Pseudomonas syringae* pv. *actinidiae* NZV13) was firstly inoculated in KB (Kings Broth) media and grown for 18 hrs in planktonic mode at 22°C. 500µl of overnight culture was used to inoculate flow cells filled with KB media and allowed to attach for 4 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°C for 72 hrs. For treatment with ClO2, each flow cell chamber were either incubated with normal saline (negative control), with 10 ppm, 20 ppm or 50 ppm of ClO2 for 1 h. After treatment with ClO2, KB media was allowed to flow through the flow cells for 15 min for washing. The ClO2 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 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. Image analysis indicated that 2-3% of dead cells (or 97-98% live cells) did naturally occur in the PSA biofilm. The green cells in the figure represent the viable cells whereas dead cells are stained red (Fig. 1).

PSA biofilms were exposed to 10 ppm of chlorine dioxide for 1h and then analysed by Confocal Scanning Laser Microscopy. This treatment resulted in a significant reduction of live cells to about 3.5% of the biofilm cell population (Fig. 2).

To assess whether a further increase in ClO2 concentration would improve killing of PSA in biofilms, the concentration was increased two-fold to 20 ppm. Biofilms were analysed by Confocal Scanning Laser Microscopy and a further reduction of live cells to about 2.5% of the biofilm population was found. Variation in killing efficiency is most likely due to variation in cell density, polymer matrix density and biofilm thickness throughout the biofilm (Fig. 3).

The concentration of was increased to 50 ppm to assess whether a further increase in killing efficiency i.e. more dead cell of PSA in biofilms would be achievable. Biofilms were analysed by Confocal Scanning Laser Microscopy and a further reduction of live cells to about 0.6% of the biofilm population was observed. In some cases even a total killing was observed (Fig. 4). Variation in killing efficiency is most likely due to variation in cell density, polymer matrix density and biofilm thickness throughout the heterogeneous biofilm (Fig. 4).

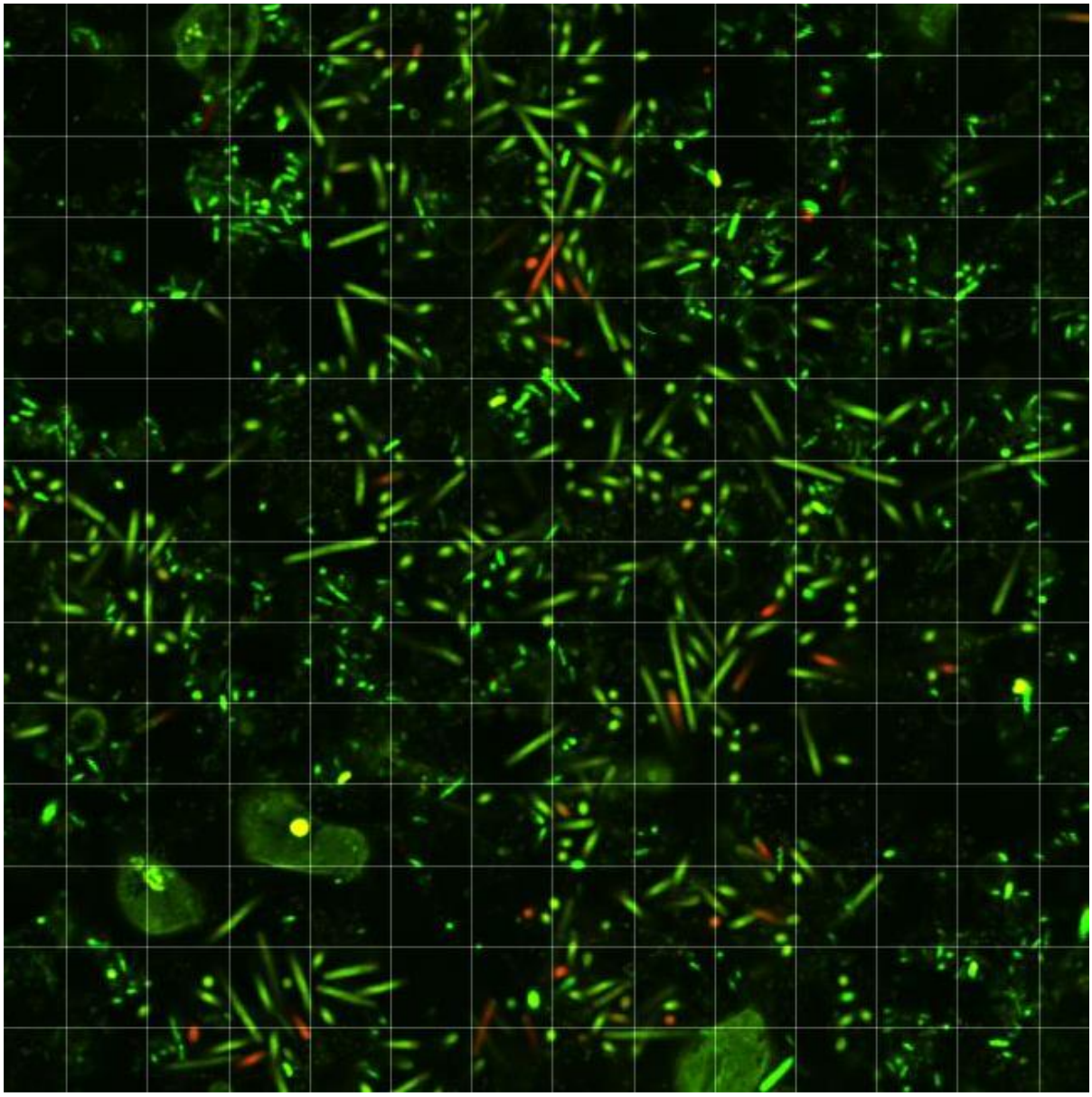
References

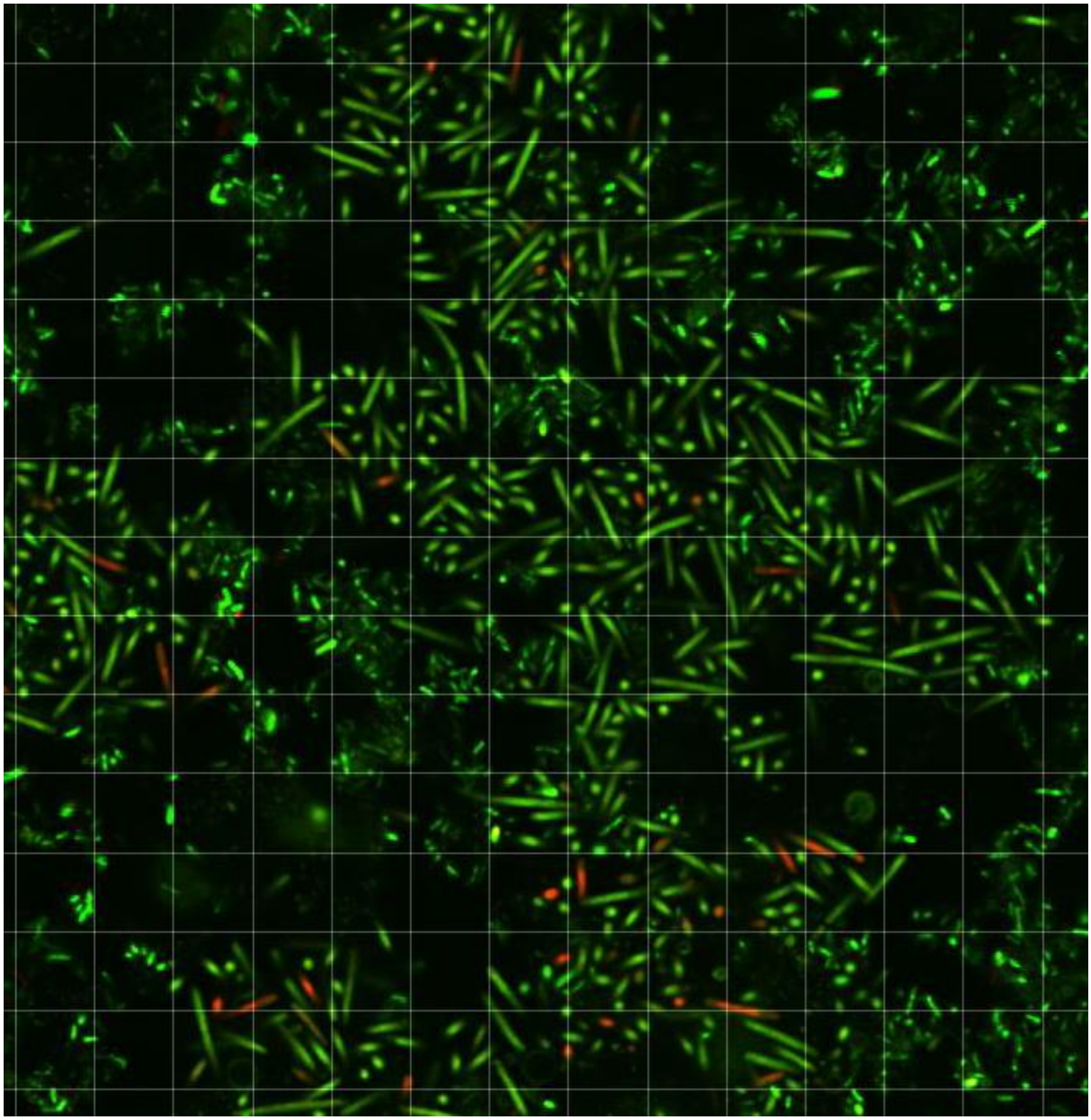
1. Behnke, S., and A. K. Camper. 2012. Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells. Biofouling 28:635-47.

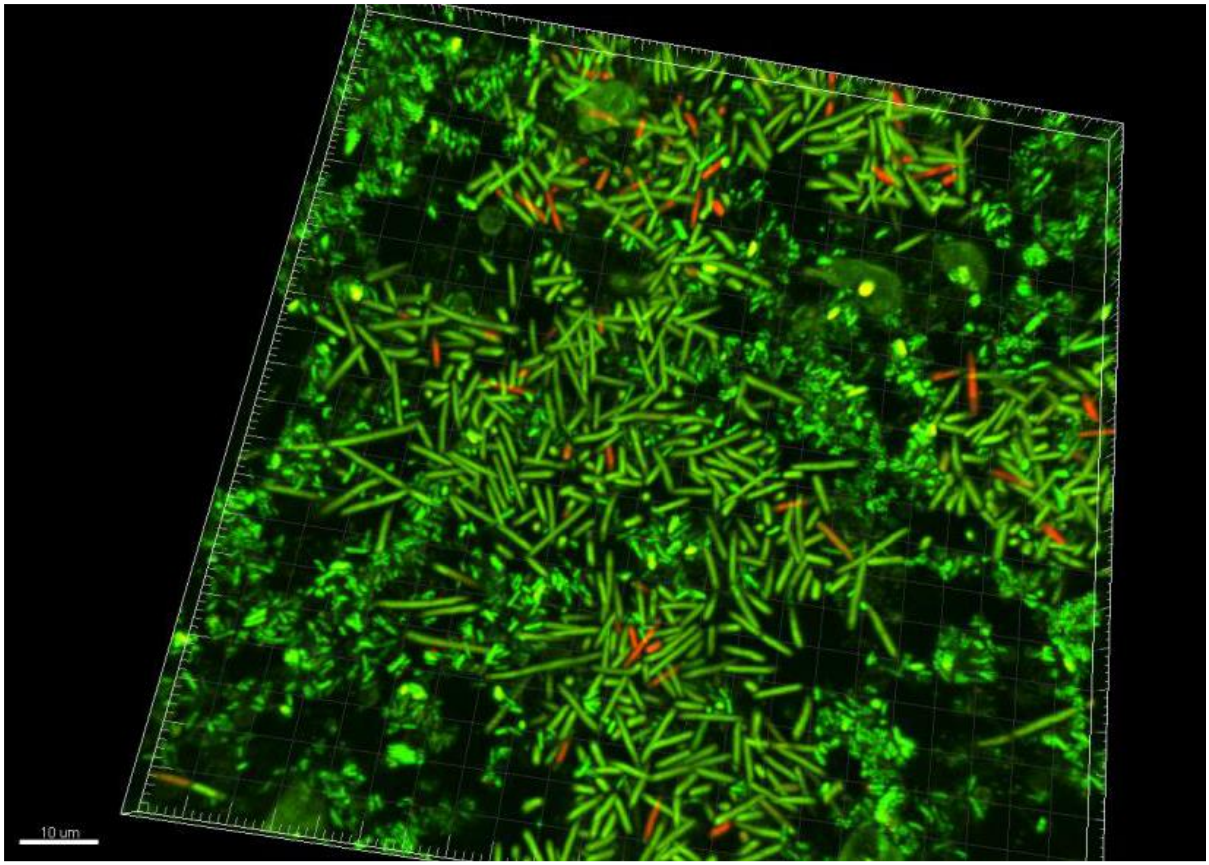
2. Chapman, J. R., R. K. Taylor, B. S. Weir, M. K. Romberg, J. L. Vanneste, J. Luck, and B. J. Alexander. 2012. Phylogenetic relationships among global populations of Pseudomonas syringae pv. actinidiae. Phytopathology 102:1034-44.

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.



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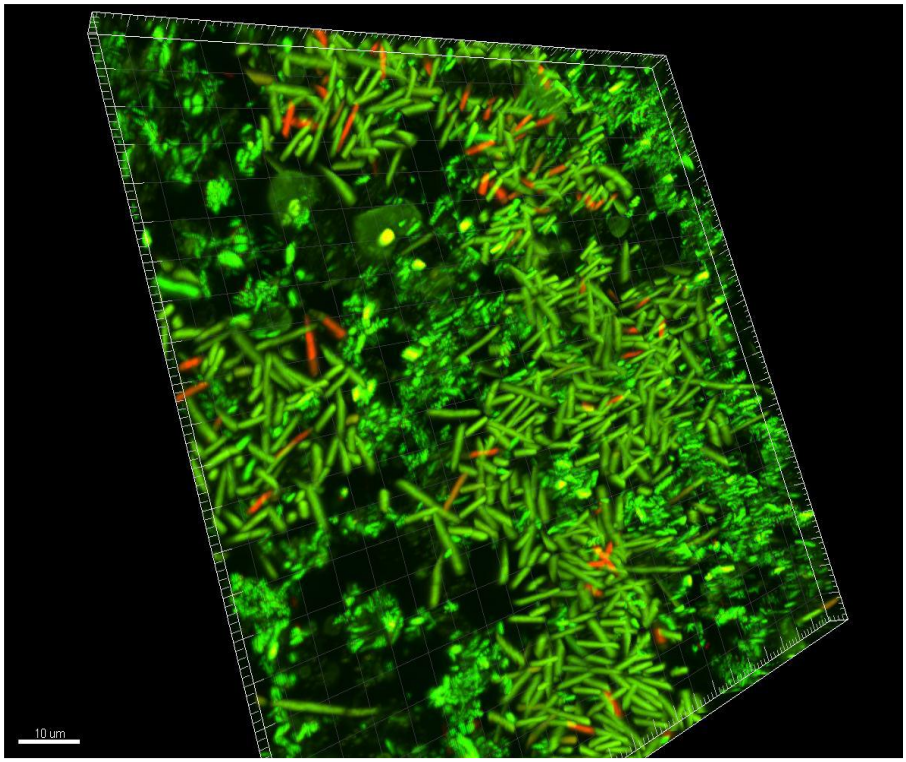
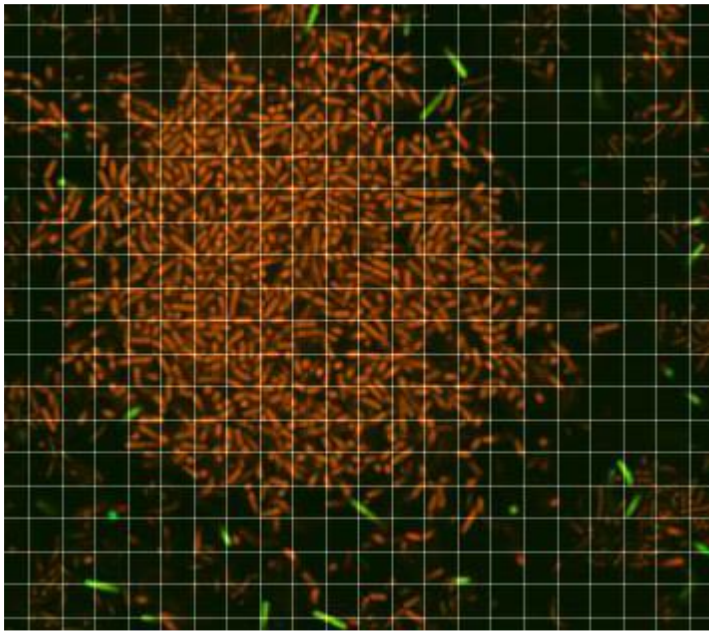
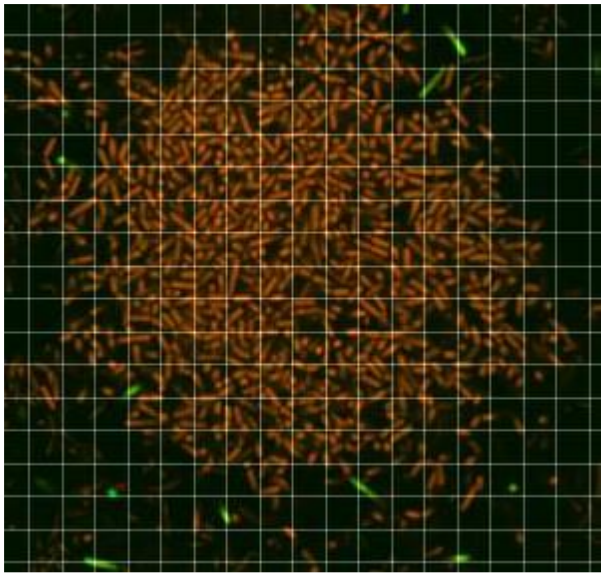
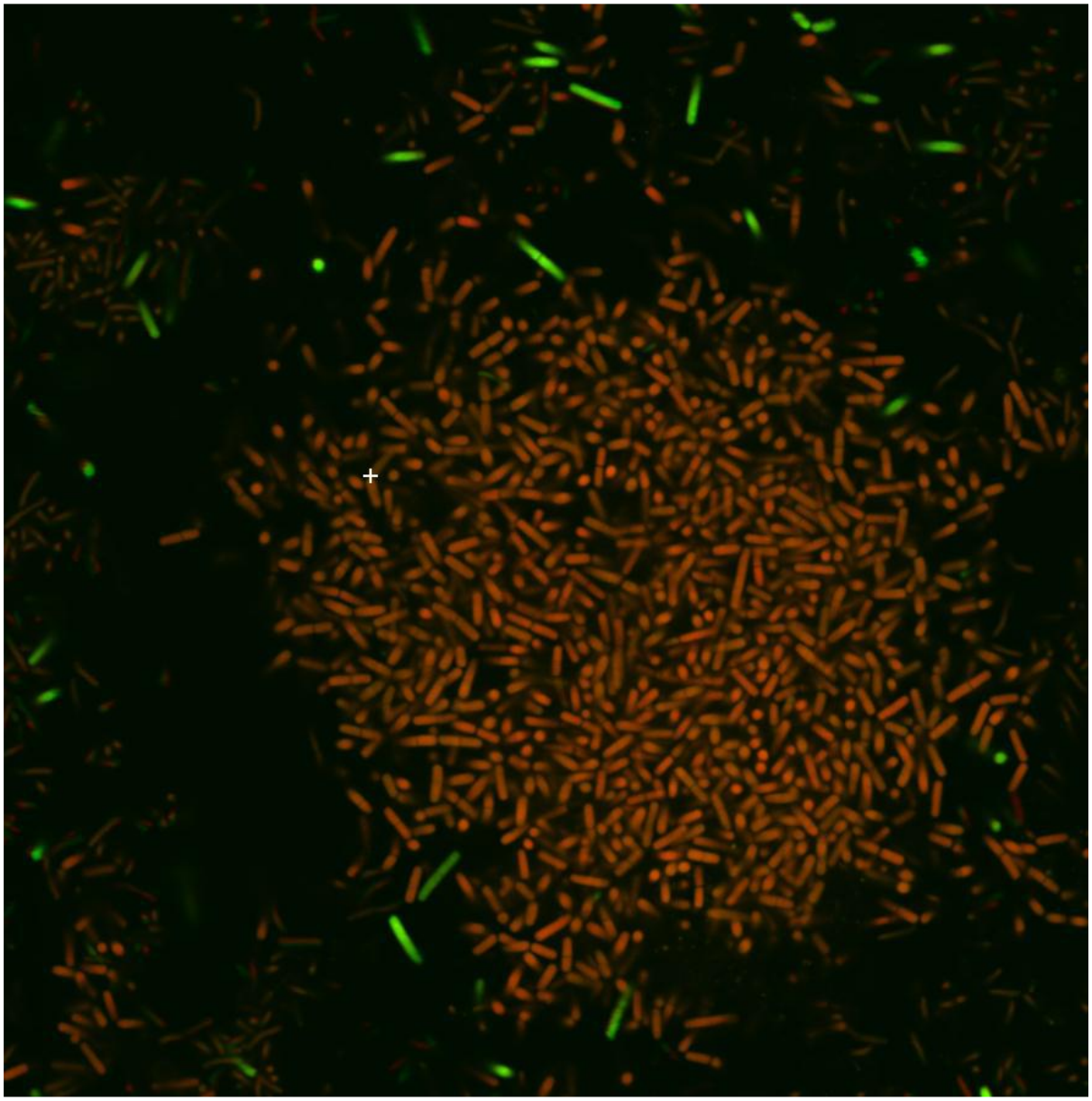
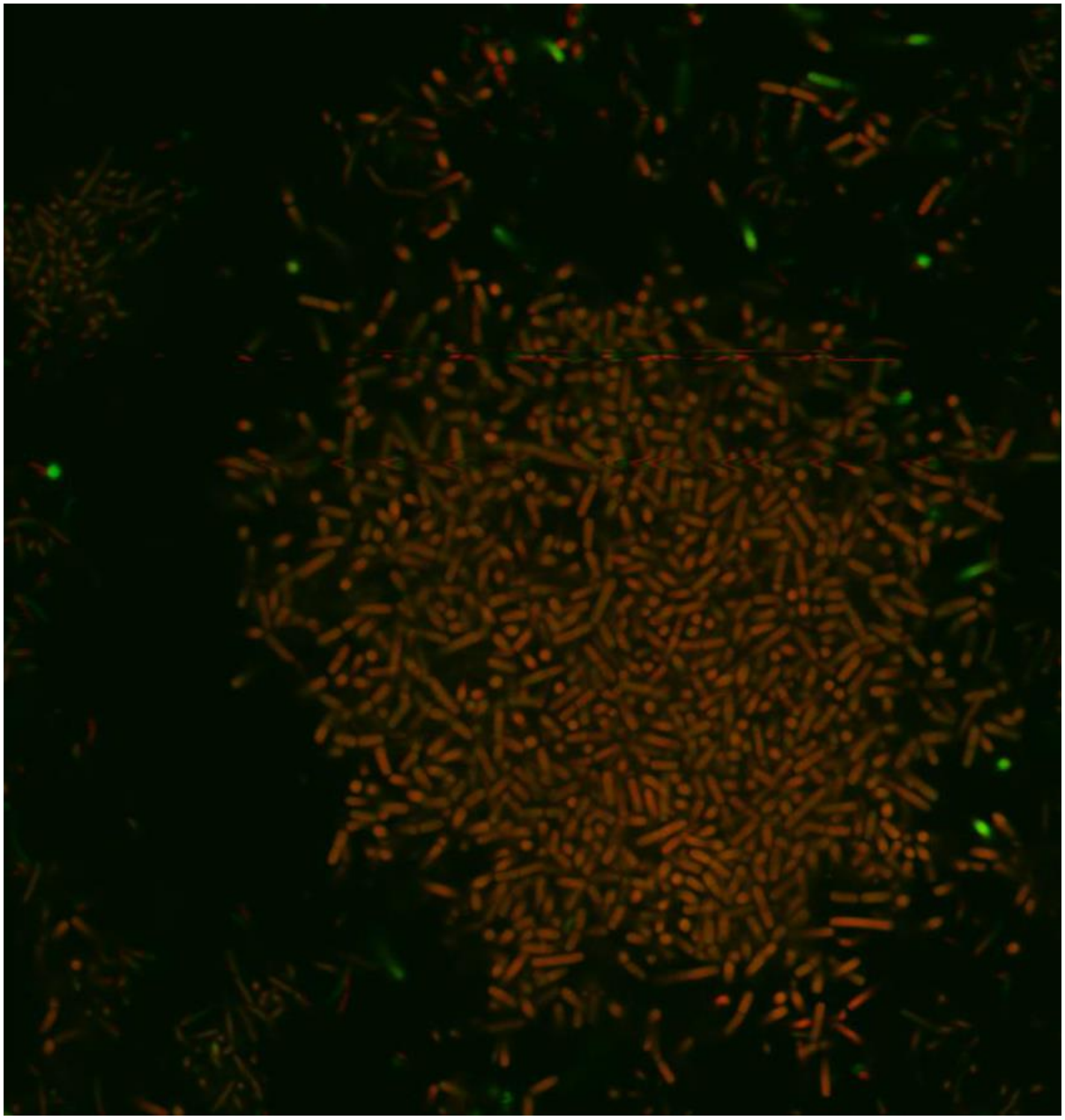


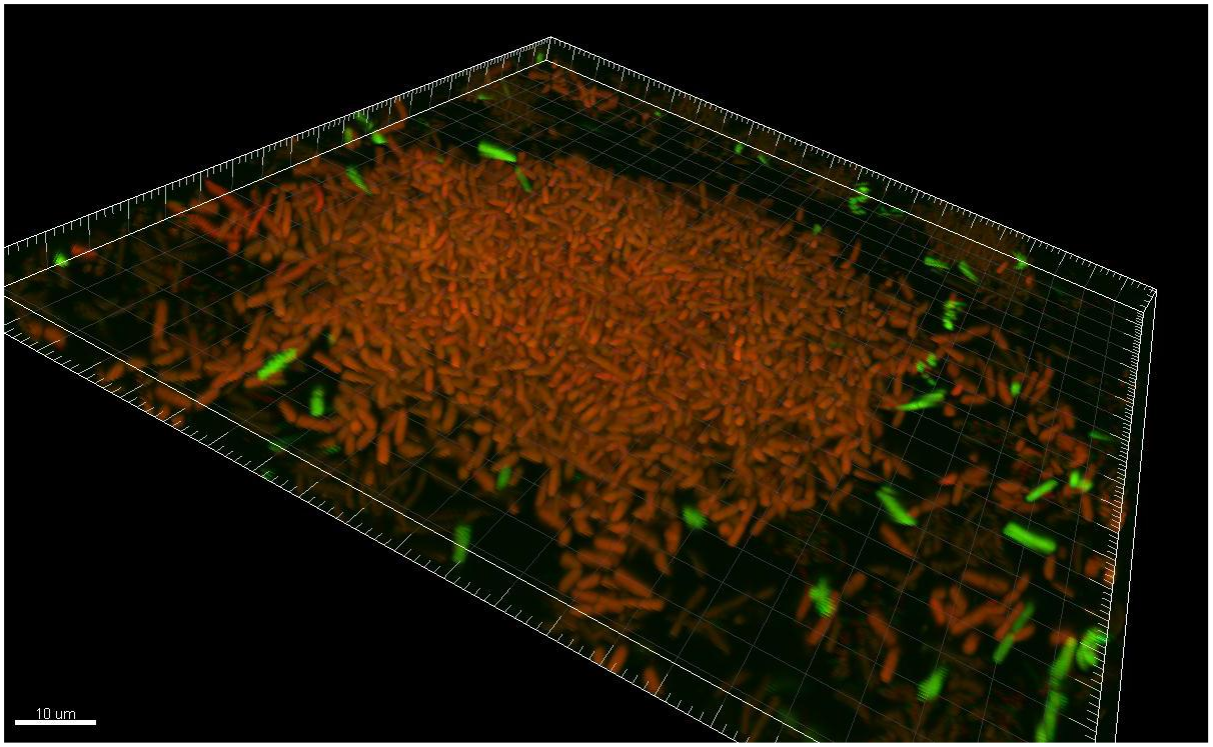
Fig. 2: Confocal scanning microscope images of PSA grown for 72 h in the biofilm mode and treated for 1 h with 10 ppm of Southwell IP Ltd ClO2. The viable cells are bright green in colour and dead cells are red in colour. **3.5% live cells**







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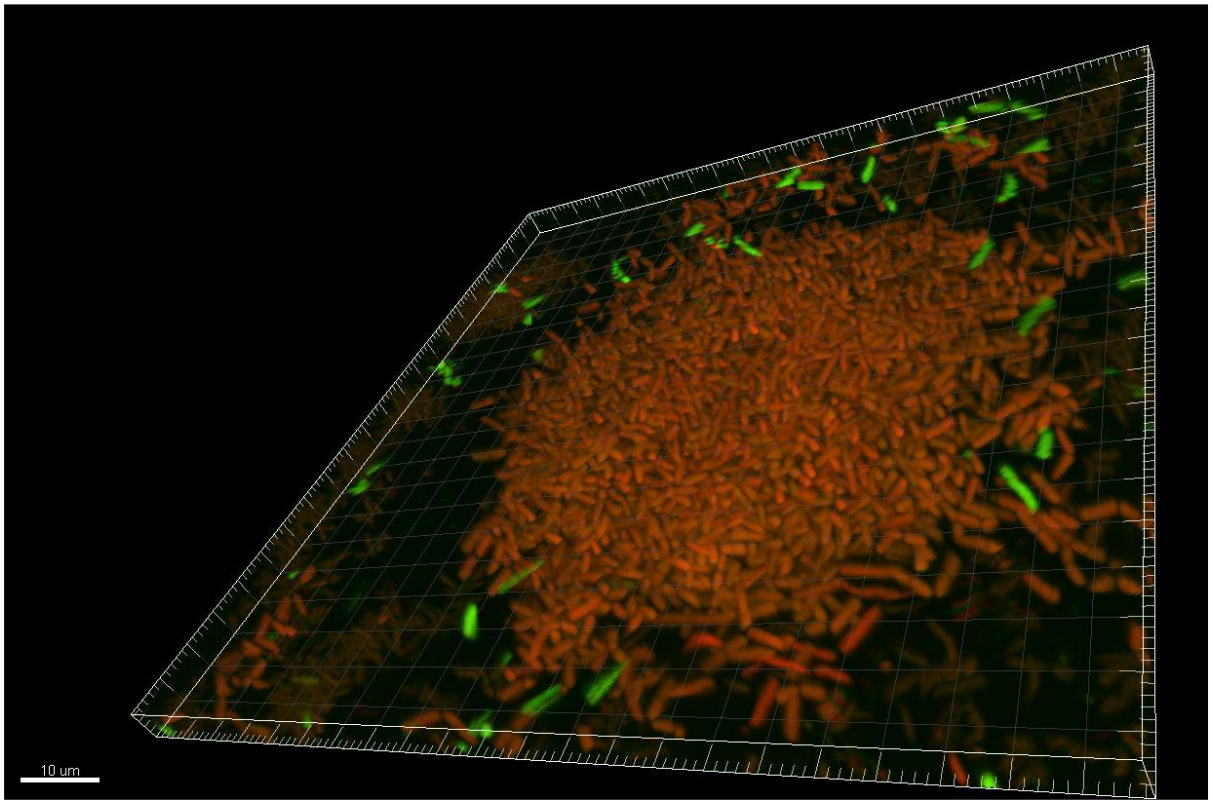
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