The Quantitative Assessment of Antimicrobial Agents: The Case of Sub-Inhibitory Concentrations of *Eucalyptus globulus* Essential Oil Against *Staphylococcus aureus*

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Running title: Antimicrobial activity of *Eucalyptus globulus* against *S. aureus*

Abstract
We have applied techniques that enable us to measure quantitatively the action of an antimicrobial agent upon the five characteristic growth parameters of a microbial culture. The methods were used to study the action of *Eucalyptus globulus* (Myrtaceae) essential oil upon a suspension of *Staphylococcus aureus* in a standard growth medium. We showed that the *Eucalyptus* oil produced a cultivable population reduction during both the adaptation phase and the exponential growth phase with the same specific reduction rate. This reduction rate was determined as an explicit function of the oil concentration. The oil does not affect the adaptation time. Our results suggest that the oil inactivates the bacteria via a "target" that is accessible to the oil whilst the internal cell transformation prepare the cell for its exponential growth period, but later, as the cells transform themselves into the stationary phase, this target ceases to be "accessible" to the oil. This process is reversible; the change from "accessible" to "inaccessible" occurs when the bacteria are transforming into the stationary phase.

Keywords: antimicrobial, conductancemetry, essential oil, *Eucalyptus globulus*, growth parameters, *Staphylococcus aureus*,

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Introduction

Many plant oils and extracts have been traditionally used as topical antiseptics or have been reported to have antimicrobial properties. With the resurgence of interest in natural therapies and increasing consumer demand for effective, safe, natural products quantitative data on the antimicrobial effects of plant extracts are required. This interest is reflected by the large number of articles concerned with the screening of potential antimicrobial agents published in this journal such as Da Silva *et al.* (2006), Dob *et al.* (2006), Dulger (2006), Kuçuk *et al.* and Mahid (2006) to cite a few. These, and many similar articles, all use either the agar disc diffusion technique or the broth dilution method to test the possible antimicrobial activity via the determination of a minimum inhibitory concentration (MIC). The results obtained by the disc diffusion and dilution methods for the same microbe and antimicrobial agent may vary between assays and there is often only a weak correlation between the results obtained by the two methods (Donaldson *et al.* (2005)). The usefulness of these methods is limited to the generation of preliminary qualitative data and they cannot give any more precise information than the estimation of a concentration threshold that delimits an "inhibition – no inhibition" effect. It is very important to screen a large number of potential antimicrobial agents before undertaking more sophisticated investigations.

In order to progress in our understanding of the action of an antimicrobial agent we must have quantitative information about how the agent modifies the principal parameters that characterize the growth of a microbial suspension in a medium containing the potential antimicrobial agent:

The adaptation time of the microbe to the medium.

A specific reduction rate of the cultivable cell population during the adaptation phase due to the action of the antimicrobial agent.

A specific growth rate of the cell population during the exponential growth phase.

If the antimicrobial agent does not totally inhibit growth, the maximum cell concentration attained at the beginning of the stationary growth phase.

A specific reduction rate of the cultivable cell population during the stationary phase due to the action of the antimicrobial agent.

Certain agents, depending upon their concentration, might modify one or more parameters, others might only affect the adaptation time and yet others might only act upon dividing cells and so affect only the exponential growth phase. An antimicrobial agent is deemed to have an action if it modifies at least one of the above parameters.

In naturally occurring situations when antimicrobial action is required, the bacteria are in one of two forms: as a planktonic suspension or as a sessile biofilm. The study of antimicrobial action against biofilm bacteria is certainly very important, but it also requires very specific techniques. Biofilm bacteria proffer unexpected resistance to standard prophylaxis, due partly to the protection afforded by the extra-cellular polymeric substances that form the biofilm. See Caubet *et al.* (2004) for a technique using antimicrobial molecules combined with electric fields and for further references. Here, we restrict our attention uniquely to the investigation of the action of antimicrobial agents against microbes in suspension.

We have developed new original methods (Ellison *et al.* (2007)), that can be used with automatic multi-cell conductance or multi-cell light absorbance techniques. Compared with the usual applications of these techniques our methods give precise quantitative information about the action of an antimicrobial agent upon the five suspension growth parameters as a function of the antimicrobial concentration. Our methods have a light work-load, avoid most of the traditional CFU plate-counts and can provide real-time specific growth rates. This latter possibility is useful when investigating the effects of physical and chemical stresses upon microbial growth.

As an illustration of these methods, we present a study of the antimicrobial activity of a commercially available *Eucalyptus* (Myrtaceae) essential oil against *Staphylococcus aureus*. *Eucalyptus* extracts are often used in oral hygiene products (mouth rinses, toothpastes, anti-plaque chewing gums), surface cleaning 'wipes' etc. Many screening reports, using disc diffusion and dilution techniques, have established an antimicrobial activity of *Eucalyptus* extracts from various species against a number of pathogens including Inouye *et al*. (2001) (*Haemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus*), Farah *et al*. (2001) (*Escherichia coli, Staphylococcus aureus, Aspergillus niger*), Takarada *et al.* (2004) *(Porphyromonas gingivalis, Streptococcus sobrinus, Streptococcus mutans*), Wilkinson and Cavanagh (2005) (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Pseudomonas aeruginosa*), Mounchid *et al.* (2005) (*Escherichia coli*) and Schelz *et al*. (2006) (*Escherichia coli, Staphylococcus epidermidis*). To our knowledge, there is no published investigation of the effect of *Eucalyptus* oil on any of the parameters characterizing the growth phases of a microbial suspension.

Our growth paradigm is derived from the "three linear phase" model described by Buchannan *et al*. (1997), in which $T_{Adapt}(c)$ and $\mu_{Exp}(c)$ are precisely defined. We have the following possible actions when *Staphylococcus aureus* cells are inoculated in a medium containing a concentration c of *Eucalyptus* oil:

The oil modifies the adaptation time, $T_{Adapt}(c)$, of the cells to the medium.

The oil causes a *Cultivable Population Reduction* (CPR) during the adaptation phase (CPR_{Adapt}). Thus, during the adaptation time, the cell concentration will decrease with a specific rate $\mu_{Adapt}(c)$.

The oil modifies the specific exponential growth rate $\mu_{Exp}(c)$ of the suspension.

The oil modifies the maximum cell concentration, $CFU_{Max}(c)$, of the suspension.

The oil causes a *Cultivable Population Reduction* during the stationary phase (CPR_{Stat}). Thus, during the stationary phase, the cell concentration will decrease with a specific rate $\mu_{Stat}(c)$.

We shall determine $T_{Adapt}(c)$, $\mu_{Adapt}(c)$, $\mu_{Exp}(c)$, $CFU_{Max}(c)$, $\mu_{Stat}(c)$ for a suspension of *S. aureus* in a standard growth medium containing a concentration *c*, $0 \leq c \leq 1600$ ppm, of *Eucalyptus* oil and discuss the implications of this data and directions for further studies designed to elucidate the mode of action of *Eucalyptus* essential oil against this bacteria.

Material and Methods

Apparatus
The conductance data were obtained with the Rapid Automatic Bacterial Impedance Technique (RABIT), Don Whitley Scientific, Shipley, U.K.

Conductance growth medium
Whitley Impedance Broth (W.I.B.), a non-selective medium designed to have relatively large conductivity changes as a function of microbial growth, (Don Whitley Scientific).

Bacteria and temperature conditions
Staphylococcus aureus (C.I.P. 103429) was stored in a glycerol peptone medium at -80°C. Freshly thawed aliquots were used for each experiment. The growth temperature was 37°C.

Suspended bacteria culture
A pre-culture was made by inoculating frozen cells in 10 mL of Müller-Hinton broth. After incubation for 24 h at 37°C, 2 mL of the pre-culture was inoculated in 150 mL Müller-Hinton broth contained in a 250 mL Erlenmeyer flask and shaken at 120 rpm for 24 h in a water bath (type SW23, Julabo, Germany) at 37°C. Samples (0.1 mL) of different cell concentrations were then inoculated into 4 mL of W.I.B. medium at 37°C containing known a known concentration of antimicrobial agent.

CFU/ml counts
The CFU/ml counts were made using the spiral colony counting method (Whitley Automatic Spiral Plater, Don Whitley Scientific). The inoculated plates of Plate Count Agar (PCA), Difco, U.S.A., were incubated for 24 h at 37°C before counting the colonies.

The antimicrobial agent
The *Eucalyptus* (*Eucalyptus globulus*) was a commercial essential oil. No further refinement or chemical analysis was performed. It was stored at 4 °C. A 10% (volume-volume) mother solution was prepared using DMSO. *Eucalyptus* oil concentrations in the W.I.B. medium were in the range 0 to 1600 ppm.

The suspension growth parameters protocol
The protocols and the mathematical analysis behind our methods are fully described in Ellison *et al*. (2007). We recall here the minimum explanation necessary to understand how the results are obtained. We use an automatic conductance method to monitor the microbial growth. With modern devices one can continuously monitor several hundred suspension samples simultaneously. The principle of the conductance method is that a growing microbial suspension, by the excretion of ionic metabolic substances into the surrounding liquid medium,

modifies the conductivity of the growth medium. We have shown that, in the case under consideration, the conductance changes for *S. aureus* are proportional to the cell concentration. Thus, automatic monitoring of the conductance of the suspension gives real-time information about the microbial cell concentration. For very low cell concentrations the conductance change is not detectable, as growth continues the cell concentration increases to a certain value N_{Det}, at which point the apparatus does detect a conductance change. The time, T_{Det}, after inoculation of the microbes in the growth medium at which this occurs is called the 'detection time'. For suspension growing in a growth medium containing a concentration *c* of antimicrobial agent there is a relationship between the initial cell concentration C_0, the adaptation time $T_{Adapt}(c)$, the CPR reduction rate $\mu_{Adapt}(c)$, the specific exponential growth rate $\mu_{Exp}(c)$ and the detection time:

$$\text{Log } C_0 = -\mu_{Exp}(c)T_{Det} + \beta(c),$$

where the adaptation factor $\beta(c)$

$$\beta(c) = (\mu_{Exp}(c) + \mu_{Adapt}(c))T_{Adapt}(c) + \text{Log } N_{Det}.$$

The quantities $\mu_{Exp}(c)$, $\beta(c)$, $T_{Adapt}(c)$, $\mu_{Adapt}(c)$, $CFU_{Max}(c)$ and $\mu_{Stat}(c)$ can be determined by a series of experimental protocols involving the automatic measurement of detection times. They are described in detail in Ellison *et al.* (2007). The only plate-counting necessary is for the determination of the initial suspension cell concentrations. The first step is to determine $\mu_{Exp}(c)$ and $\beta(c)$ for a series of inhibitor concentrations. The second step is to determine the adaptation time $T_{Adapt}(c)$, for the same concentrations. Once the adaptation time is know one can determine $\mu_{Adapt}(c)$ either from the above formula, since N_{Det} is known. The penultimate step consists in the determination of $CFU_{Max}(c)$ and whether or not there is a CPR during the stationary phase for each concentration. If there is a CPR, then one must determine $\mu_{Stat}(c)$.

Acquired microbial resistance protocol
We shall see that *S. aureus* cells in contact with *Eucalyptus* oil and grown until a stationary phase is reached are resistant to inactivation by the oil. The resistance may be due to a temporary adaptation or to a more permanent modification of the cells. To distinguish between these two possibilities we evaluate this resistance by growing suspensions of *S. aureus* in W.I.B. or W.I.B + *Eucalyptus* oil until the stationary phase is reached, then inoculating stationary phase bacteria into either pure W.I.B. or into W.I.B. + *Eucalyptus* oil and recording the conductance data. The cycle inoculation, growth to stationary phase is repeated several times. The degree of acquired microbial resistance to the *Eucalyptus* is reflected in the detection times.

Statistical analysis
Linear regressions were performed using a maximum likelihood analysis rather than a least squares method, as the latter is very sensitive to the occasional and inevitable outlaying points. Non-parametric significance tests for differences between mean values were used, since the necessary hypotheses required for using Student's t-test could not be verified.

Results

The relationship between the initial cell concentration C_0 and the conductance detection time T_{Det} for a *S. aureus* suspension in pure W.I.B. is given by:

$$\text{Log } C_0 = -\mu_{Exp}(0)T_{Det} + \beta(0), \text{ where } \mu_{Exp}(0) = 1.97, \beta(0) = 21.18, \text{ with } r^2 = 0.997.$$

The adaptation time is given by the formula $T_{Adapt}(0) = (\beta(0) - \text{Log}N_{Det})/\mu_{Exp}(0)$, where N_{Det} is the cell concentration that produces a conductance change detection. For this species of *S. aureus* $N_{Det} = 5.2\text{x}10^6$ CFU/ml. This gives an adaptation time of $T_{Adapt}(0) = 2.9$ h for stationary phase *S. aureus*, cultured in Müller-Hinton broth inoculated into pure W.I.B. medium.

Determination of $\mu_{Exp}(c)$ and $\beta(c)$
The specific exponential phase growth rate $\mu_{Exp}(c)$ and the factor $\beta(c)$ for *Eucalyptus* oil concentrations in the range $0 \leq c \leq 1600$ ppm are shown in Fig. 1. A maximum likelihood regression analysis gives $\mu_{Exp}(c) = 1.97 - 0.0007c$, with a linear correlation coefficient $r^2 = 0.98$ and $\beta(c)$ is constant and equal to 22.267, with sd = 0.392. Thus, $\beta(c)$ is independent of the oil concentration. This is a special situation and, as shown in Ellison *et al.* (2007), suggests that for the oil concentration range 0 to 1600 ppm there is a CPR during both the adaptation and the exponential phases with the same specific reduction rate = $\mu_{Exp}(0) - \mu_{Exp}(c) = 0.0007c$ and that the adaptation time $T_{Adapt}(c)$ of *S. aureus* from the stationary phase in the Müller-Hinton broth to the W.I.B. + *Eucalyptus* mixture does not depend upon the oil concentration. Thus $T_{Adapt}(c)$ should be equal to 2.9 h for all c up to 1600 ppm.

Adaptation phase results
In figure 2 we have the results of the adaptation phase experiments for 500, 1000 and 1500 ppm oil concentrations. After inoculation, the detection time differences between samples with and without oil decrease, this corresponds to the CPR during the adaptation phase. Once the bacteria have adapted to the medium the exponential growth phase begins with a linear relationship between contact time and detection time difference. The increasing straight line begins when the bacteria have been in contact with the *Eucalyptus* oil for slightly less than 3 h for each concentration. This represents the adaptation time, which is in very good agreement with the 2.9 h obtained from the previous experiments and confirms that the *Eucalyptus* oil concentration has no notable effect upon adaptation time.

As an independent check upon the estimation of $T_{Adapt}(c)$ we carried out CFU plate-counts every 30 min during the period from inoculation up to detection time for an oil concentration of 1000 ppm. This enabled us to determine independently the CPR during the adaptation phase and so determine $T_{Adapt}(1000)$. The results are shown in figure 3. Again, the slope change occurs slightly less than 3 h after the inoculation.

Stationary phase results.
The conductance changes as a function of time and *Eucalyptus* concentration are shown in Fig. 4. In our conditions the CFU/ml cell concentration is proportional to the conductance change and so the relative cell concentrations are the same as the relative conductance changes (Ellison *et al.* (2007)). The maximum CFU concentration is always the same. The only variability is the time taken to reach this maximum value. Thus $CFU_{Max}(c)$ does not depend upon c.

The detection times of suspension samples taken during the first 24 h of the stationary phase and inoculated into pure W.I.B. are shown in figure 5. The detection times show no significant differences between all the oil concentrations and all stationary phase samples taken over the 24 h period. This means that the stationary phase cell concentrations are not significantly different. Hence, there is no CPR during the first 24 h of the stationary phase.

The microbial resistance persistence
The results for a *Eucalyptus* oil concentration of 1600 ppm are shown in table 1. The significance of the inoculation growth cycle notation is as follows: For example, "W+E $\rightarrow$ W $\rightarrow$ W+E" means:

Samples are inoculated in W.I.B. containing a 1600 ppm *Eucalyptus* concentration and allowed to grow to a stationary phase.
Samples are then inoculated in pure W.I.B. and allowed to grow to a stationary phase.
Samples are inoculated in W.I.B. containing a 1600 ppm *Eucalyptus* concentration and allowed to grow until a conductance change is detected.

The detection time of the final suspension is recorded. Each of the cycle experiments was performed with n = 8 samples.

The detection time for *S. aureus* growing in pure W.I.B. is 1.4 h. When the cells reach a stationary phase and are re-inoculated into W.I.B. containing 1600 ppm of *Eucalyptus* oil the detection time is 6.4 h. Cells cultivated in W.I.B. + 1600 ppm *Eucalyptus* until they reach a stationary phase and then re-inoculated into pure W.I.B. again have a detection time of 1.4 h. Thus *Eucalyptus* resistant cells, when inoculated into pure W.I.B. have a normal growth pattern. *S. aureus* cells that have undergone two or three growth cycles in W.I.B. + 1600 ppm *Eucalyptus* oil do not show any change in the corresponding detection times. Thus, the acquired resistance of the stationary phase cells is not modified by successive contacts with *Eucalyptus* oil.

In all samples, there was no significant difference in the microbial growth characteristics from the detection time up to the resulting stationary phase. This means that once the exponential growth phase begins, the past history of the cells does not influence the subsequent microbial growth.

Discussion

It is obviously important to have some information about the *Eucalyptus* oils' composition, but large variations exist, both in extraction methods and in the plants themselves. For example, Della Port *et al*. (1999) analyzed the composition of *Eucalyptus globulus*. They found that with one type of extraction method the principal components were 1,8-cineole 62.6%, α-pinene 10.5%, aromadendrene 8%, p-mentha-1,3,5-triene 1.6%. A different extraction method, using the same source of *Eucalyptus* leaves, gave the components: 1,8-cineole 48.2%, α-pinene 6.9%, p-mentha-1,3,5-triene 3.8% and aromadendrene 13.7%. Other published composition

analyses naturally vary in the percentages of each component. This illustrates one of the major difficulties working with essential oils, namely the difficulty of obtaining reproducible samples. In our case this is not a problem.

The principal component is not necessarily the active antimicrobial component. One cannot draw hasty conclusions about the active component of the oil uniquely from a standard chromatography analysis. The antimicrobial effect of *Eucalyptus* essential oils has sometimes been ascribed to the presence of 1,8-cineole, its principal component. This is not necessarily true. Lis-Balchin and Deans (1997) tested *Eucalyptus globulus, Eucalyptus radiata, and Eucalyptus citriodora* against 20 different strains of *Listeria monocytogenes*. The major component of *Eucalyptus globulus* was 1,8-cineole (90.8%), in *Eucalyptus radiata* it was present at 84% and in *Eucalyptus citriodora* it was only present at 0.005%. However, the *Listeria* strains used in the study did not correlate with the 1,8-cineole concentration. The *Eucalyptus globulus* inhibited 6 out of 20 strains, the *E. radiata* and *E. citriodora* inhibited all 20 strains. The above results indicate that 1,8-cineole is not necessarily the principal active agent, at least for *Listeria* strains. Belaiche *et al.* (1995) studied the antimicrobial effects of three components of *Eucalyptus* oil: 1,8-cineole, linalool and eugenol upon *Staphylococcus aureus*. They found that 1,8-cineole had no effect, but the other two terpenes, used individually, had significant effects, but used at the same time had antagonistic effects. Farah *et al*. (2001, 2002) also confirmed the lack of antimicrobial activity of 1,8-cineole against *Staphylococcus aureus, Escherichia coli* and *Aspergillius niger*.

In this report we are concerned with the general effect of the oil upon the growth parameters of a *S. aureus* suspension. The detailed study of each of the principal components of *Eucalyptus* essential oil will be the object of a separate study.

The adaptation and exponential phase results

For *Eucalyptus globulus* oil concentrations in the range 0 to 1600 ppm there is a CPR during the adaptation phase, with a specific reduction rate $\mu_{Adapt}(c)$ proportional to the oil concentration. During the exponential growth phase the observed specific growth rate is the difference between the specific growth rate in pure W.I.B. and $\mu_{Adapt}(c)$. Thus, the reduction in the specific growth rate during the exponential phase is the same as the CPR during the adaptation phase. This suggests that the action of the *Eucalyptus globulus* oil does not depend upon the cell states during the adaptation and exponential phases.

The adaptation time of *S. aureus* to the W.I.B. + oil growth medium over the concentration range is practically constant and corresponds to the adaptation time of *S. aureus* pre-cultured in Müller-Hinton broth and inoculated into W.I.B. This suggests again that the presence of the *Eucalyptus* oil in the growth medium does not affect in a substantial way the internal metabolic cell transformations involved in the adaptation process.

The stationary phase results

During the adaptation and exponential phases *Eucalyptus globulus* produces a *CPR*, with a specific reduction rate $\mu(c)$ that depends upon the oil concentration c. If this specific reduction rate is less than the cell division rate, then it is to be expected that, once the stationary phase is reached, $CFU_{Max}(c) = CFU_{Max}(0)$. In fact, we do observe that there is no effect upon CFU_{Max} (Figure 4). Whatever the initial concentration in cells or concentration in *Eucalyptus*, the final cell concentration in the medium is always the same. The only factor that varies is the time necessary to reach this maximum cell concentration.

During the stationary phase there is no CPR. This is slightly surprising. The hypothesis that there is some component of the oil that has a non specific destructive effect upon the integrity of the cell membrane is frequently evoked, often with little or no convincing experimental evidence. If there is some such agent in *Eucalyptus* oil, then one would also expect it to continue acting during the stationary phase, i.e. one should observe a CPR and this is not the case.

A possible explanation is that this hypothetical non specific inactivating agent is depleted as the suspension grows. If this is the case then once the inactivating agent has been reduced to a concentration below a threshold level, the instantaneous specific growth rate $\mu(t,c)$ in the medium containing a concentration *c* of *Eucalyptus*, should become equal to $\mu(t,0)$ at some point before the stationary phase is reached. The time at which this occurs corresponds to the moment at which all the 'inactivating factor' in the *Eucalyptus* oil has been reduced to below its threshold level. The instantaneous specific growth rates as a function of time and as a function of instantaneous cell suspension concentration are shown in figures 6 and 7. We observe that $\mu(t,c)$ does not converge to $\mu(t,0)$ at some time before the beginning of the stationary phase and that in figure 7, $\mu(t,c)$ is equal to $\mu(t,0)$ for all *Eucalyptus* oil concentrations only when the cell suspension concentration is close to the

stationary phase cell concentration. This indicates that the lack of CPR during the stationary phase is not due to some inhibiting factor in the *Eucalyptus* that has been depleted.

This leaves us with the possibility that it is the bacteria themselves have changed in some way so as to be resistant to the *Eucalyptus* oil in the medium. The second representation of the specific growth rates (figure 7) shows that this adaptation is achieved, for all the *Eucalyptus* oil concentrations used, when the cell concentration has reached about 70% of its final value. This is very near to the final cell concentration and the microbes are either in, or close to, a stationary phase physiological state.

The resistance of the stationary phase *S. aureus* to the *Eucalyptus* oil suggests the following phenomenological scenario:

Suppose that *S. aureus* cells possess a "target" via which some component of the *Eucalyptus* oil inactivates the cell.

When the target is "accessible" the cell is inactivated. If, due to physiological transformations within the cell, the target becomes "inaccessible", then the oil does not inactivate the cell.

When such cells are inoculated into a medium containing *Eucalyptus* there is a CPR during the initial adaptation phase and during the exponential growth phase, so the target is accessible.

As the stationary phase is reached, internal physiological transformations make the target inaccessible and so the cells are not inactivated by the *Eucalyptus*.

This scenario is, of course, very simplified, but it has the merit of being testable experimentally. We tested the existence of this phenomenon.

The results indicate that there is no residual effect of the acquired resistance to inactivation of the stationary phase cells by *Eucalyptus* oil when these cells are re-inoculated into the growth medium containing *Eucalyptus* oils and that successive growth cycles of cells in a medium containing *Eucalyptus* oils do not modify the acquired resistance of the stationary phase cells. These observations suggest that the resistance to the *Eucalyptus* is not due to some mutation effect but that the accessibility – inaccessibility of the *Eucalyptus* target is the result of reversible physico-biochemical transformations within the cells as they enter the stationary state.

Conclusion

We have applied techniques that enable us to measure quantitatively the action of an antimicrobial agent upon the five characteristic growth parameters of a microbial culture. The methods were used to study the action of *Eucalyptus globulus* essential oil upon a suspension of *Staphylococcus aureus* in a standard growth medium. We showed that the *Eucalyptus* oil produced a cultivable population reduction during both the adaptation phase and the exponential growth phase with the same specific reduction rate. This reduction rate was determined as an explicit function of the oil concentration. The oil does not affect the adaptation time. Our results suggest that the oil inactivates the bacteria via a "target" that is accessible to the oil whilst the internal cell transformation prepare the cell for its exponential growth period, but later, as the cells transform themselves into the stationary phase, this target ceases to be "accessible" to the oil. This process is reversible; the change from "accessible" to "inaccessible" occurs when the bacteria are transforming into the stationary phase.

The next step in the elucidation of the action of *Eucalyptus* oil against *S. aureus* would be to test individually, and in combination, different fractional components of the oil in order to determine those that do have an effect upon the bacteria.

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TABLE

Table 1: Detection times for the various growth to stationary phase cycles (W = pure W.I.B., W + E = pure W.I.B. + 1600 ppm *Eucalyptus* oil).

Cycle	Mean Detection time (h)	sd
W	1.4	0
W → W+E	6.4	1.7
W+E→W	1.4	0
W+E→W→ W+E	6.3	1.3
W+E→W+E→W+E	6.3	0.8

FIGURE CAPTIONS

Fig. 1. Specific growth rate $\mu_{Exp}(c)$ (♦) and $\beta(c)$ (■) as functions of *Eucalyptus* oil concentration.

Fig. 2. Contact time v Difference in detection times between growth in pure W.I.B. and for bacteria in contact with *Eucalyptus* oil (♦ = 500 ppm, ▲ = 1000 ppm, ■= 1500 ppm).

Fig. 3. CFU/ml counts v Contact time for a concentration of 1000 ppm *Eucalyptus* oil.

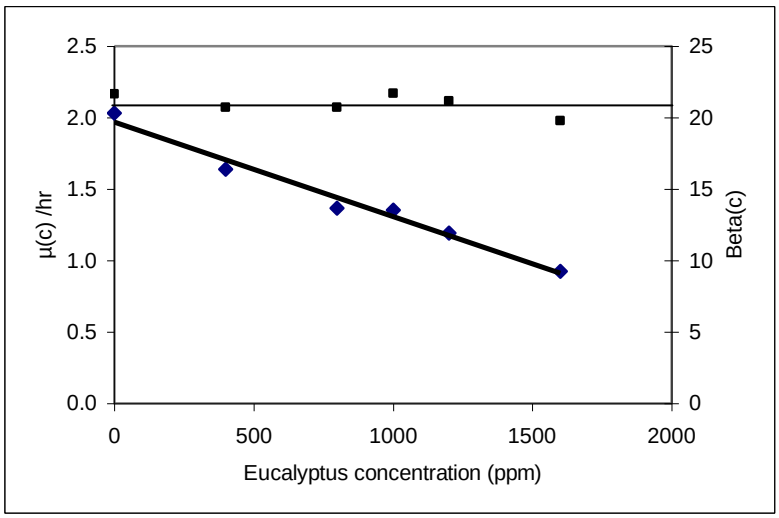
Fig. 4. The conductance changes of the *S. aureus* suspension as a function of the contact time for each *Eucalyptus* oil concentration.

Fig. 5. Detection times of samples taken during the stationary phase and inoculated into pure growth medium.

Fig. 6. Instantaneous specific growth rate, as function of time, for different oil concentrations.

Fig. 7. Instantaneous specific growth rate as function of the maximum cell concentration percentage for various oil concentrations.

SEVEN FIGURES, ONE PER PAGE



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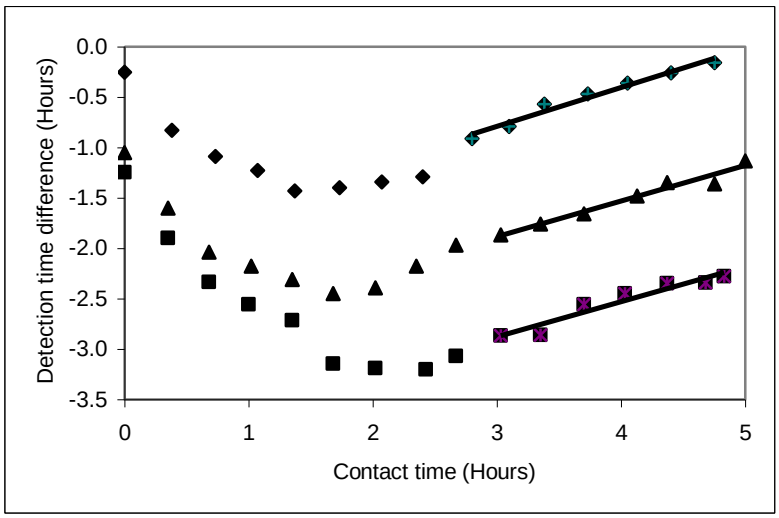


Fig. 2. Contact time v Difference in detection times between growth in pure W.I.B. and for bacteria in contact with *Eucalyptus* oil (♦ = 500 ppm, ▲ = 1000 ppm, ■= 1500 ppm).

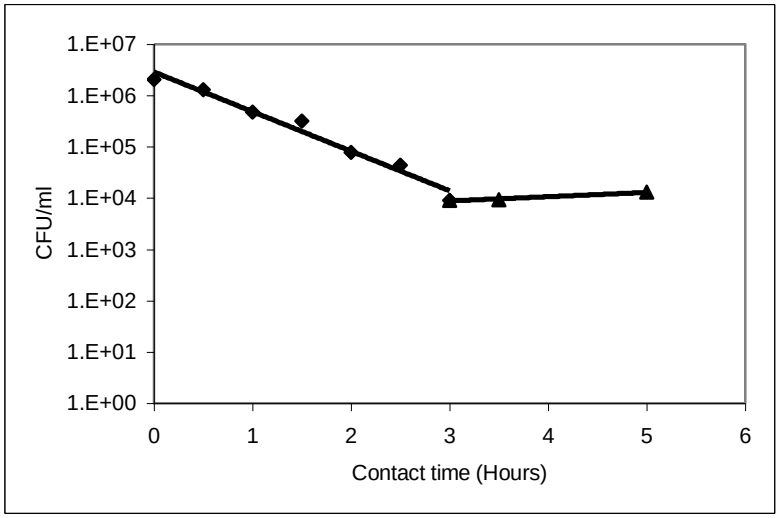


Fig. 3. CFU/ml counts v Contact time for a concentration of 1000 ppm *Eucalyptus* oil.

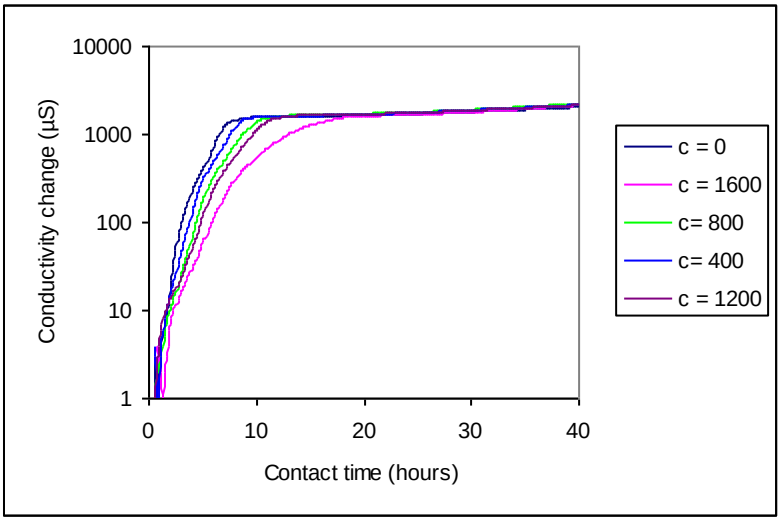


Fig. 4. The conductance changes of the *S. aureus* suspension as a function of the contact time for each *Eucalyptus* oil concentration

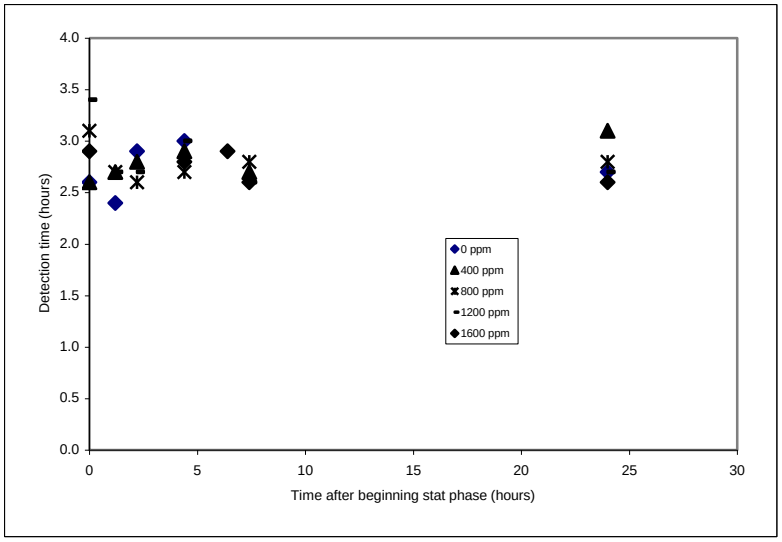


Fig. 5. Detection times of samples taken during the stationary phase and inoculated into pure growth medium.

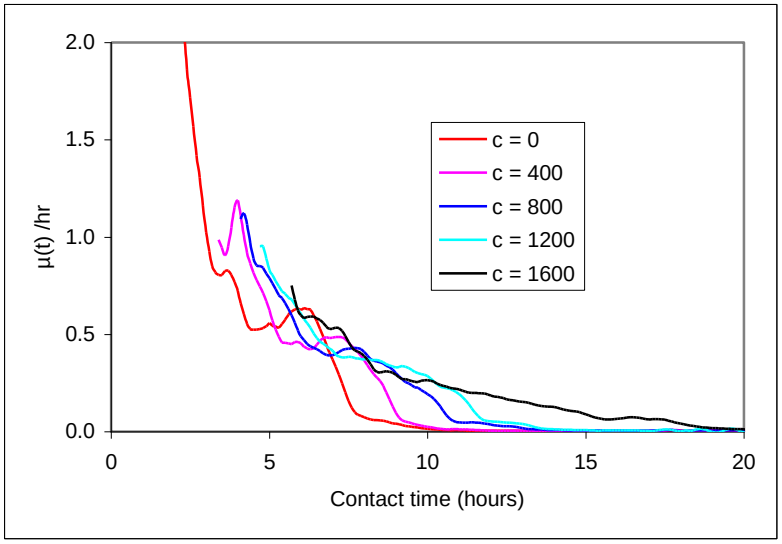
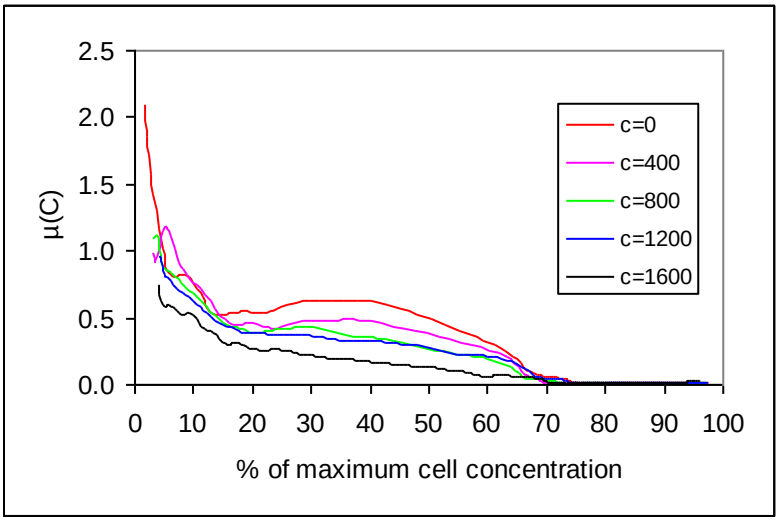


Fig. 6. Instantaneous specific growth rate, as function of time, for different oil concentrations



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