Appl Microbiol Biotechnol (2009) 82:773–783
DOI 10.1007/s00253-009-1879-x

METHODS

Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm

Alberto Mariscal · Rosa M. Lopez-Gigosos · Manuel Carnero-Varo · Joaquin Fernandez-Crehuet

Received: 28 November 2008 / Revised: 15 January 2009 / Accepted: 16 January 2009 / Published online: 6 February 2009

Abstract A new, quick method, using the resazurin dye test as a bacterial respiration indicator, has been developed to assay the antibacterial activity of various substances used as disinfectants against bacterial biofilm growth on clinical devices. Resazurin was used to measure the presence of active biofilm bacteria, after adding disinfectant, in relation to a standard curve generated from inocula in suspension of the same organism used to grow the biofilm. The biofilm was quantified indirectly by measuring the fluorescent, water-soluble *resorufin product* produced when resazurin is reduced by reactions associated with respiration. Four products used as disinfectants and the biofilm growth of five bacterial species on carriers made of materials commonly found in clinical devices were studied. Under test conditions, chlorhexidine, NaOCl, ethanol, and Perasafe at concentrations of 0.2, 0.01, 350, and 0.16 mg/ml, respectively, all produced 5-log reductions in biofilm cell numbers on the three different carriers. The redox-driven test depends on bacterial catabolism, for which reason resazurin reduction produces an analytic signal of the bacterial activity in whole cells, and therefore could be used for determining disinfectant efficacy in an assay based on the metabolic activity of microorganisms grown as biofilm or in suspension.

Keywords Biofilm · Resazurin · Disinfectant · Fluorescence

A. Mariscal (✉) · R. M. Lopez-Gigosos · J. Fernandez-Crehuet
Department of Preventive Medicine and Public Health,
Faculty of Medicine, Malaga University,
Boulevard Louis Pasteur, 12,
29071 Malaga, Spain
e-mail: mariscal@uma.es

M. Carnero-Varo · J. Fernandez-Crehuet
Service of Preventive Medicine, University Clinic Hospital,
Malaga, Spain

Introduction

Resazurin is a nontoxic, water-soluble dye which is reduced by electron transfer reactions associated with respiration, producing resorufin, a water-soluble product easily measured by fluorescence or visible light spectrophotometry (O'Brien et al. 2000). Upon reduction, resazurin changes color from blue to pink to clear, as oxygen becomes limiting within the medium (Twigg 1945). The first stage of this reduction is due to the loss of an oxygen atom loosely bound to the nitrogen of the phenoxazine nucleus. This change to pink resofurin is not reversible by atmospheric oxygen and is largely independent of both reduction potential and oxygen content. The second stage of reduction to the colorless state is reversible by atmospheric oxygen (Guerin et al. 2001). Resazurin is very stable in a culture medium without cells, but is rapidly reduced in the presence of living cells. Several reductases, such as diaphorases and NADPH dehydrogenase, could use resazurin as an electron acceptor and be responsible for the reduction of resazurin into resofurin (O'Brien et al. 2000).

Resazurin has been used to assess viability and bacterial contamination, to test for antimicrobial activity, and as an indicator of bacterial cell numbers (Shiloh et al. 1997; Sarker et al. 2007). Over the last few years, several assays for biofilm quantification in microtiter plates have been described. In a recent study, a resazurin assay was optimized and evaluated for the quantification of biofilms formed in microtiter plates and compared with another six assays previously described in the literature (Peeters et al. 2008a). In this study, the resazurin assay and a similar assay using fluorescein diacetate were the best alternatives for microbial biofilm quantification. On the other hand, the same method based on resazurin was used to evaluate the efficacy of the reduction of biofilm by several procedures for the disinfection of clinical materials (Peeters et al. 2008b). These authors

emphasize the importance of the use of viability assays such as resazurin to evaluate the effect of disinfectant in relation to crystal violet staining.

Biofilms can be defined as an adherent matrix-enclosed bacterial population. Many microorganisms adhere naturally to solid surfaces in the solid–liquid interphase boundary, forming microcolonies which later produce extracellular polymeric substances, giving rise to biofilm. These biofilms can include other bacteria, in addition to diverse residues, and are usually more resistant to disinfectants and antibiotics than planktonic bacteria (Costerton 1999). Although biofilms are currently a focus of interest for researchers in the field of bioremediation of contaminated sites and wastewaters (Singh et al. 2006), they are regarded as a serious problem both in medical and nonmedical (industrial) settings. They are associated with infections related to medical devices, potable water distribution systems, and the food processing industry (Jessen and Lammert 2003; van der Wende et al. 1989; Reid 1999). Lately, different methods for rapid biofilm quantification have been developed. Some methods are based on the quantification of total biomass, including the matrix and both live and dead cells. Other methods just estimate viable cells or the matrix (Peeters et al. 2008b). A large number of methods for quantifying biofilm use conventional plating or are based on a staining method, such as the crystal violet assay (Stepanovic et al. 2000; Christensen et al. 1985; O'Toole and Kolter 1998).

It has been known for some time now that methods for evaluating biocide activity in a liquid medium are inadequate when bacteria are attached to surfaces or form biofilm (Buckingham-Meyer et al. 2007). The nature of biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents (antibiotics, disinfectants, or germicides), with remarkable differences in susceptibility of planktonic and biofilm organisms to antimicrobial agents (Donlan and Costerton 2002). Some procedures designed to determine the activity of detergents and disinfectants on biofilm bacteria use methods that are generally rather laborious. These methods, based on electronic microscopy techniques, or which use crystal violet dye or plate counts, are not normally very useful when it is necessary to process a large number of samples, as in studies on disinfectant efficacy (Buckingham-Meyer et al. 2007; Marion et al. 2006; Perret-Vivancos et al. 2008).

Likewise, when the aim is to determine the "real life" efficacy of a disinfectant against biofilm bacteria, other factors ought to be taken into account. The composition of the biofilm growth medium, the material to which it adheres, the hydration level of the surface or the biofilm at the time of quantification, and the static or dynamic growth environment of the biofilm, in addition to many other factors, are all critical (Buckingham-Meyer et al. 2007; Mariscal et al. 2007).

The aim of this study was to evaluate the efficacy of a biofilm disinfectant test based on resazurin. Unlike other tests, the biofilm-forming capacity of surfaces made of different materials, like those that are commonly used in clinical devices, can be evaluated using the assay developed in this study. In addition, this assay makes it possible to study a broad variety of microorganisms and chemical and physical conditions for biofilm growth. Likewise, our work also includes a comparative study of the resazurin-based method with two other methods habitually used to quantify biofilm microorganisms, which are based on sonication and violet crystal dye, respectively. Lastly, the potential standardization of the proposed method and—once developed—its use as a candidate method for analyzing the efficacy of disinfectants against bacterial biofilm, was analyzed. Five microorganisms commonly used in studies on disinfectants, four frequently used disinfectants, and three carriers made of different materials were included in this study.

Materials and methods

Bacterial strains and inoculum preparation

Pseudomonas aeruginosa ATCC27853, *Enterococcus hirae* CIP5855, *Escherichia coli* ATCC25922, *Morganella morganii* ATCC 25830, and *Staphylococcus aureus* CIP53154 were grown in tryptic soy broth (TSB) for 18–24 h at 37°C. To ensure that a uniform number of bacteria were always used, a graph of absorbance at 620 nm/colony forming unit (cfu) was prepared for each organism in TSB, and a final concentration of 10^6cfu/ml was adopted as the inoculum. The number of microorganisms was determined by conventional plating in tryptic soy agar (TSA).

Disinfectant assay on planktonic bacteria

The disinfectants used in this study were chlorhxidine digluconate, ethanol and sodium hypochlorite (all from Sigma), and Perasafe®, a commercial product employed in the chemical sterilization of clinical devices, whose activated solution is equivalent to peracetic acid at 0.26%. The bactericidal activity of these disinfectants was determined using the dilution–neutralization method against bacteria in suspension (AFNOR 1995). The neutralizing agent employed was D/E neutralizing broth (Difco). A 10-min contact time was used for Perasafe and a 30-min one for the other disinfectants. The contact time for the neutralizer was 10 min in all cases. The neutralizer was tested in accordance with norms UNE-EN 1040 and AFNOR NF T 72-150 (AFNOR 1995; European Standardization Committee 1997).

Carriers

The solid surfaces used as carriers in this study were pieces of tubing made of the following materials: polyvinyl chloride (PVC) with a 5-mm internal diameter (ID), and an 8-mm outside diameter (OD); polypropylene plastic (PPP, 5 mm ID, 6 mm OD); and silicone (SIL, 5 mm ID, 9 mm OD). The tubes were cut into fragments of the same length (10 mm), cleaned with alcohol–acetone (1/1), and then soaked in a 70% (*v*/*v*) solution of ethanol for 15 min. The carriers were then rinsed three times with sterile distilled water at room temperature, before being dried and stored in Petri dishes. The surface areas available for biofilm growth on the different tubing samples were calculated as cm^2, adding the outer and inner face of each tube and twice the cross-section surface according to the thickness of the tube wall.

Biofilm culture

Biofilms were produced by pipetting one hundred microliters of inoculum into 10 ml glass tubes containing 5 ml of TSB, into which a carrier had been previously introduced. After mixing, the tubes containing the carriers were incubated for 48 h at 37°C. During this period, the spent culture medium was pipetted out of the tubes at 12-h intervals and replaced with fresh TSB. Afterwards, the carriers were removed and introduced into tubes containing 5 ml of sterile water. Next, the water was extracted using a Pasteur pipette to stir up bacteria that had not adhered to the carriers and spent nutrients, after which the tubes were refilled with 5 ml of sterile saline solution (0.9% NaCl). All tubes containing biofilm carriers were rinsed twice more in this way immediately before each analysis.

Biofilm quantification

Crystal violet assay

A slightly modified method based on those previously described was adapted so as to quantify biofilm on the carriers used in this study (Peeters et al. 2008b). In this particular case, 2 ml 99% methanol was added to the tubes containing biofilm carriers, immediately after carrying out the three previously described rinses using sterile saline solution, for biofilm fixation. After 15 min, supernatants were removed, and the carriers were air-dried. Then, 2 ml CV at 0.05% in water (m/v) was added to the tubes and incubated at room temperature. Uninoculated carriers were used as controls. After 15 min, the CV was removed by washing the tubes with 5 ml of sterile water. The tubes containing biofilm carriers were rinsed twice more in this way. Finally, the adherent CV was extracted with 3 ml of 33% acetic acid, and the absorbance of the extract measured by spectrophotometry at 590 nm (Milton Roy Spectronic 301. Rochester, NY, USA). The background staining was corrected by subtracting the value for CV bound to controls. All experiments were carried out in triplicate.

Sonication procedure

Biofilms in triplicate were removed from their carriers by sonication using a Vibracell Sonicator (Sonic and Materials, Danbury, CT, USA), fitted with a 3.2-mm stepped microtip and operating at 40 kHz for 60 s, as has been previously described (Mariscal et al. 2007). The number of microorganisms was determined, after dilutions in saline solution, by conventional plating in TSA. In previous experiments, the efficacy of the sonication method used to detach bacteria from carrier was tested. For this reason, several sonication times (30, 60, 90, 120, and 180 s) were assayed for each microorganism and carrier. Once sonicated, biofilm carriers were analyzed according to the CV assay previously described with respect to carriers without biofilm. In all cases, after 60 s of sonication, the differences between the sonicated biofilm carriers and control carriers without biofilm were not statistically significant.

Resazurin biofilm assay

After rinsing, 3 ml of TSB-RS solution [TSB diluted 1:10 in saline solution containing 1:125 (*v*/*v*) resazurin solution, obtained from Sigma, Cat. No. R-6892] was added to each tube containing biofilm carriers. After thorough mixing (Vortex mixer), the tubes were incubated in a water bath at 37°C, and then fluorescence (λex 520 λem 590) was measured during 6 h at 15-minute intervals, using a LS30 fluorometer (Perkin Elmer, Beaconsfield, UK). In each assay, a standard growth curve (standard curve) for each microorganism, based on resazurin fluorescence activity, was simultaneously generated from a series of bacterial suspensions containing decimal dilutions of viable cells. To this end, for each microorganism, 10 ml of a 16–24 h suspension culture in TSB were centrifuged (6 min, 4,500 rpm) and the resulting pellet washed and resuspended in 10 ml of saline solution. Individual cultures were diluted in saline solution to a cell concentration of about 7–8 log cfu/ml. Afterward, the microorganism suspension was tenfold diluted nine times in TSB-RS and aliquoted in glass tubes containing 3 ml each (working cultures). As in the biofilm assay, the tubes were then incubated in a water bath at 37°C, and fluorescence measured under the same conditions. Simultaneously, 100 μl of the last four dilutions were plated in TSA and the cfu counted after 24 h of incubation at 37°C.

Disinfectant activity assay on biofilm carriers using RBA

The disinfectants used in this study were 2% chlorhexidine gluconate, 70% ethanol, 1% NaOCl, and Perasafe® (16.2 g/L). The effective concentration was determined by performing a dilution range in 1:10 TSB solutions of these disinfectants on carrier biofilm. Sterile distilled water was used as a control. Five milliliter4 of each one of the disinfectant dilutions were added to the tubes containing bacterial biofilm. After the contact time, the disinfectant was extracted using a Pasteur pipette, and 10 ml of D/E neutralizing broth was added. After 10 min at room temperature, the neutralizer was extracted, and the tubes containing the carrier biofilm were each washed three times with 5 ml of saline solution to remove the neutralizing agent. Once the wash solution had been extracted, 3 ml of TSB-RS were added to each tube containing the carrier biofilm and carrier control (without biofilm). After mixing (Vortex), the tubes were incubated in a water bath at 37°C and the fluorescence measured as already described.

Cell density in each assay with disinfectant using RBA was calculated from the regression line obtained from the corresponding standard curve and then $\log_{10}$-transformed. As before, only values between 10% and 90% of maximum resazurin reduction activity were used in the initial exponential growth phase of fluorescence emissions. For each of the assays, the logarithms of densities were converted into a measure of efficacy by logarithmic reduction (LR). LR is the average logarithmic density of control carriers minus that of the carrier treated with the corresponding disinfectant, in accordance with the previously described protocol (Buckingham-Meyer et al. 2007; Zelver et al. 2001).

Statistical analysis

Statistical differences among the results obtained for biofilm quantification by CV, sonication and resazurin assays were examined using the nonparametric Friedman test.

A *t* test of the slope for a linear least squares regression of the resazurin response on the log-transformed disinfectant concentrations was used to test for trend. A one-sided Dunnett's many-to-one test was conducted to determine the different concentrations of the same disinfectant for each one of the carriers and assayed bacterial species. *P* values of <0.05 were regarded as significant in all cases.

Results

Disinfectant assay on planktonic bacteria

The minimum inhibitory concentration (MIC), determined as the lowest concentration inhibiting bacterial growth, for the four test disinfectants is given in Table 1. Ethanol test concentrations were five serially double-diluted samples from an initial dilution of 70% during 30 min. Five serially decimal-diluted samples during 10 min were tested for 2% chlorhexidine, 1% ethanol, and 1.62% Perasafe. None of the bacterial strains showed growth in use-concentrations of the four test disinfectants.

Standard curve of bacteria using the resazurin assay

The average fluorescence signals, expressed as relative fluorescence units (RFU), obtained in the experiments with diluted working cultures containing resazurin solution after 5 h of incubation are shown in Fig 1. As can be seen, each regression line comprised a first phase with a high slope line, an intermediate phase of stability, whose duration varied depending on the strain, and a final phase of decline. For the same inoculum size, *E. hirae* and *S. arureus* generated a quicker fluorescence response than other microorganisms tested with the resazurin-based fluorometry method. The other microorganisms needed approximately 3 h to produce a maximum fluorescence signal. The fluorescence response of *P. aeruginosa* was slower, since inocula smaller than 10^8ufc/ml required between 4 and 5 h to reach the maximum fluorescence response. Using the RFU values obtained during the initial phase of exponential fluorescence emission of the inocula, the time needed for the fluorescence signal of each one of them to reach 50% of maximum fluorescence was calculated by regression

Table 1 Minimum inhibitory concentration (mg ml^{-1}) for clorhexidine, ethanol, NaClO, and Perasafe on the five bacterial test strains in suspension

	Clorhexidine	Ethanol	NaClO	Perasafe
Enterococcus hirae CIP5855	0.002	87.5	0.001	0.16
Escherichia coli ATCC25922	0.002	175	0.001	0.016
Morganella morganii ATCC 25830	0.002	175	0.01	0.016
Pseudomonas aeruginosa ATCC27853	0.0002	175	0.001	0.016
Staphylococcus aureus CIP53154	0.002	87.5	0.01	0.16

A 10-min contact time was used for Perasafe and a 30-min one for the other disinfectants

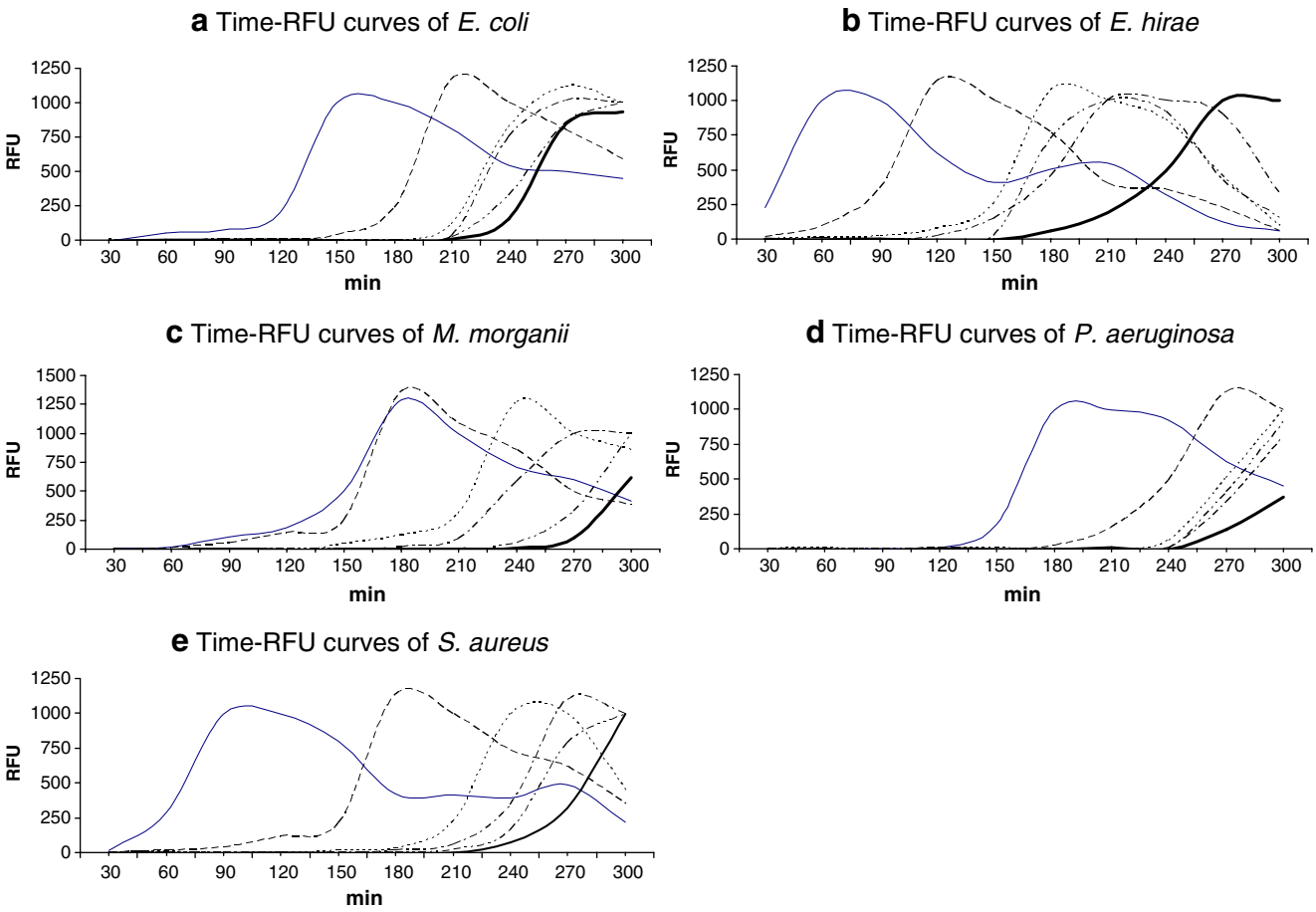

Fig. 1 Standard growth curves for different inoculum suspensions of *E. coli* (a), *E. hirae* (b), *M. morganii* (c), *P. aeruginosa* (d), and *S. aureus* (e) based on resazurin fluorescence activity expressed as relative fluorescence unit (RFU). Inocula in cfu/ml of 10^8(———), 10^7(- - - - - -), 10^6(.....), 10^5(-·-·-·-), 10^4(—··—··), 10^3(▬▬)

analysis. For calculating the 50% value, only emission values between 10% and 90% of the maximum value were estimated. Figure 2 shows the correlation between the inoculum size of each of the strains and the time needed for the fluorescence signal to reach 50%, together with the resulting equations and their R^2 values.

Biofilm quantification

The results of the three different methods for quantifying 48-h biofilms of the five test microorganisms grown on SIL, PVC, and PPP carriers are shown in Fig. 3. The average absorbency values obtained with the CV assay for the five biofilm-forming strains were normalized to square centimeters and are shown in Fig. 3a. The repeatability of the assay was high, since the standard deviation was between 4% and 11% in relation to average values. The absorbance values obtained in this assay for the two Gram-positive microorganisms, *E. hirae* and *S. arureus*, were significantly lower in comparison with those of the three Gram-negative microorganisms. Nonetheless, these differences were not as pronounced in the results of the other two quantification methods used, which are shown in Figs. 3b and c. As can be seen, the microorganism count per square centimeters obtained when using the sonication method was very similar for all five test species, and although the *Pseudomonas* counts, as with those of the other two Gram-negative microorganisms, were slightly higher, differences were not statistically significant.

Figure 3c shows the results obtained from the quantification of 48-h biofilm for each of the test strains, based on the time required to reach 50% fluorescence values in the resazurin assay. In all cases, the number of microorganisms estimated by means of this assay was slightly higher than those obtained using sonication, *E. hirae* and *S. aureus* being the microorganisms with the lowest counts. The differences were not, however, as pronounced as those observed in the CV assay. Likewise, on comparing the growth of each of the test bacterial species in relation to the carrier material in both methods—sonication and the fluorescence assay—no statistically significant differences were observed.

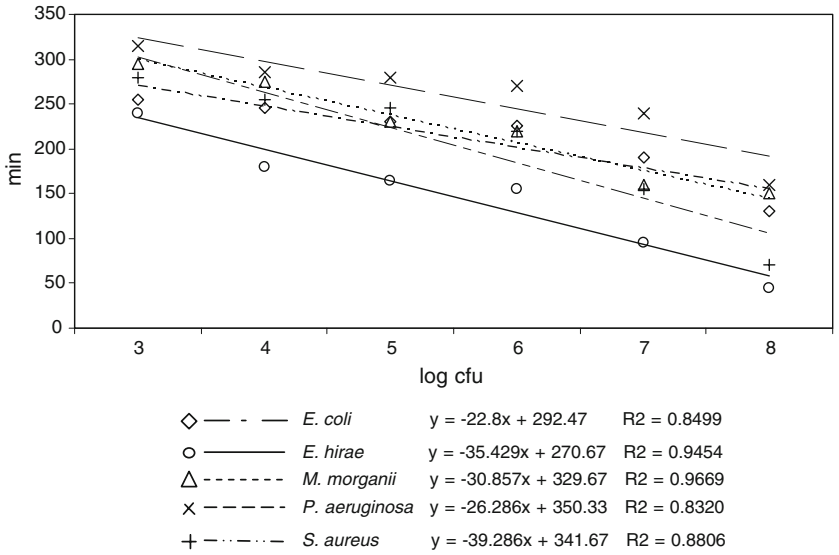

Fig. 2 Correlation between inoculum size expressed as log-cfu, and the time required to reach a 50% fluorescence level. Each point represents the average from five independent assays

The cell density of carrier biofilms in the resazurin assay was estimated on the basis of the hypothesis that a given number of biofilm cells will emit the same amount of fluorescence as that of the same number of cells grown in suspension. In each biofilm assay, a curve was simultaneously generated from the decimal dilutions of each one of the strains, in accordance with the method described for generating the standard curve of bacteria using the resazurin assay. Biofilm cell density was estimated from the RFU values emitted by each biofilm and the corresponding linear equations of 50% RFU values estimated from the standard curve. Figure 4 shows the fluorescence curves obtained over time for each one of the microorganisms forming biofilm on the three test materials. Figure 4 only shows the time needed for the fluorescence signal of each biofilm to reach maximum values (100% RFU). As can be seen, the two Gram-positive microorganisms and *E. coli* were the quickest in generating a maximum fluorescence signal (60 min), whereas *P. aeruginosa* needed double the time to generate the same signal. The number of microorganisms in each biofilm was established in terms of the time required to reach a 50% fluorescence signal and their comparison by means of interpolation with the values obtained from the standard curve generated from known inocula of each one of the strains. For estimating the corresponding equations of the biofilms and standard curves, only values between 10% and 90% of the RFU signal were taken into account.

Disinfectant activity assay on carrier biofilm using RBA

Table 2 shows the average LR values obtained from the action of each one of the disinfectants against the five test strains adhering to the PPP, PVC, and SIL carriers. Disinfectant concentrations whose LR values were 0 or >5 for all the microorganisms, and materials are not included in this table. As can be appreciated, concentrations of chlorhexidine at 0.2 mg/l, NaClO at 0.1 mg/l, ethanol at 350 mg/l, and Perasafe at 0.16 mg/l produced under test conditions an inhibition close to, or greater than, 99.999% of the metabolic activity measured with resazurin on all carrier biofilms. In the majority of cases, these concentrations turned out to be greater than the MIC obtained in tests conducted with cultures in suspension of the same strains shown in Table 1. Likewise, as can be seen in Table 2, some of the strains adhering to the SIL carrier showed a greater resistance to the disinfectants than when adhering to carriers made of other materials. These differences were most pronounced for chlorhexidine.

Discussion

Bacteria grown in suspension generally show less resistance to biocides than when grown on surfaces or forming biofilm (Costerton 1999; Carpentier and Cerf 1993; Maillard 2007/6). In addition, the methods generally used for determining the efficacy of disinfectants against cells in suspension are considered inadequate for determining their efficacy against cells adhering to surfaces or growing as biofilm, for which reason it is necessary to develop a standard disinfectant test for attached or biofilm cells (Luppens et al. 2002). Among the means employed, the plate count is probably one of the most common and simple methods to interpret when dealing with microorganisms grown in suspension. When microorganisms grow adhering to surfaces or as biofilm, the plate count method requires much more handling, does not ensure complete cell separation (both on the surface and in between), and the difference in biofilm cell concentrations can be so high (Ceri et al. 1999; Jackson et al. 2001) as to make tests difficult to standardize.

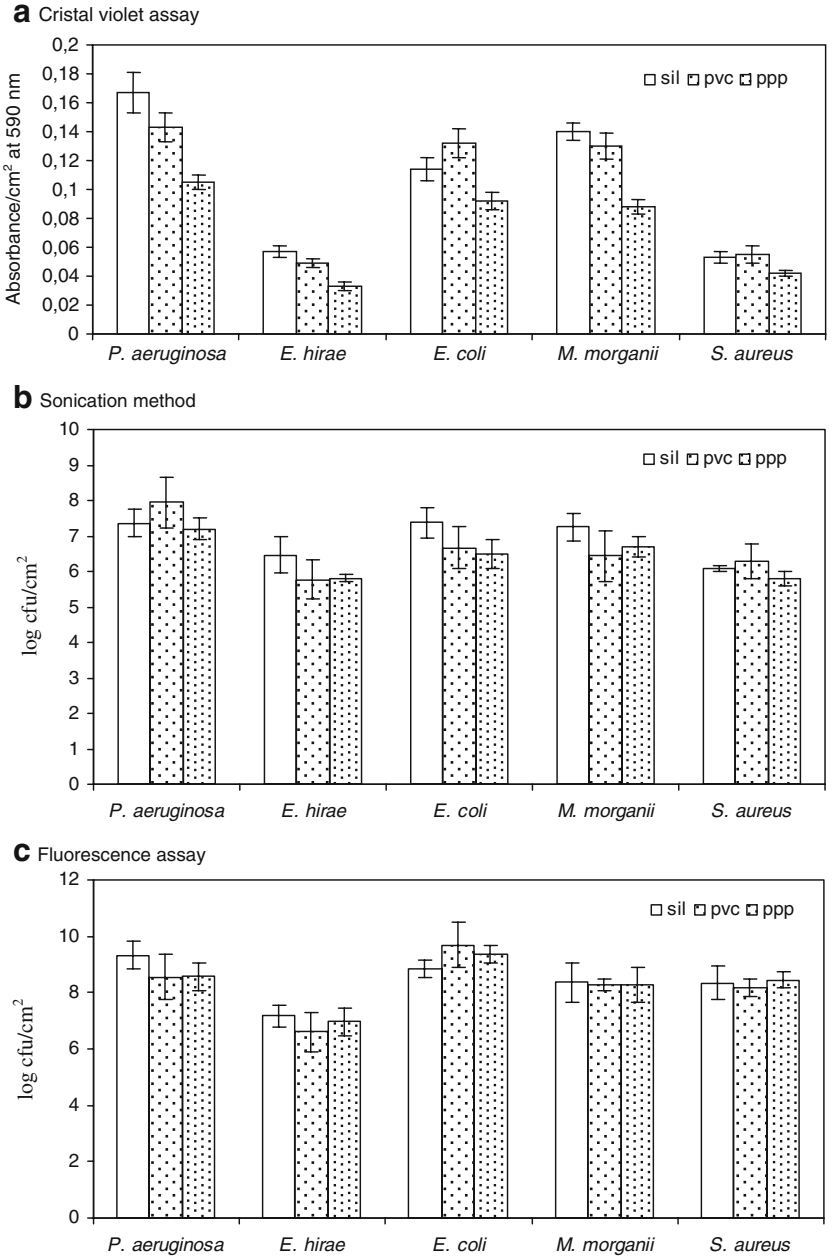

Fig. 3 A 48-h biofilm quantification of five bacterial strains on SIL, PVC, and PPP carriers by means of three test methods. **a** Crystal violet assay; **b** Sonication method and; **c** Fluorescence assay. *Bars* represent the SD of five independent assays

In this study, a fluorescence method based on the metabolic activity of several bacterial strains was studied to verify its usefulness for determining the efficacy of disinfectants against biofilm bacteria, and to propose a standard test. The relation of this fluorescence method with the other two methods, one based on crystal violet staining and the other on sonication, with regard to its capacity for quantifying the number of biofilm microorganisms, was also studied. Since evaluating and comparing the efficacy of disinfectants against different species was not the aim, except in the case of Perasafe, solutions of commercial disinfectant for hospital or domestic use were not employed.

Carriers made of PVC, SIL, and PPP, all of which are materials frequently found in clinical devices, were included in this study; nevertheless, the method's design allows for the use of any other material of clinical interest or with other uses, like for example in the food processing industry. The aim was to test disinfectant efficacy against 48-h biofilms of five microorganisms, grown in a static culture in which the medium was replaced at specific intervals. However, the method can be used for biofilm formed by means of a variety of procedures, like for example using dynamic culture media, different incubation periods, or any organism capable of metabolizing resazurin. In the proposed method, disinfectant activity was estimated from the

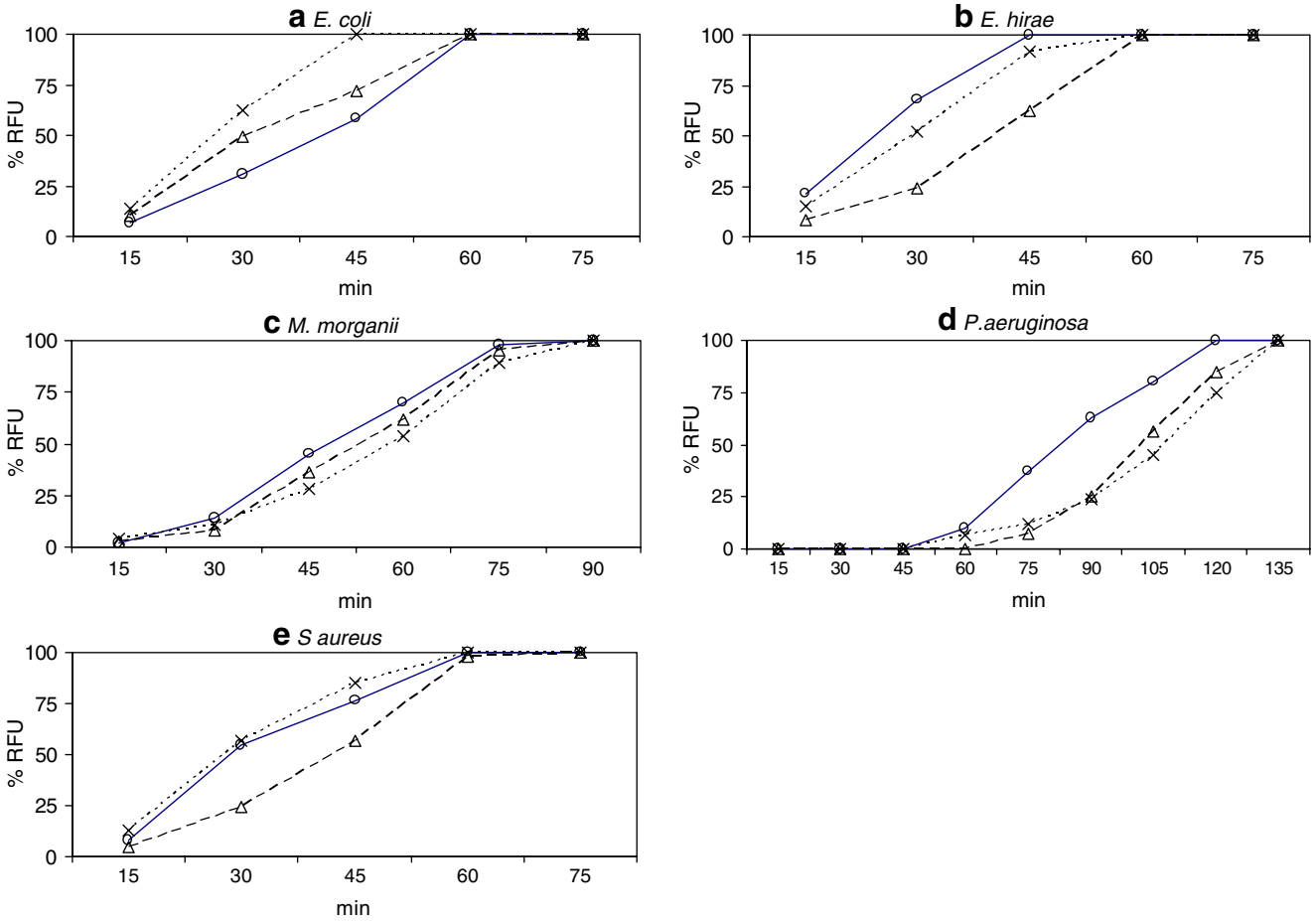

Fig. 4 Correlation between the percentage of RFU and time obtained for bacterial biofilms grown on SIL, PVC, and PPP carriers. Only the time required for biofilm of each one of the strains to reach a maximum fluorescence signal (100% RFU) is shown. Each point represents the average from five independent assays. SIL(—○—), PVC(- - △ - -), PPP(·····×·····) carriers

percentage of fluorescence decrease (estimated as RFU) in relation to a control without disinfectant. In the assays on each strain of the test microorganism, it was possible to estimate the number of cells both on the control biofilm carrier (without disinfectant) and in the samples after being treated with disinfectant by using a standard curve for known inocula of the same microorganism in suspension.

One of the chief limitations of microorganism quantification methods based on metabolic activity like that described here, is usually the fact that they require a relatively high number of microorganisms, generally speaking, greater than 5- or 6-log cfu, to obtain results reasonably quickly (O'Brien et al. 2000; Shiloh et al. 1997; Peeters et al. 2008b). In this study, something similar occurred with resazurin. The difference in the time required for the smallest and the biggest inoculum of the strains to reach maximum resazurin reduction rates was approximately 3 h, as can be observed in Fig. 1 (*S. aureus* or *E. hirae*). For the 10^8 inoculum, out of the five strains, the two Gram-positive microorganisms—*S. aureus* and *E. hirae*—were those that reached a greater resazurin reduction rate more quickly than the rest. The Gram-negative strains required between 1 and 2 h more to reach the same rate. In a previous study on strains of different bacterial species using a biofilm resazurin assay, the *S. aureus* strains required the shortest incubation period, which was attributed to the formation of extremely high amounts of resorufin, which accelerates its secondary reduction to the colorless, nonfluorescent hydro-resorufin (Peeters et al. 2008b).

As can be seen in Fig. 1, all the inocula of the test strains between 3- and 8-log cfu had reached a good reduction rate of resazurin after 5 h, which was sufficient to establish a correlation between the cfu value and resazurin activity, and to obtain a standard curve for biofilm quantification.

As has already been mentioned, the number of microorganisms on the biofilm carriers estimated by fluorescence, and in the controls—and therefore in the samples before being treated with biocide—varied between 7 and 10 log cfu/cm^2 (Fig. 3c). Generally speaking, a 5-log reduction is required in studies for determining disinfectant efficacy. With regard to this requirement, the carrier sizes and their corresponding microorganism load, as well as the incubation

Table 2 Logarithmic reduction values for each combination of microorganism, disinfectant, and carrier materials using the biofilm resazurin assay

	Chlorhexidine (mg ml^{-1})			NaOCl (mg ml^{-1})		Ethanol (mg ml^{-1})			Perasafe (mg ml^{-1})		
	0.2	0.02	0.002	0.1	0.01	350	175	87.5	0.16	0.016	0.0016
PPP											
E. coli	>5	>5	5.0	>5	2.0	>5	1.0	0	4.7	0	0
E. hirae	>5	5.0	4.2	>5	5.0	>5	3.6	0	>5	2.1	0
M. morganii	>5	>5	5.0	>5	2.9	5.0	0.3	0	4.2	1.6	0
P. aeruginosa	>5	>5	4.1	>5	4.0	>5	4.3	0	5.0	1.0	0
S. aureus	>5	>5	>5	>5	>5	>5	5.0	3.0	>5	4.8	1.2
PVC											
E. coli	>5	5.0	2.9	>5	>5	5.0	1.9	0	5.0	0	0
E. hirae	>5	>5	4.8	>5	>5	>5	3.1	0	>5	3.4	0
M. morganii	>5	5.0	2.1	>5	4.6	>5	2.6	0	5.0	0	0
P. aeruginosa	>5	>5	3.2	>5	5.0	>5	3.9	0	>5	0	0
S. aureus	>5	>5	5.0	>5	>5	>5	4.2	0	>5	4.0	0
SIL											
E. coli	4.9	3.8	0	>5	3.2	>5	2.0	0	>5	3.0	0
E. hirae	4.8	3.2	0	>5	4.0	>5	5.0	1.0	>5	3.9	0
M. morganii	5.0	3.0	0	5.0	2.1	5.0	1.5	0	4.8	2.0	0
P. aeruginosa	>5	>5	3.6	>5	5.0	>5	5.0	0	5.0	4.3	0
S. aureus	>5	5.0	4.3	>5	>5	5.0	4.8	0	5.0	>5	1.8

times used in this study, proved to be sufficient, as can be observed in Table 2. Smaller carrier sizes, or species with a slower growth or metabolism, would probably need longer incubation times. Nonetheless, the assay's flexibility means that it could be adapted to other situations, since it is necessary to generate a standard curve for each assay.

As we can see in Tables 1 and 2, the biofilm microorganisms proved to be more resistant to disinfectant than those grown in suspension, as has been shown in a large number of previous studies (Costerton 1999; Carpentier and Cerf 1993; Maillard 2007/6). According to these results, the disinfectant concentrations required to reach a LR greater or equal to 5 were generally higher in the case of the bacterial biofilms than the MICs obtained by means of the standard method for cells in suspension; although as different techniques were employed, the results cannot be interpreted in the same way, nor was this the aim of the study.

A great many methods described for quantifying biofilm cell density include laborious processes such as mechanical separation or sonication (Surdeau et al. 2006/4). These methods are very complex to standardize, cannot be applied in the same way to any bacterial species, and require an excessive amount of time and material when used in assays needing a large number of samples, as is the case in studies on disinfectant efficacy. A number of methods using biofilm growth on test tubes or microtiter-plates have been developed. Generally speaking, they are quick and reproducible and can be applied simultaneously to a large number of samples (Peeters et al. 2008b; Stepanovic et al. 2000; Djordjevic et al. 2002; Pitts et al. 2003). However, although these methods make it possible to obtain valuable data on strain sensitivity, many other parameters present in real-world conditions cannot be included in the assays. For example, the influence of the physiochemical characteristics of the materials on which microorganisms form biofilm or that of the dynamic conditions of the fluid in which biofilms have been constituted (Buckingham-Meyer et al. 2007; Ceri et al. 1999), is very difficult to evaluate. Staining microorganisms with vital dyes, such as crystal violet or fluorescent dyes (Shi et al. 2006), has also been proposed. Crystal violet is probably one of the dyes most used for quantifying biofilm. However, it is unsuitable for determining disinfectant efficacy, due to the fact that it stains both the biofilm microorganisms and the accompanying matrix, making it impossible to establish that partial discoloration after biocide treatment is due to a reduction in the number of microorganisms. Neither does it provide data on the viability of remaining microorganisms. As can be seen in Fig. 3, practically no correlation was observed between the three methods used for quantifying biofilm. According to the results of the CV method, biofilms formed by *S. auresus* and *S. hirae* proved to be significantly smaller than those constituted by the Gram-negative microorganisms. By means of the method described here, not only the microorganisms present are quantified, but also the biofilm matrix. Generally speaking, Gram-positive microorganisms form finer biofilms (Peeters et al. 2008b), which tallies with the results obtained in this study. These differences, however, were not visible when using the other two test methods, in which pronounced differences between

the results obtained with the different test strains and materials were not observed. Although the biofilms formed by the Gram-positive organisms proved to have a lower number of microorganisms than the other three organisms, these differences were not statistically significant. As can be seen in Fig. 3, according to the results obtained with the fluorescence method, the number of cfu/cm^2 was generally greater than that obtained with sonication. Although not statistically significant, the cause of this difference is not clear; however, lower cell density could be attributed to incomplete detachment or alterations in the metabolic/reproduction capacity of the cells due to sonication, among other reasons. In a recent study, Peeters et al. (2008a) did not find any relationship between the removal of biofilm biomass tested by a CV assay and the potential to kill biofilm cells tested by a similar resazurin assay. These authors suggest that a CV assay should not be used to assess the ability of a disinfectant to eradicate viable sessile cells, although it provides useful information on the efficacy of the disinfection to remove biofilm remnants. On the other hand, resazurin and its fluorescent product seem to be perfectly membrane permeable with regard to the biofilm matrix. The fluorescence emitted by a specific cell density in the biofilm does not show significant differences in relation to fluorescence emissions of the same number of cells growing in suspension. In addition, since biofilm quantification does not require any manipulation, the resazurin method seems to be very suitable, especially when a large number of samples, with their corresponding replicates, are required.

Other previously developed biofilm quantification methods include indirect measurements based on cell metabolism in which a direct correlation is assumed between the number of microorganisms and the magnitude of a specific reaction due to the said cell metabolism (Peeters et al. 2008b; Abbondanzi et al. 2003); although they have not been designed to study the efficacy of disinfectants. A similar fluorescence method also based on cell metabolism, which has been previously described, was developed for testing the efficacy of disinfectants against biofilm. Nevertheless, since it is based on glucuronidase activity, this method can only be used with *E. coli* and a few other species (Mariscal et al. 2007). In spite of this, resazurin has indeed been used to measure the cell viability of a great many microorganisms, including Gram-negative bacteria, Gram-positive bacteria such as some species of *Mycobacterium* and even fungus like *Candida albicans* (Shiloh et al. 1997; Tizzard et al. 2006).

Previous studies suggest that assays based on bacterial metabolism, such as bioluminescence or chemical luminescence, can be employed to determine the viable cells of a simple cell, but, depending on assay conditions and the type of test microorganism, cannot always be used to determine CFU (Yamashoji et al. 2004). The fluorescence assay is simple and quick in comparison with other methods, although its detection limit is not like that of plate count methods, generally being between 10 and 10^2 cfu. Nonetheless, due to the cell density of carrier biofilm, the resazurin assay proved to be sufficient for measuring reductions of 5-log cfu, which is enough for the biocide to be regarded as efficient.

The resazurin assay's repeatability and applicability for quantifying biofilm and the effect of biocides was also evaluated. The five replicates that were carried out showed a good repeatability, which was confirmed by the variation coefficient obtained in biofilm quantification (CV 5–12%) and LR value estimates (CV 2–14%).

The resazurin-based method developed in this study was fairly simple, did not required special equipment, and showed enormous potential for screening tests. Since each microorganism has different resazurin reduction kinetics, it is necessary to generate a standard curve for each assay. Nevertheless, the assay can be used with any type of carrier biofilm—regardless of how it has been obtained; for example, by means of a different fluid velocity—provided that the test microorganism metabolizes resazurin.

Acknowledgments This work was supported by the Consejería de Educación of the Junta de Andalucía and the Ministerio de Ciencia y Tecnología in the Programa General del Conocimiento. The authors wish to thank T. MacFarlane for his helpful suggestions while translating the manuscript.

References

Abbondanzi F, Cachada A, Campisi T, Guerra R, Raccagni M, Iacondini A (2003) Optimisation of a microbial bioassay for contaminated soil monitoring: bacterial inoculum standardisation and comparison with Microtox® assay. Chemosphere 53:889–897

AFNOR. (1995) Antiseptiques et désinfectants. NF T 72-150

Buckingham-Meyer K, Goeres D, Hamilton M (2007) Comparative evaluation of biofilm disinfectant efficacy tests. J Microbiol Methods 70:236–244

Carpentier B, Cerf O (1993) Biofilms and their consequences, with particular reference to hygiene in the food industry. J Appl Bacteriol 75:499–511

Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A (1999) The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 37:1771–1776

Christensen GD, Simpson WA, Younger JJ (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 22:996–1006

Costerton JW (1999) Introduction to biofilm. Int J Antimicrob Agents 11:217–221

Djordjevic D, Wiedmann M, McLandsborough LA (2002) Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol 68:2950–2958 doi:10.1128/AEM.68.6.2950-2958.2002

Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193 doi:10.1128/CMR.15.2.167-193.2002

European Standardization Committee (1997) Norma española UNE—EN 1040. Antisépticos y desinfectantes químicos. Actividad bactericida básica. Método de ensayo y requisitos (fase 1). AENOR, Madrid

Guerin TF, Mondido M, McClenn B, Peasley B (2001) Application of resazurin for estimating abundance of contaminant-degrading microorganisms. Lett Appl Microbiol 32:340–345

Jackson G, Beyenal H, Rees WM, Lewandowski Z (2001) Growing reproducible biofilms with respect to structure and viable cell counts. J Microbiol Methods 47:1–10

Jessen B, Lammert L (2003) Biofilm and disinfection in meat processing plants. Int Biodeterior Biodegrad 51:265–269

Luppens SBI, Reij MW, Van der Heijden RWL, Rombouts FM, Abee T (2002) Development of a standard test to assess the resistance of *Staphylococcus aureus* biofilm cells to disinfectants. Appl Environ Microbiol 68:4194–4200

Maillard J (2007/6) Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? J Hosp Infect 65:60–72

Marion K, Freney J, James G, Bergeron E, Renaud FNR, Costerton JW (2006) Using an efficient biofilm detaching agent: an essential step for the improvement of endoscope reprocessing protocols. J Hosp Infect 64:136–142

Mariscal A, Carnero-Varo M, Gutierrez-Bedmar M, Garcia-Rodriguez A, Fernandez-Crehuet J (2007) A fluorescent method for assessing the antimicrobial efficacy of disinfectant against Escherichia coli ATCC 35218 biofilm. Appl Microbiol Biotechnol 77:233–240

O'Brien J, Wilson I, Orton T, Pognan F (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267:5421–5426

O'Toole GA, Kolter R (1998) Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28:449–461

Peeters E, Nelis HJ, Coenye T (2008a) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 72:157–165

Peeters E, Nelis HJ, Coenye T (2008b) Evaluation of the efficacy of disinfection procedures against *Burkholderia cenocepacia* biofilms. J Hosp Infect 70:361–368 doi:10.1016/j.jhin.2008.08.015

Perret-Vivancos C, Marion K, Renaud FNR, Freney J (2008) Efficient removal of attached biofilm in a naturally contaminated colonoscope using detachment-promoting agents. J Hosp Infect 68:277–278

Pitts B, Hamilton MA, Zelver N, Stewart PS (2003) A microtiter-plate screening method for biofilm disinfection and removal. J Microbiol Methods 54:269–276

Reid G (1999) Biofilms in infectious disease and on medical devices. Int J Antimicrob Agents 11:223–226

Sarker SD, Nahar L, Kumarasamy Y (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 42:321–324

Shi Z, Neoh KG, Kang ET, Wang W (2006) Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles. Biomaterials 27:2440–2449

Shiloh M, Ruan J, Nathan C (1997) Evaluation of bacterial survival and phagocyte function with a fluorescence-based microplate assay. Infect Immun 65:3193–3198

Singh R, Paul D, Jain RK (2006) Biofilms: implications in bioremediation. Trends Microbiol 14:389–397 doi:10.1016/j.tim.2006.07.001

Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40:175–179

Surdeau N, Laurent-Maquin D, Bouthors S, Gelle MP (2006/4) Sensitivity of bacterial biofilms and planktonic cells to a new antimicrobial agent, Oxsil(R) 320N. J Hosp Infect 62:487–493

Tizzard AC, Bergsma JH, Lloyd-Jones G (2006) A resazurin-based biosensor for organic pollutants. Biosens Bioelectron 22:759–763

Twigg RS (1945) Oxidation-reduction aspects of Resazurin. Nature 155:401–402

van der Wende E, Characklis WG, Smith DB (1989) Biofilms and bacterial drinking water quality. Water Res 23:1313–1322

Yamashoji S, Asakawa A, Kawasaki S, Kawamoto S (2004) Chemiluminescent assay for detection of viable microorganisms. Anal Biochem 333:303–308

Zelver N, Hamilton M, Goeres D, Heersink J (2001) Development of a standardized antibiofilm test. Methods Enzymol 337:363–376