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ORIGINAL ARTICLE

A new microtitre plate screening method for evaluating the viability of aerobic respiring bacteria in high surface biofilms

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Abstract

Aims: It is difficult to determine the effects of bactericidal compounds against bacteria in a biofilm because classical procedures for determining cell viability require several working days, multiple complicated steps and are frequently only applicable to cells in suspension. We attempt to develop a compact, inexpensive and versatile system to measure directly the extent of biofilm formation from water systems and to determine the viability of respiring bacteria in high surface biofilms.

Methods and Results: It has been reported that the reduction of tetrazolium sodium salts, such as XTT (sodium 3,3′-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]Bis(4-methoxy)-6-nitro)benzene sulfonic acid hydrate), during active bacterial metabolism can be incorporated into a colorimetric method for quantifying cell viability. XTT is reduced to a soluble formazan compound during bacterial aerobic metabolism such that the amount of formazan generated is proportional to the bacterial biomass.

Conclusions: We show here, for the first time, that this colorimetric approach can be used to determine the metabolic activity of adherent aerobic bacteria in a biofilm as a measure of cell viability. This technique has been used to estimate viability and proliferation of bacteria in suspension, but this is the first application to microbial communities in a real undisturbed biofilm.

Significance and Impact of the Study: This simple new system can be used to evaluate the complex biofilm community without separating the bacteria from their support. Thus, the results obtained by this practice may be more representative of the circumstances in a natural system, opening the possibility to multiple potential applications.

Introduction

A biofilm is a complex aggregation of micro-organisms characterized by the excretion of a protective adhesive matrix. The cells living in a biofilm are embedded in this polymeric substance, which permits them to adhere and colonize the surfaces of different materials (Flemming 2002). At the biofilm matrix, the micro-organisms are highly protected from the action of several toxic substances, such as biocides and antibiotics (Bhinu 2005; Harrison *et al.* 2007; Kim *et al.* 2008).

Micro-organisms are often exposed to fluctuations in their local environment that can affect their survival and influence their community dynamics. Therefore, the effects of several stress factors on bacterial viability in biofilms have received considerable interest (Trachoo *et al.* 2002; Krumbein *et al.* 2004; Queck *et al.* 2006). Different tools and techniques for measuring respiratory activity as

a surrogate for cell viability have been developed; however, these techniques can generally only be applied to cells in suspension (Roszak and Colwell 1987). In addition, classical procedures (colony counting) for determining the number of viable cells require several working days and sometimes multiple processes.

Variations in respiratory activity per unit time can be interpreted as a reflection of the metabolic behaviour of the micro-organisms residing in a particular environment (Roszak and Colwell 1987). In the last decades, the validity of estimating cell viability based on colony counts has been questioned; this method is increasingly being replaced with new techniques that are based on the evaluation of cellular metabolic activity (Roslev and King 1993; Bhupathiraju *et al.* 1999; Créach *et al.* 2003; Keep *et al.* 2006). Plate counts estimate the numbers of culturable active bacteria that are able to initiate cell division at a sufficient rate to form colonies (Boulos *et al.* 1999). However, a large proportion of bacteria encountered in environmental samples do not grow on conventional substrates (Keep *et al.* 2006).

To assess and monitor biofilm build-up, the development of simple nonintrusive methods for the detection of these structures and to measure its cell viability is becoming of paramount importance. Different approaches have been used to determine the active biomass. To this end, respiration has been measured by both direct and indirect methods, such as by evaluating electron transport and using the reduction in tetrazolium coloured salts as a measure of electron transport (Roslev and King 1993; Bhupathiraju *et al.* 1999; Créach *et al.* 2003; Hatzinger *et al.* 2003). It has been reported that the reduction in tetrazolium sodium salts, such as XTT and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), during active bacterial metabolism has been used as a colorimetric indicator for quantifying cell viability in both aerobic and anaerobic bacteria. Créach *et al.* 2003 reviewed the utilization of the redox dye CTC for evaluating the metabolic activity of aerobic and anaerobic bacteria. CTC indicates respiratory activity and is therefore a direct indicator of oxidative metabolism as well as viability. CTC is a colourless, membrane-permeable compound that produces a red-fluorescing precipitate in the cell when it is reduced by the electron transport system of bacterial cells. This approach for estimating active bacterial cells has been used in a wide range of studies and is comparable in simplicity and reliability to any epifluorescence technique commonly used for the enumeration of total bacterial density (del Giorgio *et al.* 1997; Sieracki *et al.* 1999; Gruden *et al.* 2003). However, it has not been determined if there could be a direct relationship between the amount of actively respiring cells, that are capable of growing and dividing, and the amount of cells that reduce sufficient dye to be detected as CTC positive. Besides these experimental troubles that may be corrected with further studies, the simplicity and rapidity of this method makes them of interesting value. Bhupathiraju *et al.* (1999) examined the utility of CTC for evaluating the metabolic activity of anaerobic bacteria. In addition, the factors contributing to abiotic reduction of CTC were also examined. Facultative and obligate anaerobic bacteria actively reduced CTC during all phases of growth to intracellular CTC-formazan crystals. CTC-formazan production by all cultures examined was proportional to biomass production, and CTC reduction was observed even in the absence of added nutrients. Abiotic reduction of CTC was observed in the presence of ascorbic acid, cysteine hydrochloride, dithiothreitol, NADH, NADPH, $Fe(II)Cl_2$, sodium thioglycolic acid and sodium sulfide, suggesting that while CTC can be used to capture the metabolic activity of anaerobic bacteria, care must be taken to avoid abiotic reduction in CTC.

Alternatively, XTT is also reduced to a soluble coloured product (formazan) by different cell types during *aerobic* metabolism as a result of active electron transport, such that the amount of formazan generated is proportional to the bacterial biomass. This technique has been used to estimate cell viability and proliferation in suspension bacteria (Hatzinger *et al.* 2003; Cerca *et al.* 2005; Peeters *et al.* 2008), but to our knowledge, its application to the study of microbial communities in a biofilm, *i.e.* adhered fixed bacteria, has not yet been evaluated.

Our efforts have been directed to adapt the XTT colorimetric method to determine the metabolic activity of adherent respiring bacteria in a biofilm as measure of cell viability. In this paper, we tested for the first time the ability of this colorimetric approach to be used for the determination of the metabolic activity in adherent bacteria in a biofilm, as a parameter associated with cell viability.

We attempt to develop a compact, inexpensive and versatile system to measure directly the extent of biofilm formation from water systems and to determine the viability of respiring bacteria in biofilms. To this end, a standardized attachment media (Siporax®; Tropiacuarium, Bilbao, Spain) has been used, in a similar approach to previous work based on a packed-bed biofilm reactor for the determination of the potential of biofilm accumulation from water systems (Morato *et al.* 2005). The use of Siporax® cylinders as standard attachment media, which have a very high surface-to-volume ratio and allow the growth of large microbial numbers, improves the obtaining of large sample volumes.

On the other hand, the method was developed to provide easy operation and sampling of undisturbed biofilms, as well as high sensitivity when using conventional microbiological methods. The method allows nonintrusive

sequential sampling and provides the possibility of analyzing the kinetics of biofilm formation in different conditions.

Coupled to a microtitre plate, this colorimetric approach based on the reduction of tetrazolium salts may also be used as a fast, simple and inexpensive screening method for the evaluation of viability in respiring cells in a biofilm. Special interest has been directed to evaluate the effects of different biocides on biofilms and viable bacteria. In this sense, the effect of biocides commonly used for biofilm control in cooling towers was evaluated.

Materials and methods

Biofilm production

Biofilms were produced in a batch reactor (a laboratory pilot scale cooling tower) during a minimum of 15 days, using submerged Siporax®, a fine-pore filtering material with a large surface area that can be colonized by micro-organisms. Each Siporax® cylinder has approximately 0·9 m^2 of specific surface, i.e. a large area to be colonized by micro-organisms (20–50 μm amplitude among its micropores). Briefly, the system was formed by a water bath filled with 30 l of tap water plus 1 l of municipal treated wastewater. The reactor was connected to a pump that permitted the recirculation of wastewater, and the temperature was maintained at 30°C, with the continuous recirculation of water, covering the solid material. Sampling of attached biomass required periodical removing of individual Siporax® cylinders for off-line nondestructive biofilm analyses. For each sample and as a routine approach, three independent assays were performed, with triplicate analyses for each one. Where data means are given, the standard deviation (SD) associated with the mean and sample number (n) is also indicated. Statistical comparisons were made by ANOVA using SPSS statistical software (version 9·0, SPSS, Chicago, IL, USA), and differences reported significance at the 5% level.

Measurement of cell viability by a colorimetric method

Cell viability was evaluated using a commercially available kit (Cell Proliferation Kit II XTT – #11 465 015 001; Roche Diagnosis, Mannheim, Germany) according to the manufacturer's recommendations. This colorimetric, non-radioactive assay measures the metabolic activity of viable cells and can be fully adapted to a microtitre plate reader. A decrease in the number of live cells correlates with a decrease in overall activity of the dehydrogenases responsible for transforming the sodium salt of tetrazolium XTT into formazan, a compound that can be determined colorimetrically at 490 nm. Briefly, Siporax® (Fig. 1) colonized cylinders (biofilms) were loaded in a 24-well plate and exposed to different concentrations (12·5–100 ppm) of 2,2-dibrome-3-nitrilopropionamide (DBNPA 96%; Sigma–Aldrich, St Louis, MO, USA), chlorine 1 ppm (Sigma–Aldrich), Sanosil S015® 50 ppm (a Ag/H_2O_2 mixture; Sanosil Ltd, Hombrechtikon, Switzerland), bismuth ethanedithiol (Bis-EDT) 0·1 ppm (kindly provided by Dr P. Domenico, Winthrop-University Hospital, Mineola, USA) or Cu^{2+}/Ag^{+} solution (0·4 ppm Cu^{2+}/0·04 ppm Ag^{+}; all reagent from Sigma-Aldrich), in saline solution containing XTT (final concentration 0·3 mg ml^{-1}, *i.e.* 0·45 mmol l^{-1}) during 10–12 h at 37°C. Formazan was continuously quantified spectrophotometrically at 490 nm during this period, using a microplate reader ELISA (GENios-Tecan, Männedorf, Switzerland). Sterile Siporax® cylinders were used as negative controls.

Figure 1 24-well plate utilized for the incubation of Siporax® cylinders and to colorimetrically evaluate the viability of respiring bacteria in biofilms.

DBNPA is a commonly used biocide for biological control of bacteria, fungi and algae in cooling tower systems (Kim *et al.* 2002). Chlorine is without doubt the most widely used industrial biocide for disinfection of domestic water supplies and for the removal of tastes and odours from water. The silver/copper combination is another effective biocide against a wide range of organisms and can be applied to many applications because of its residual long-lasting effect. Although silver alone has limited efficacy when used in conjunction with copper ions, its efficacy is dramatically improved because the positively charged copper ions (Cu^{2+}) are attracted to, and attack, the negatively charged protective walls of the bacterial cells. At this point, it is interesting to note that the presence of copper and silver ions in the order of 0·4 mg l^{-1} (ppm) copper and 0·04 mg l^{-1} (ppm) silver at the point of water storage or supply with residual concentrations of 0·2 and 0·02 mg l^{-1}, respectively, at the outlet point is generally considered as sufficient to provide an effective kill rate and thus comprehensive protection (Kim *et al.* 2002, 2008).

Sanosil® disinfectants are highly effective and universally applicable for pathogenic bacteria, amoeba, biofilms, fungi, mould and virus control. Finally, Bis-EDT is a member of a new generation of biocides that have been tested in *in vitro* experiences and have showed a high efficacy against attached bacteria. Because of its enormous biocide potential, Bis-EDT could be widely utilized for the disinfection of industrial refrigeration systems (Codony *et al.* 2003).

Results

Respiration in a biofilm

Our first aim was to develop a simple method for determining cell viability in a complex biofilm. We allowed in a microtitre plate Siporax® sterile rings and Siporax® rings colonized with bacteria after incubation in the biofilm reactor and compared formazan production as an indicator of metabolic activity. Optical density (OD) values were normalized, and therefore at time zero OD was 1 for each sample. As seen in Fig. 2, only the Siporax® rings colonized with a biofilm demonstrated an increase in OD_{490nm} (i.e., formazan production), validating this approach.

Under the conditions used in this experiment, the presence of attached respiring bacteria (viable cells) on biofilms could be colorimetrically detected and easily quantified through the formazan production. At this point, it is interesting to notice that no formazan was detected during the initial phase of the experiments (around the first 4 h). This delay is explained by the time required for the reagent (XTT) to reach the bacteria in the Siporax® cylinders and to be metabolized by the dehydrogenases, and mostly by the detection limit of the spectrophotometer, *i.e.*, until certain concentration of formazan is generated, it would not be detected by the microplate reader.

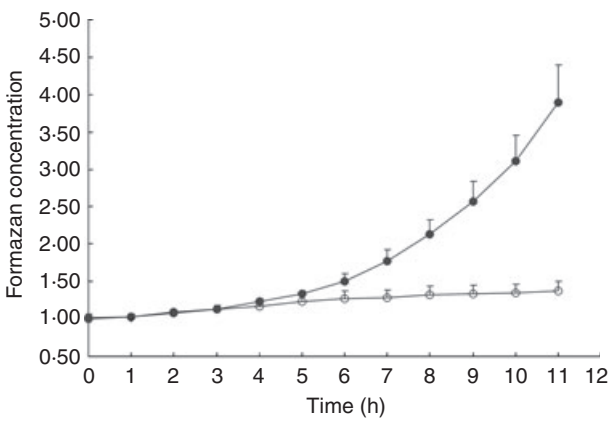

Figure 2 Evaluating respiration in a biofilm. Formazan production was assessed spectrophotometrically at 490 nm as a measure of cell viability every hour during 10–12 h at 37°C in a microplate reader. Black filled circle (●) represents formazan production from Siporax® rings colonized with a biofilm; white filled circle (○) represents formazan production from Siporax® sterile rings. As we can see, only viable cells in the Siporax® colonized cylinders were responsible from formazan production. Bars = standard deviation of the mean. Only positive bars are represented for better visual comprehension of the figure. Formazan concentration values are normalized to 1 at time zero to avoid possible differences related with the total bacterial initial mass.

Biocide dose–response in attached respiring bacteria

We next asked whether our method could be applied to dose–response studies by evaluating formazan production of the biofilm following the addition of different concentrations of DBNPA. This fungicide is used to control bacteria, fungi and algae in cooling systems (Kim *et al.* 2002).

As shown in Fig. 3, formazan production showed a dose-dependent sensitivity to DBNPA. A concentration of 12·5 ppm DBNPA showed only a slight decrease in respiring activity. The effect was significantly higher with 25 ppm, and especially with 50 ppm, where maximal respiratory inhibition has been reached.

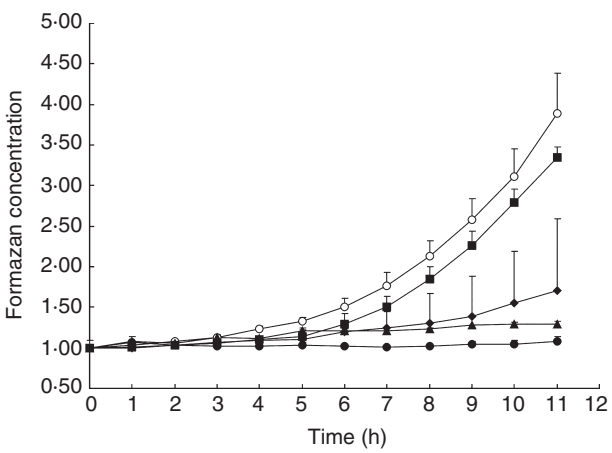

Figure 3 Respiration in a biofilm in the presence of a biocide. Siporax® cylinders colonized with biofilm were exposed to several concentrations of 2,2-dibrome-3-nitrilopropionamide (DBNPA). Formazan production was assessed spectrophotometrically at 490 nm every hour as a measure of cell viability during 10–12 h at 37°C in a microplate reader. White filled circle (○) represents formazan production from Siporax® rings colonized with a biofilm, not exposed to DBNPA during the test (Control); black filled symbols represents formazan production from Siporax® colonized rings exposed to (■) 12.5 ppm, (◆) 25 ppm, (▲) 50 ppm, or (●) 100 ppm DBNPA. As can be seen, increasing the biocide concentration diminishes cell viability of the biofilm, represented as a lower formazan production. Bars = standard deviation of the mean. Only positive bars are represented for a better visual comprehension of the figure. Formazan concentration values are normalized to 1 at time zero to avoid possible differences related with the total bacterial initial mass.

Viability following exposure to different biocides

We wanted to determine whether the observed changes in biofilm respiration were independent of the type of biocide applied. Therefore, we evaluated formazan production after treating the biofilm with four different commonly used antimicrobial compounds for cooling tower biofilm control: Sanosil®, silver and copper ions, chlorine and Bis-EDT.

The results of the experiments to determine the extent of attached respiring bacteria are shown in Fig. 4. The different biocides affected the respiration of the biofilm to different degrees, indicating that this method can be used to determine the biocidal activity of various agents against bacteria in a biofilm, regardless of their mechanisms of action.

The experiments performed have not shown significant decrease during the first 6 h, on respiratory activity with 1 ppm chlorine exposure, and no differences with the saline solution (without biocide) control samples were observed. However, the respiratory activity of attached cells began to decrease after 7 h of exposure to 1 ppm chlorine. The effect of chlorine on viable attached bacteria slightly increased until the end of the experiment. A similar trend was observed with the exposure to copper (0·04 ppm) and silver (0·4 ppm) ions.

Compared to 1 ppm chlorine and copper (0·04 ppm) and silver (0·4 ppm) ions, 50 ppm Sanosil® and 0·1 ppm Bis-EDT displays a higher efficacy and a significant decrease in respiring attached cells after 5 h of exposure to the biocide.

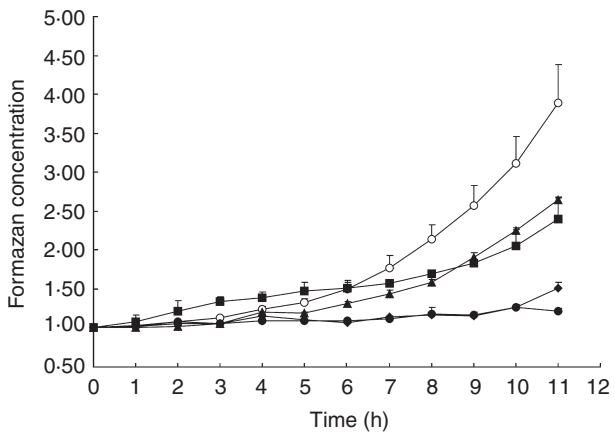

Figure 4 Biofilm respiration in the presence of different biocides. Siporax® cylinders colonized with biofilm were exposed to various biocides and formazan production was assessed spectrophotometrically at 490 nm every hour as a measure of cell viability during 10–12 h at 37°C in a microplate reader. White filled circle (○) represents formazan production from Siporax® rings colonized with a biofilm, not exposed to any biocide during the entire test (Control); black filled symbols represents formazan production from Siporax® colonized rings exposed to: (■) a 0.04 ppm Ag^{+}/0.4 ppm Cu^{2+} solution, (▲) 1 ppm Chlorine, (●) 50 ppm Sanosil, or (◆) 0.1 ppm bismuth ethanedithiol. As can be seen, biocide treatment diminishes cell viability of the biofilm at different extents, represented as a lower formazan production. Bars = standard deviation of the mean. Only positive bars are represented for a better visual comprehension of the figure. Formazan concentration values are normalized to 1 at time zero to avoid possible differences related with the total bacterial initial mass.

Discussion

The method described in this paper presents a number of advantages for biofilm research. The lack of standard procedures negatively affects the quality of information generated by biofilm researchers and makes the comparison of results produced in different laboratories difficult or impossible (Heydorn *et al.* 2000). We suggest this method as an advantageous tool for regular use to study undisturbed biofilms.

First, we test the ability of the XTT colorimetric method used for determining metabolic activity as a parameter associated with cell viability in adherent aerobic bacteria from biofilms. This approach gave significant results to evaluate the specificity for attached bacteria on biofilms. At this point, it is important to note that Hatzinger *et al.* (2003) showed that XTT concentrations about 3 mmol l^{-1} can underestimate cell activity and in some cases may be toxic to cells. In our experimental conditions, moderate levels of XTT were used to prevent metabolic inhibition (0·45 mmol l^{-1}) but, if this potential toxicological effect already exists, the comparative results obtained here are still valuable because it will affect all samples at the same way.

Later, first positive tests were conducted on dose–response studies to evaluate the metabolic activity of attached micro-organisms. In this sense, the first experimental trial was developed using different concentrations of DBNPA, a commonly used biocide for biological control of bacteria, fungi and algae in cooling tower systems (Kim *et al.* 2002). Afterwards, other biocides commonly used for cooling tower biofouling control were used, such as chlorine, silver and copper ions, Sanosil® and Bis-EDT (Kim *et al.* 2002, 2008; Codony *et al.* 2003).

We also test the independence of the colorimetric method on Siporax® attachment media, from the different types of biocides, obtaining different results from several biocide compounds. In all cases, an approximately 4-h delay in formazan production was observed. This delay could be related with the biofilm density or complexity, but also with the number of metabolic active cells and other factors such as: (i) the time required for XTT to reach the cells at the biofilm because the dye does not penetrate directly, (ii) the respiratory taxes of the cells to

produce XTT and (iii) the time required for the instrument to detect a significant signal. Compared to the control, the different treatments show information about the disinfection effect in the real biofilm with all the potential problems that this may involve (such as interaction with the bacterial EPS (extracellular polymeric substances)), but this is not a problem in the comparative studies because all samples will be equally affected. It is also important to notice that in the presence of different biocides XTT may penetrate differently, probably related with the different damage in the biofilm. Besides the direct or indirect biocide effect over the cells or the biofilm structure, or both, the use of XTT will allow us to improve the knowledge and the extent of disinfection.

In the second place, one important advantage of this new approach is the utilization of cylinders or rings as colonization supports (Siporax®). The use of specific packing material, such as Siporax® cylinders, increases the ratio of surface area to volume for biofilm formation, achieving the maximum surface with the least volume. Owing to the large surface of each cylinder, the present method has a good sensitivity and permits the securing of considerable biofilm samples. Despite its reduced dimensions (15 cm long, 15 cm outer diameter and 8 cm inner diameter), each cylinder has a considerable sampling surface of 0·9 m^2.

In addition to being a practical material that permits effective microbial colonization, these rings are compatible with the use of a microtitre plate and plate reader. The internal diameter of the rings allows the beam of light from the plate reader to pass through making possible the colorimetric lectures. The combination of the rings as colonization supports and the use of a microtitre reader will facilitate the performance of several complex studies, including the estimation of metabolic rates and kinetic parameters, and its use as a general screening tool on different fields, from the clinical, industrial or environmental microbiology.

It is important to notice that the present experimental design (*i.e.*, the supporting material and the selected dye) does not allow the evaluation of the biofilm anaerobic metabolism, but this may be technically possible using other specific dyes for anaerobic metabolism such as Sudan dyes (Xu *et al.* 2007) or the redox dye CTC (Bhupathiraju *et al.* 1999) with a liquid paraffin cap in each well, to achieve anaerobic conditions.

The method presented, independently of the dye, is a feasible, simple and practical method to determine the bacterial viability in a biofilm without the need to separate the micro-organisms from its supporting material, with the disadvantages and troubles that the sonication process implies to cell viability.

In conclusion, these results open the possibility for a potential application of this approach as a useful tool for the study and evaluation of compounds with different biocide activity; the evaluation of synergistic and antagonistic effects between them and as a suitable tool for the discovering of newly unidentified agents with antimicrobial capacity or non informed antibacterial ability of already known compounds.

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