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Physiological methods to study biofilm disinfection

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This report reviews the development of a rapid *in situ* approach to study the physiological responses of bacteria within biofilms to disinfectants. One method utilized direct viable counts (DVC) to assess the disinfection efficacy when thin biofilms were exposed to chlorine or monochloramine. Results obtained using the DVC method were one log higher than plate count (PC) estimates of the surviving population after disinfection. Other methods incorporated the use of fluorogenic stains, a cryotomy technique to yield thin (5-μm) sections of biofilm communities and examination by fluorescence microscopy. The fluorogenic stains used in this approach included 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which indicates cellular electron transport activity and Rhodamine 123, which responds specifically to proton motive force. The use of these stains allowed the microscopic discrimination of physiologically active bacteria as well as heterogeneities of active cells within thicker biofilms. The results of experiments using these techniques with pure culture and binary population biofilms on stainless steel coupons indicated biocidal activity of chlorine-based disinfectants occurred initially at the bulk-fluid interface of the communities and progressed toward the substratum. This approach provided a unique opportunity to describe the spatial response of bacteria within biofilms to antimicrobial agents and address mechanisms explaining their comparative resistance to disinfection in a way that has not been possible using traditional approaches. Results obtained using this alternative approach were also consistently higher than PC data following disinfection. These observations suggest that traditional methods involving biofilm removal and bacterial enumeration by colony formation overestimate biocide efficacy. Hence the alternative approach described here more accurately indicates the ability of bacteria surviving disinfection to recover and grow as well as demonstrate spatial heterogeneities in cellular physiological activities within biofilms.

Keywords: biofilm control; biofouling; biocides; disinfection; biofilm structure; physiological activity; fluorescent stains; cryosectioning; epifluorescence microscopy

Introduction

Biofilms contribute to a range of costly problems within industrial environments. These interfacial microbial communities are directly involved in biofouling and biocorrosion phenomena that cause significant reductions in system performance and contribute to the accelerated deterioration of components. Biofilms are found on the surfaces of natural and engineered aquatic systems and account for most of the microbial activity that is seen in those environments. Problems associated with biofilm buildup on engineered surfaces include reduced heat transfer efficiency within heat exchangers, increased frictional resistance in pipes, deterioration of water quality within municipal drinking water systems, plugging, petroleum souring and accelerated biodeterioration of system components [7]. The magnitude and pervasive character of these impacts emphasize the importance of efforts to control biofilms in industry.

The control of biofilms represents one of the most persistent challenges within industrial systems where these microbial communities are problematic. There is a wealth of literature documenting the resistance of attached microorganisms and biofilms to antimicrobial agents [1,5,6,8, 14–16,27]. Difficulties in formulating efficient control strategies are related to our incomplete understanding of biofilm processes and structure. Mechanisms have been proposed to explain this enhanced resistance in bacteria that are associated with surfaces. These include reduced penetration of agents into biofilms because of diffusion limitations or reactions with community components and possible unique physiological characteristics of bacteria within biofilms [27]. Despite our unclear understanding of the basic mechanisms involved in this process, there is a practical need for methods to provide accurate information concerning the efficacy of biocides used to treat biofilms.

Methodological approaches that have been applied to study biofilm disinfection have relied on the quantitative removal of the community from the substratum followed by disaggregation and enumeration by colony formation. This traditional approach is limited in a number of ways. Cultural methods fail to detect some autochthonous bacteria in environments where natural biofilm communities are composed of a wide range of different organisms [31] and allochthonous bacteria can exhibit reduced culturability following environmental exposure [23]. In addition, bacteria exposed to sublethal conditions of antimicrobial treatment often become injured and are incapable of forming colonies on some media [18,19]. These limitations are particularly acute when studying biofilms where spatial heterogeneities may be complex. In addition, accurate data describing these structural relationships might be crucial in gaining an understanding of the mechanism of antimicrobial action

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within the undisturbed community. Methods to remove and disaggregate biofilms can also be inefficient. Therefore, the traditional approach of describing microbial populations within biofilms on the basis of biomass removal and colony formation is inadequate and often results in underestimations of the actual viable population of bacteria. In addition, results obtained using conventional methods are community-wide averages and yield no spatial information describing structure and physiological heterogeneities within biofilms following treatment with an antimicrobial agent. These are some of the difficulties that have hindered work characterizing the viability and activity of surface-associated bacteria. Hence, alternative approaches are needed to provide more complete information concerning the action of biocides in these important bacterial populations. The information resulting from such improved methods could also be used to expand our understanding of the mechanisms by which surface-associated bacteria gain enhanced resistance to antimicrobial agents. Ideally, such methods should allow a physiological assessment of individual bacterial cells within a native biofilm with minimal disruption.

The application of specific fluorogenic dyes and fluorescence microscopy provides a strategy by which the physiological heterogeneity of bacteria within biofilms can be observed. Epi-illuminated fluorescence microscopy is of particular utility since biofilms stained with appropriate fluorochromes may be viewed directly on the substratum without any physical disruption [35]. This application, however, is limited to very thin biofilms such as the ones typically found in drinking water distribution pipes. The structural analysis of thicker biofilms is also possible through the use of thin-sectioning techniques [33] and scanning confocal laser microscopy (SCLM). The application of SCLM offers a non-disruptive imaging capability of vertical sections within biofilms [2,3,13]. However, some problems have been encountered in the use of SCLM with very thick or opaque biofilms and the instrumentation is not routinely available. Both SCLM and conventional fluorescence microscopy can be used to visualize biofilm specimens stained with fluorescent stains. There is an impressive array of fluorogenic stains that have been used by cell biologists in physiological studies [17]. Of these, only a few have been applied in microbiological studies [21]. Collectively, the application of physiological assessment methodologies including specific fluorogenic stains with fluorescence microscopy provide a powerful new capability to study the action of antimicrobial agents and spatial physiological heterogeneities within biofilms with minimal disruption.

This review summarizes the findings from an ongoing study of biofilm disinfection using a suite of new methods to follow the action of biocides. The analytical approaches used in these studies allow the spatial resolution of physiological activities and viability of individual cells within biofilms. This kind of information provides a unique opportunity to address questions concerning the mechanism(s) of biocide action and resistance as well as spatial physiological gradients within biofilm communities.

Viability assessment using the Direct Viable Count Method and Image Analysis

The Direct Viable Count (DVC) technique is widely used by microbiologists. Originally proposed for applications in marine systems [12], this method determines the viability of individual bacteria on the basis of cell elongation in a suitable growth medium containing a quinolone antibiotic such as nalidixic acid. However, inaccuracies are not uncommon due to the subjective nature of results obtained by the traditional DVC method which involves discriminating elongated and normal length cells. This is also a tedious and time-consuming procedure making it difficult to process large numbers of samples. In addition, many workers adopt the original formulation of the reaction medium proposed by Kogure [12] without considering the influence of variables such as incubation time, nalidixic acid concentration and nutrient content on the cellular elongation process with different bacteria. As a result, variable results that are often significant underestimates of the actual population of viable bacteria have been reported. Some of those problems were addressed in a report by Singh *et al* [26]. That study, which included a range of planktonic Gram-negative bacteria, demonstrated that optimal assay conditions are often somewhat different for various bacteria and that Image Analysis (IA) technology is useful in providing an objective analysis of larger data sets that are more statistically robust. The optimized DVC method can provide an efficient and quantitative technique for counting viable bacteria when coupled with IA technology. That approach was subsequently adapted to the detection and measurement of injured bacteria following sublethal exposure to chlorine [25]. When compared with similar data where colony formation was used as the criterion for viability, the optimized DVC method with IA was more efficient in the detection of chlorine-stressed bacteria and yielded comparable levels of bacterial injury.

The DVC method was also applied to the problem of enumerating attached bacteria and assessing disinfection efficacy with thin bacterial biofilms [36]. Monolayer biofilms of *Klebsiella pneumoniae* were grown on stainless steel coupons and treated with either free chlorine (0.25 mg L^{-1}) or monochloramine (1.0 mg L^{-1}). The effects of biocide exposure were then measured by three different methods. Those techniques included the conventional plate count (PC) method after biofilm removal and disaggregation, a conventional DVC method applied to the removed cell suspensions and an *in situ* DVC method where cell elongation was observed directly on the coupons that were incubated in the DVC medium. All three methods provided similar results when control biofilms, that were not exposed to disinfectant, were examined. However, the *in situ* DVC method yielded at least one log higher viable cell densities than the PC method after 10 min of biocide treatment. That finding indicates that the *in situ* DVC technique is probably more efficient in the enumeration of biocide-exposed cells and may provide a more accurate evaluation of how bacteria in biofilms respond to disinfection although the method is probably limited to very thin biofilms. In addition, the removal of attached bacteria by biocides could be determined with this analytical approach. These results are consistent with other reports describing low proportions

of viable allochthonous bacteria that are capable of colony formation following environmental stress [18,19,23]. Another interesting observation concerning the comparative performance of the two methods was the magnitude of their variances. Error estimates obtained from the PC data were consistently at least 10-fold larger than those for DVC values.

Assessment of physiological activities of bacteria in biofilms with fluorescent stains

Traditional enumerative methods are inadequate to determine the viability and activity of bacteria in biofilms because of the dependence on enumeration by colony formation and the accompanying physical disruption of spatial relationships of the natural community structure. Our historical reliance on these approaches has resulted in imprecise concepts of the potential viability and activity of bacteria in biofilms that lack spatial inference. However, direct microscopic observations of biofilms stained with fluorogenic stains can provide a description of the physiological architecture of biofilms with minimal physical disruption [21].

Acridine orange (AO) is a fluorogenic dye that is widely applied by microbiologists in the acridine orange direct count (AODC) method and has been interpreted with putative physiological implications. As reviewed elsewhere [20], numerous workers have used the resulting color of AO-stained bacteria to distinguish between active and inactive cells. While that concept is based on the well established differential spectrofluorescence of single-stranded versus double-stranded nucleic acids complexed with AO, there was a paucity of specific published information to test that hypothesis by correlating established physiological indices with the AO staining reaction in bacteria of defined physiological activity. The results of such a study were eventually reported using *Escherichia coli* under controlled laboratory conditions [20]. The findings indicate that the AO staining reaction was influenced by a number of variables including undefined characteristics of other bacteria in environmental systems as well as staining and fixation alternatives. However, AO is potentially useful as an index of physiological activity in carefully controlled experiments.

CTC (5-cyano-2,3-ditolyl tetrazolium chloride) [22] and rhodamine 123 (Rh 123) [10] have only recently been used by microbiologists as cellular indicators of bacterial activity. Active cellular respiration results in the reduction of CTC and the accumulation of intracellular fluorescent crystals that can be detected microscopically [22,24,32,34] and by flow cytometry [11]. Intracellular rhodamine 123 fluoresces in proportion to cellular proton motive force. Biofilms of *K. pneumoniae* were used to determine the utility of CTC and Rh 123 as indices of interfacial bacterial activity by comparing those results against PC and *in situ* DVC data [34]. Our findings demonstrated that the response of bacteria within actively growing biofilms using the DVC method, Rh 123 and CTC were statistically comparable and approximately 90% of the total cell population appeared active. Bacterial population densities determined using these indices of cellular activity were also at least two-fold higher than with PC enumeration data [34]. A similar result was reported by another group studying the use of CTC to describe autochthonous biofilms in drinking water although they observed a lower fraction of the bacteria with active respiration [24]. These results collectively indicate that staining with CTC and Rh 123 accomplish the rapid and accurate (ie comparable with DVC data) *in situ* enumeration of active bacteria. They also allow the assessment of specific physiological properties of bacteria within biofilms to an extent that is not possible with conventional techniques. Hence, these fluorescent probes can be useful in revealing physiological insights when applied to bacterial biofilms and may be used in studies of biofilm control through the use of biocides.

Cryosectioning of biofilms for microscopic examination

Most structural studies of biofilms have relied on light or electron microscopy [27]. Conventional preparatory techniques cause the disruption or distortion of the biofilm structure or introduce serious artifacts resulting in the loss of the native community architecture. While very thin biofilms may be readily observed microscopically, thicker biofilms that are typical in many industrial and environmental systems are more difficult to study. The application of SCLM is a useful approach to visualize undisturbed biofilms *in situ* [2,3,13]. In this technique, the reconstruction of two-dimensional and three-dimensional images is based on optical sections collected through the community. However, this approach has been adopted in only a relative few laboratories.

Physical sectioning techniques have been extensively used with great success in the microscopic study of many biological tissues. For example, cryoembedding and cryotomy (cryosectioning) is routinely applied as a rapid clinical procedure in the diagnosis of certain diseases. This approach was applied in the microscopic examination of biofilm structure through the use of fluorogenic dyes [34]. Binary population biofilms of both *K. pneumoniae* and *Pseudomonas aeruginosa* grown on stainless steel coupons were used in the initial development of this method. Coupons with stained biofilm were placed on a block of dry ice and commercial tissue embedding medium was added to the surface. The frozen, embedded biofilms could then be easily and completely removed from the substratum by gently flexing the coupon. Thin (5-μm) vertical sections of the original biofilm were then obtained with a cryostat operated at $-19°$ C. These sections were mounted on microscope slides and examined by epifluorescence microscopy. This technique preserved the structural features of the biofilm including an irregular upper surface, water channels or voids and a well defined substratum interface. These same features have been observed using SCLM [2,3]. This technique has subsequently been applied successfully to the microscopic examination of biofilms of different thicknesses on various substrata. Hence, this method provides a relatively rapid and simple approach for examining individual cells and structural features within biofilms.

Studies on biofilms exposed to biocides

Experiments designed to provide community-wide estimates of biocidal effects within biofilms yield useful albeit

somewhat limited information. Such a study was conducted using binary population biofilms composed of *P. aeruginosa* and *K. pneumoniae* grown in an annular reactor with a minimal medium containing glucose as the only added energy source [28]. The thickness of the biofilms after one week of growth ranged between 30 and 60 μm. After exposure to monochloramine, the activity and viability of the cells was determined following physical removal from the coupons and compared with community-wide glucose utilization and oxygen uptake levels. The ability of the bacteria to form colonies on both nonselective and optimal selective media declined much more rapidly (3-log reduction) after one hour of disinfection than did cellular respiratory activity, measured by CTC reduction, which decreased by only one log. At the same time, there was only a slight change in the total number of bacteria within the biofilms. These results indicate that the disinfection treatment had little effect in removing the bacteria from the substratum and that PC data yielded the most optimistic estimate of biofilm disinfection. It was also not surprising that estimates of cellular activity using the CTC reduction assay were much higher than PC data following monochloramine exposure since the ability of such bacteria to form colonies often underestimates the actual viable population. However, the results of the oxygen and glucose utilization produced much more conservative appraisals of biocidal activity. These results clearly demonstrate that very different estimates of cellular viability and physiological activity are obtained when a range of analytical tools are used following biocide treatment. Specifically, enumeration results obtained using methods assessing physiological activities, such as respiration, indicate much less biocidal efficacy and are likely to more accurately reflect the ability of a surviving and injured sub-population to recover and resume growth following biocide treatment. Cultural techniques are known to be inefficient in the detection of disinfectant-injured bacteria [18]. Therefore, the findings of this study do provide useful information describing the effects of disinfectants on an entire biofilm community.

A similar study was done to examine the physiological response of bacteria in thin biofilms (ie 1–2 μm) to disinfection [36]. Biofilms of *K. pneumoniae* were grown on stainless steel coupons in a reactor for 36 h using dilute medium and exposed to either chlorine (0.25 mg L^{-1} free chlorine, pH 7.2) or monochloramine (1 mg L^{-1}, pH 9.0) for 10 min. Biocide effects were determined using CTC and Rh 123 as *in situ* fluorogenic indicators of individual cellular physiological activity, incorporation of tritiated uridine to reveal community-wide RNA turnover as well as PC and DVC estimates of bacterial viability following biomass removal. As in our previous report [35], results obtained using CTC, DVC and Rh 123 methods yielded bacterial densities that were comparable when examining untreated biofilms. These values were all in relatively close agreement with microscopic enumerations of the total bacterial population. Following treatment with both disinfectants, PC data were at least one log lower than results obtained using DVC and the fluorogenic indices of physiological activity. However, tritiated uridine incorporation and Rh 123 activity were approximately one log higher than CTC-positive and DVC-responsive cell numbers which were in turn, approximately one log higher than PC results following disinfection for 10 min. As in the previous study [28], large variations were observed between the responses of different physiological activities after exposure to low concentrations of biocides. Again, these results indicate that reliance on PC determinations of viable bacteria overestimates the efficacy of biocidal activity. However, the question of which physiological index is the most valid indicator of potential subsequent bacterial recovery and growth is a question that merits further consideration. It could be argued that respiratory activity (CTC reduction) is a useful physiological criterion when studying the biocidal activity of chlorine since bacterial respiration is one of the primary targets of that biocide [4] and the ability of bacteria to reduce CTC closely follows the DVC response, a well established criterion of bacterial viability. Likewise, bacterial membrane potential, as assessed by staining with rhodamine 123, is a useful criterion of viability. Hence, these indicators of physiological activity provide sensitive information describing the response of specific physiological processes in individual bacteria within biofilms to sublethal biocide expsure *in situ*. However, the results of this and the previous study [28] lack spatial resolution within the biofilm structure and do not allow critical questions concerning specific mechanisms of biocide action to be addressed.

More recently, we conducted studies intended to describe spatial and temporal patterns of respiratory activity within mature biofilms during disinfection [9]. Binary population biofilms of *P. aeruginosa* and *K. pneumoniae* were grown on stainless steel coupons in an annular reactor using mineral salts medium plus glucose for 7–10 days and exposed to low concentrations (2 and 4 mg L^{-1}) of monochloramine. Bacteria in treated biofilms were evaluated for viability by colony formation on a nonselective medium and for physiological activity by CTC reduction followed by cryosectioning and image analysis. Epifluorescent micrographs of CTC-stained sections of biofilms exposed to monochloramine for various times clearly revealed gradients of respiratory activity. Cells near the biofilm-bulk fluid interface lost respiratory activity first and that effect progressed with depth into the biofilms as the biocide treatment continued. After 2 h of disinfection, there was a small residual zone of bacteria capable of respiration deep within the biofilm structure(s). These gradients of respiratory activity with depth in the biofilm community were quantified by the ratio of CTC-positive cells to the total cell population, stained with DAPI, using IA. The resulting quantitative data supported the qualitative observations from the micrographs and suggested a possible mechanism explaining the increased bacterial resistance seen within thick biofilms. Therefore, by coupling the CTC probe for bacterial respiratory activity [35] with the cryosectioning technique [33], we were able to resolve spatial patterns of bacterial respiratory activity within a disinfected heterogenous biofilm community using microscopic observations and image analysis.

In summary, alternative measurements of physiological activity, including DVC [12,25,26,36], staining with fluorescent probes (2,9–11,20–22,24,29,30,32,34,35], and tritiated uridine incorporation [35] provide information distinct from the traditional plate count. In all cases, these alterna-

tive measures indicate higher levels of residual microbial activity after treatment with disinfectants, suggesting that PC results may overestimate biocide efficacy. When coupled with cryoembedding and cryosectioning, fluorescent stains can be used to visualize spatial patterns of physiological activity within biofilms [9]. As more intracellular probes are adapted for use in assessing different physiological processes and activities within bacteria, investigators will be more able to choose the appropriate probe(s) to describe heterogeneities in structured bacterial communities such as disinfected biofilms. Selection of the optimal probe for each application will depend on physiological knowledge concerning the phenomenon of interest.

Related techniques for the rapid assessment of physiological activities including energy metabolism and membrane potential are emerging [21,29,30]. In our quest to understand the underlying mechanisms that render microorganisms in biofilms more resistant to biocides than their planktonic counterparts, we are also coupling engineering tools, such as microelectrodes and computer models, with specific cellular physiological assays. In addition, the results obtained using these analytical approaches will lead to a more accurate understanding of biofilm structure and function.

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