

Comparison of Respiratory Activity and Culturability during Monochloramine Disinfection of Binary Population Biofilms

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Biofilm bacteria challenged with monochloramine retained significant respiratory activity, even though they could not be cultured on agar plates. Microbial colony counts on agar media declined by approximately 99.9% after 1 h of disinfection, whereas the number of bacteria stained by a fluorescent redox dye experienced a 93% reduction. Integrated measures of biofilm respiratory activity, including net oxygen and glucose utilization rates, showed only a 10 to 15% reduction. In this biofilm system, measures of microbial respiratory activity and culturability yielded widely differing estimates of biocide efficacy.

The efficacy of chemical biocides used to control microbial fouling has traditionally been measured by colony formation on microbiological media following removal of the bacteria from the substratum. Drawbacks to this approach are that it is slow and the ability of a bacterium to grow on a particular agar medium may not reflect its activity or viability in the environment from which it was removed. Colony formation assays tend to overestimate antimicrobial potency because they underestimate exposed bacterial populations (4, 6, 10, 15, 17). Alternative approaches to evaluating biocide efficacy include bioluminescence (14), impedimetry (3), direct viable counting (17), respiratory activity (12), and the use of fluorescent physiological probes (16). This article reports a comparison of various measures of respiratory activity with colony-forming ability as indicators of the efficacy of biofilm disinfection. Binary biofilms were exposed to monochloramine and biocidal efficacy was evaluated by plate counts, microelectrode-measured oxygen concentration profiles across the biofilms, overall glucose consumption rates, and ability of individual bacteria to reduce a redox stain.

Binary population biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were grown on a glucose minimal medium in a continuous-flow annular biofilm reactor. We chose to work with a binary population biofilm because thicker biofilms, more like those encountered in actual fouling environments, develop with the combination of microorganisms than with either one in pure culture (11). The reactor system and operating conditions have been described elsewhere (1, 2, 11). Biofilm was sampled from removable stainless steel slides that fit into grooved recessions in the reactor body. Biofilms were 1 week old when treated, at which time they were approximately 50 μm thick with total cell areal densities of 2×10^{12} to 8×10^{12} cells per m^2 . Monochloramine (4 mg of Cl per liter) was applied in a pulse-step treatment for 1 or 2 h (1). Neither the reactor system components nor the medium exhibited any

monochloramine demand (1), but the biofilm did consume monochloramine.

Biofilm bacteria were assayed by scraping biofilm from sample slides into 100 ml of phosphate buffer and dispersing cells by using a tissue homogenizer (Tekmar). The scraping procedure removed 95 to 98% of biofilm organisms. The homogenized cell suspension was plated in triplicate on R2A (7) and MT7 (5) agar (Difco). R2A is a nonselective medium; MT7 is selective for coliforms. Colonies were distinct, indicating that the biofilm was effectively disaggregated. Total cells were enumerated by direct counting of 4',6-diamidino-2-phenylindole (DAPI)- and acridine orange-stained samples by epifluorescence microscopy (1).

Residual microbial activity measured by colony counts on both agar media declined by approximately 4 to 5 orders of magnitude after 2 h of monochloramine treatment (Fig. 1). The species composition of the biofilms immediately prior to treatment ranged from 16 to 20% *K. pneumoniae*. The treatment killed the *P. aeruginosa* somewhat more quickly than it did the *K. pneumoniae*. The total number of bacteria remaining in the biofilm, as measured by acridine orange direct counts, declined by only 56% in the same interval (Fig. 1). These data suggest effective disinfection but relatively little removal of biofilm.

The number of respiring cells in the same suspension was determined by a modification of the procedure reported by Rodriguez et al. (9), which involves staining with the fluorescent redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Inc.). An aliquot was incubated in 0.05% CTC (final concentration) at 35°C for 2 h. Filtered formalin (final concentration, 5%) was added to fix the sample. Bacteria were collected by filtering onto a 25-mm-diameter, 0.2- μm -pore-size black polycarbonate membrane (Nucleopore), counterstained with 1 μg of DAPI per ml for 5 min, and then air dried prior to examination by epifluorescence microscopy. An Olympus BH-2 microscope configured with epifluorescence illumination and Olympus cubic unit B was used to enumerate total cells and respiring cells. Respiring cells were identified by the presence of intracellular red CTC-formazan crystals.

The number of respiring bacteria declined in response to monochloramine treatment but much less than did the number of culturable bacteria. Before treatment, the number of respiring cells was comparable to the number of CFU (Fig. 1). After 2 h of monochloramine treatment, the number of CTC-

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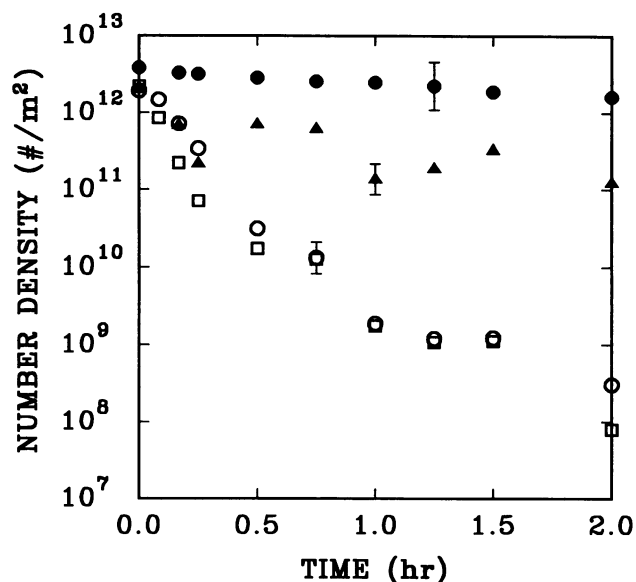


FIG. 1. Biofilm culturable, respiring, and total cell areal densities during monochloramine treatment. Total direct counts (●), CFU (○, R2A; □, MT7), and CTC-positive cells (▲) provide disparate measures of biocide efficacy. The data shown are averages of duplicate experiments; error bars indicate the constituent values of the average at a representative point.

positive bacteria exceeded the number of CFU by at least 2 orders of magnitude (Fig. 1). This comparison indicates that many of the bacteria determined to be incapable of producing a colony on an agar plate nevertheless still exhibited some respiratory activity.

Integrated measures of biofilm respiratory activity, including oxygen flux and overall glucose consumption, declined very little during monochloramine treatment. To quantify the flux of oxygen into biofilm, oxygen concentration profiles adjacent to and within a biofilm were measured during the course of monochloramine treatment by using cathode-type microelectrodes prepared as described elsewhere (8). Profiles were measured repeatedly at a single location on a biofilm-covered slide. Since the annular reactor was not configured to incorporate microelectrodes, these measurements were performed on a biofilm slide removed from the reactor and placed in a flow cell. Effluent from the annular reactor was fed to and recycled through the flow cell such that the hydraulic residence time in the flow cell was 3 min and the liquid velocity was approximately 0.01 m/s. Each profile measurement was acquired within 2.5 min.

Oxygen fully penetrated the biofilm before and during biocide treatment (Fig. 2). Oxygen profiles were used to calculate the relative flux of oxygen into the biofilm. The oxygen flux is proportional to the oxygen concentration difference between the biofilm-bulk fluid interface and the well-mixed bulk fluid. The flux measured at any time during biocide treatment has been expressed relative to the flux immediately prior to biocide treatment (Fig. 3) by taking the ratio of the measured concentration differences at the respective times. Determining these concentration differences requires that the biofilm surface be located. For the data shown in Fig. 2, the biofilm was estimated by microscopic examination to be 30 to 60 μm thick. The flux ratio was insensitive to the value of biofilm thickness over this range.

The glucose consumption rate of biofilm microorganisms was calculated by performing an overall material balance on

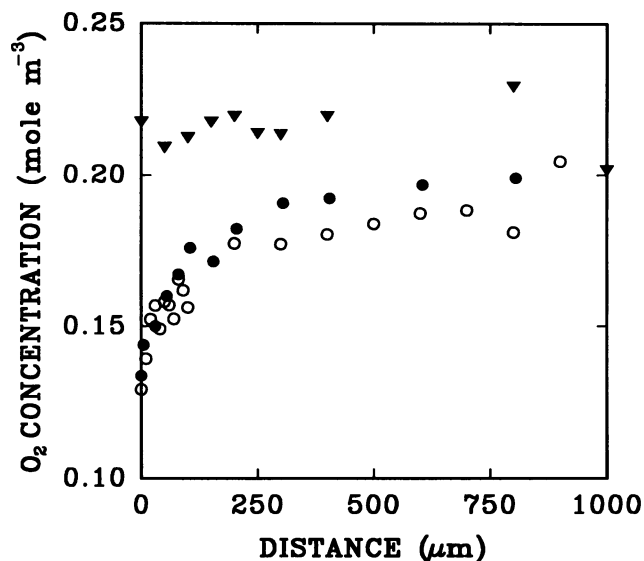


FIG. 2. Oxygen concentration profiles immediately prior to monochloramine treatment (○), after 60 min of treatment (●), and for a control treated with 2% formaldehyde (▼).

glucose around the annular reactor. Glucose in the reactor effluent was determined enzymatically with a commercial kit (Sigma). The accumulation term in the material balance equation was calculated by fitting a cubic polynomial to the concentration-versus-time data and differentiating to find the first derivative. Three replicate experiments were analyzed to determine glucose consumption rate.

Both energy source (glucose) and electron acceptor (oxygen) continued to be consumed in the biofilm at rates similar to their respective levels of utilization before disinfection (Fig. 3). The changes in glucose and oxygen utilization were consistent (10 to 15% reduction in 1 h) and indicate less loss in

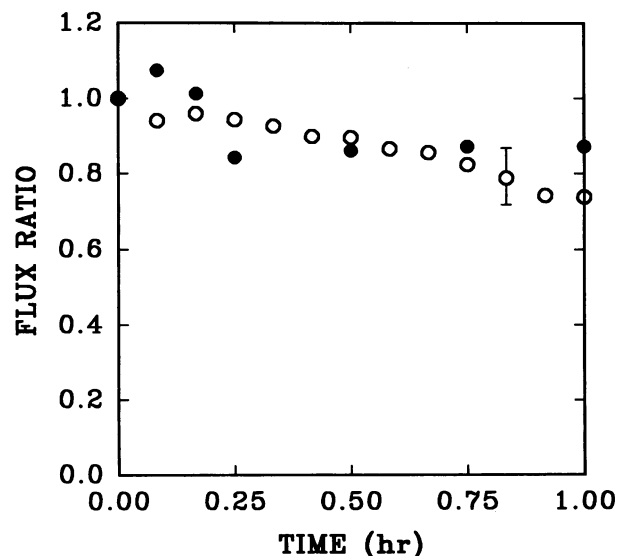


FIG. 3. Overall biofilm metabolic activity during monochloramine treatment. The oxygen flux into a point on a biofilm (●) and the overall glucose consumption rate (○) are normalized to their values immediately prior to monochloramine addition. The error bar indicates the maximum and minimum values included in the average.

TABLE 1. Summary comparison of methods for measuring the reduction in microbial biofilm activity after monochloramine treatment

Measured activity	Log ₁₀ (f) ^a at:	
	1 h	2 h
R2A agar plate colonies	-3.1 ± 0.3	-3.8 ± 0.4
CTC reducing cells	-1.2 ± 0.3	-1.2 ± 0.3
Glucose consumption rate	-0.15 ± 0.05	ND ^b
Oxygen flux	-0.05	ND
Total cells (acridine orange direct counts)	-0.21 ± 0.07	-0.37 ± 0.14

^a The results are expressed in terms of the log reduction of the fraction of pretreatment activity, *f*. The error estimate is the sample standard deviation (*n* = 2).

^b ND, not determined.

respiratory activity than does enumeration of CTC-reducing cells (93% reduction in 1 h). The reasons why CTC-reducing capacity and overall measures of glucose or oxygen consumption differ as quantitative measures of respiratory activity are not clear.

In summary, four methods for measuring the efficacy of monochloramine disinfection and removal of binary population biofilms of *P. aeruginosa* and *K. pneumoniae* were compared. Measures of microbial respiratory activity, such as reduction of the redox stain CTC, glucose consumption rate, and total oxygen flux into a biofilm, revealed much higher levels of residual microbial activity after monochloramine treatment than did plate counting with two media (Table 1). The results show that many of the microorganisms retained significant respiratory activity during monochloramine treatment even though they failed to form colonies on agar media. These findings are in agreement with previous reports demonstrating that biocidal efficacy determined by bacterial enumerations based on colony formation result in much greater estimates of antimicrobial activity than when indices such as the direct viable count (17) and bacterial luminescence (14) are used.

Our data are not adequate to discriminate between transient uncoupling of metabolism from cell growth and permanent loss of viability. That is, the experimental design did not allow resolution of whether respiring bacteria were nonviable with residual respiratory activity or merely injured and, thereby, not detected by colony formation (6). If the culture technique fails to detect injured organisms that eventually recover, the plate-counting method overestimates biocide efficacy. On the other hand, if the culture technique accurately reflects the potential for monochloramine-treated bacteria to grow, then measures of respiratory activity underestimate the effectiveness of the biocide in controlling microbial growth. Reports of very rapid biofilm regrowth (1, 13) support the interpretation of injury.

The use of respiratory activity to measure biocide efficacy has the advantage of being more rapid than traditional assays of culturability. Whereas determining colony-forming ability on plates requires at least 24 h, all three of the measures of respiratory activity we used can be completed within 3 h. Oxygen microelectrodes and substrate mass balances are probably impractical for many applications, but cellular staining with CTC should be broadly applicable in evaluation of biocides. CTC staining may not work for anaerobic biofilms or in the presence of reduced compounds that could facilitate nonspecific reduction. The CTC assay could potentially be made yet more rapid and less labor-intensive by using fluorimetry to measure total fluorescence instead of counting individual microorganisms.

These data raise the possibility that plate counts may seriously overestimate biocide efficacy in some applications. On the other hand, newer methods such as redox staining and *lux* gene assays might underestimate biocide efficacy if they underestimate the potential for growth. Critical evaluations of these methods in the context of specific applications are needed to assess their practical utility.

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