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Method for flow cytometric monitoring of *Renibacterium salmoninarum* inactivation

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ABSTRACT: The slow growth of *Renibacterium salmoninarum* limits the usefulness of culture as a research tool. Development of a 2-color flow cytometric assay to quantify the proportions of live and dead *R. salmoninarum* in a test population is described. Bacteria were simultaneously stained with fluorescein isothiocyanate-conjugated immunoglobulin and exposed to the exclusion dye propidium iodide. Propidium iodide red fluorescence profiles of control groups of untreated and killed *R. salmoninarum* were compared with those for bacteria exposed to chlorine. Bacterial inactivation was based on mean red fluorescence intensity, and analyzed by high-red fluorescence intensity (HRFI) and curve subtraction (CS) analyses. When the concentration of *R. salmoninarum* was 8.65×10^6 bacteria ml^{-1} and the bacteria exposed to chlorine at 1 mg l^{-1} for periods from 1 to 20 min (high-Rs assessment), the mean red fluorescence intensity of the profile for each chlorine-exposure group was higher than that for the untreated control ($p < 0.0001$). When the concentration of *R. salmoninarum* was reduced to 1.76×10^6 bacteria ml^{-1} and exposed to 0.8 mg l^{-1} free chlorine level for periods from 20 s to 5 min (reduced-Rs assessment), the mean red fluorescence intensities of the exposure groups were higher than that for the untreated control only when the *R. salmoninarum* was exposed to chlorine for at least 1 min ($p \leq 0.01$). On the basis of red fluorescence intensity, the proportion of dead cells generally increased with the duration of chlorine exposure. Whereas the rates of inactivation derived from the HRFI and CS analyses did not correlate with the duration of exposure in the high-Rs assessment ($r^2 \leq 0.27$), there was a correlation between these estimates and the duration of exposure in the reduced-Rs assessment ($r^2 \geq 0.92$). Because of the rapid loss of culturable *R. salmoninarum* in both assessments following chlorine exposure, neither the duration of exposure nor the inactivation estimates correlated with bacteriological culture ($r^2 \leq 0.22$). In both assessments, there was a correlation between the estimates of inactivation based upon HRFI and CS analyses ($r^2 > 0.99$). These results suggest that flow cytometry can be used as a supplementary or alternative method to bacteriological culture for monitoring the inactivation of *R. salmoninarum*.

KEY WORDS: *Renibacterium salmoninarum* · Flow cytometry · Viability assay · Propidium iodide · Chlorine

INTRODUCTION

Renibacterium salmoninarum is the causative agent of bacterial kidney disease (BKD) in wild and cultured salmonid fishes worldwide (Fryer & Sanders 1981). This Gram-positive bacterium may be transmitted vertically (Bullock et al. 1978, Evelyn et al. 1986b), but healthy susceptible fish can also become infected by waterborne *R. salmoninarum* (Mitchum & Sherman 1981, Bell et al. 1984). The disease is usually chronic, and mortality from BKD occurs among juvenile and adult fish in both fresh and salt water.

The chronic nature of the disease and the existence of more than one mode of transmission make BKD one of the most difficult bacterial fish diseases to control. Methods currently used to interrupt the cycle of vertical transmission include the injection of prespawning adult salmon with erythromycin phosphate (Bullock &

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Leek 1986, Evelyn et al. 1986a) and brood stock segregation (Elliott et al. 1989, Pascho et al. 1991). Horizontal transmission of *Renibacterium salmoninarum* to healthy fish is thought to occur while they are residing in the same water supply as infected fish. This is of great importance in hatcheries because salmon and trout are often reared in untreated river or ground water, or in the absence of adequate water sources, in recirculation systems. River water may contain resident species of fishes that are known to be susceptible to *R. salmoninarum*, or in many cases, progeny of fish from infected hatchery stocks which were allowed to spawn naturally in the river. In a water reuse hatchery, the probability of horizontal transmission is greatly increased if *R. salmoninarum* shed from the infected fish is repeatedly cycled past the healthy fish. Although the use of pathogen-free well or spring water would be most desirable, it may also be possible to disinfect or sterilize surface water. Methods to disinfect or sterilize water for fish culture include ozone, oxidizing agents, filtration, heat, ultraviolet light, quaternary ammonium compounds, and chlorine as a gas or solution (Dupree 1981, Wedemeyer 1996). There are only a few reports describing the inactivation of *R. salmoninarum* by antimicrobial compounds; iodine (Ross & Smith 1972), ozone (Wedemeyer & Nelson 1977, Wedemeyer et al. 1978), and chlorine (Pascho et al. 1995) are effective against the bacterium.

Laboratory studies examining antimicrobial treatments to interrupt vertical and waterborne transmission of *Renibacterium salmoninarum* have created a need to rapidly distinguish living from dead bacterial cells. The enzyme-linked immunosorbent assay (Pascho & Mulcahy 1987, Turaga et al. 1987) and the fluorescent antibody test (Bullock et al. 1980, Elliott & Barila 1987) are typically used to quantify the prevalence and levels of *R. salmoninarum*, but neither detection method can distinguish live from dead bacteria. Unfortunately, bacteriological culture is the only method currently available to quantify the proportion of viable bacteria in a population of *R. salmoninarum*. The slow growth of the bacterium, with incubation periods as long as 19 wk for growth on an agar medium (Benediktsdottir et al. 1991), can create substantial delays in the interpretation of experimental results.

Alternative methods have been reported for differentiating between dead and injured or living bacterial cells (Roszak & Colwell 1987, Calabrese & Bissonnette 1990, Desmonts et al. 1990, Singh et al. 1990, Byrd et al. 1991). Lethal injury of a microorganism by physical or chemical treatment is based on the severity of both the structural injury to the permeability barrier and the metabolic injury to the functional components (Ray 1979). If the lethal effects of an antimicrobial compound are initiated by damage to the cell wall, it may be possible to use an exclusion dye to distinguish cells with a lethal injury. Such dyes are considered viability stains because they do not enter cells with intact membranes and the absence of staining is considered indicative of a live cell.

Propidium iodide (PI) is a basic dye that binds to DNA and double-stranded RNA, yet is excluded by intact membranes (Parks et al. 1986, Shapiro 1988). PI staining can be used in conjunction with flow cytometry (FC) to create a rapid, quantitative fluorescence assay to describe the viability characteristics of a population of organisms. Analysis of the measurements permits one to distinguish unique or changing physical and chemical properties of a cell population. Although FC is commonly used in the analysis of eucaryotic cells, particularly in the field of immunology, it is now gaining wider use in bacterial studies. Fluorescence FC using PI has been used to identify bacteria in heterogeneous populations (Van Dilla et al. 1983, Miller & Quarles 1990), to monitor bacterial growth (Boye & Løbner-Olesen 1991), and to assess viability (Jepras et al. 1995, Nebe-von Caron et al. 1998). The ability of FC used in conjunction with PI and other fluorescent dyes to rapidly distinguish between live and dead bacteria has been used successfully to detect bacteria in viable but dormant, nonculturable states (Thorsen et al. 1992) and to monitor bacterial injury from antibiotics (Suller & Lloyd 1999) or survival following different lethal treatments (López-Amorós et al. 1995).

This report describes the development of a 2-color immunofluorescence flow cytometric assay to quantify the proportions of live and dead *Renibacterium salmoninarum* in a treatment population. Results from monitoring the inactivation of *R. salmoninarum* by chlorine suggest that FC can be used as an alternative or supplementary method to bacteriological culture in laboratory studies assessing the efficacy of antimicrobial compounds with this bacterium.

MATERIALS AND METHODS

Bacterial strain and growth conditions. A stock isolate of *Renibacterium salmoninarum* (ATCC 33209) was grown in KDM 2 broth (Evelyn 1977) as described by Pascho et al. (1995). The bacterial cells were washed 3 times, and on the basis of cell counts by the membrane-filtration fluorescent antibody test (MF-FAT), adjusted to the appropriate concentration in chlorine-demand-free 0.01 M phosphate-buffered saline (PBS) pH 7.0 (NaH_2PO_4-Na_2HPO_4 phosphate buffer in distilled-deionized water [Nanopure II; Barnstead Co., Newton, MA, USA]).

Quantification of bacteria by the MF-FAT. The concentration of *Renibacterium salmoninarum* cells in the

washed preparation was determined by the MF-FAT of Elliott & Barila (1987), as described by Pascho et al. (1995).

Fluorescent stains and microbeads. Affinity-purified goat immunoglobulin to *Renibacterium salmoninarum* conjugated with fluorescein isothiocyanate (FITC-IgG; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) was used for the MF-FAT, and the flow cytometric procedures described below. The FITC-IgG was filtered through a 0.2 µm, 13 mm polycarbonate filter (Nuclepore, Pleasanton, CA, USA) before use for the MF-FAT, or a 0.2 µm, polyvinylidene difluoride filter (Millipore, Bedford, MA, USA) before use in FC. Filtered conjugate was adjusted to the appropriate dilution in 0.01 M PBS pH 7.4.

A stock solution of propidium iodide (PI; Sigma Chemical Co., St. Louis, MO, USA) at 200 µg ml^{-1} was prepared in 0.01 M PBS pH 7.4 and aliquots were frozen at −70°C. To determine the proportion of dead *Renibacterium salmoninarum* cells in a treatment group, the bacteria were stained with a volume of freshly thawed stock PI solution at a final concentration of 5 µg ml^{-1}.

Concentrated Fluoresbrite carboxylate beads (5.75 ± 0.12 µm; Polysciences, Inc., Warrington, PA, USA) were diluted 1:24 (v/v) in sterile Nanopure water and centrifuged at 1000 × *g* for 10 min at 4°C. The pellet was resuspended in 25 ml sterile Nanopure water and stored at 4°C. To determine the concentration of beads in the final suspension, a 1 ml volume of the resuspended beads diluted to 1:1000 (v/v) in PBS-Triton was filtered through a 0.2 µm, 13 mm diameter polycarbonate membrane filter (Nuclepore, Pleasanton, CA, USA). The filter was examined with a fluorescence microscope and the bead concentration was calculated as described for the enumeration of bacteria.

Preparation of chlorine test solutions. A stock solution of sodium hypochlorite (5.2% active ingredient; J. T. Baker, Phillipsburg, NJ, USA) was prepared as described by Pascho et al. (1995) such that the free chlorine concentration was 40 mg l^{-1}. The free residual chlorine in the control and bacterial treatment solutions was neutralized with the appropriate volume of sterile 1% (w/v) sodium thiosulfate (Sigma Chemical Co., St. Louis, MO, USA).

Chlorine determinations. The free chlorine residual of the stock sodium hypochlorite solutions and treatment solutions were determined by the *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (American Public Health Association 1989).

General experimental procedure for chlorine exposures. After the washed *Renibacterium salmoninarum* were adjusted to the appropriate concentration in 0.01 M PBS, they were exposed to chlorine according to the general method described by Pascho et al. (1995). Briefly, equal volumes of a bacterial preparation were placed in 2 stirred flasks and equilibrated to 15°C in an incubator or a chilled, circulating water bath. A prescribed volume of a stock chlorine solution equilibrated to 15°C was added to one of the stirring flasks. After each exposure period, a 10 ml volume was removed and neutralized by the addition of 100 µl of 1% (w/v) sodium thiosulfate. Subsamples of control bacterial preparations and bacteria from the neutralized chlorine treatments were then cultured on agar plates as described above. In a separate study, 0.01% (w/v) sodium thiosulfate did not inactivate *R. salmoninarum* as determined by bacteriological culture and flow cytometry (data not shown). The concentration of free chlorine was measured over the duration of an experimental exposure in the second flask of bacteria after addition of the same concentration of chlorine as was used for the experiment; the bacteria were removed from each sample by passing the suspension through a 0.2 µm polysulfone membrane filter (Gelman Sciences, Ann Arbor, MI, USA), and the free chlorine concentration was measured immediately.

Reference bacterial preparations. In addition to the chlorine exposure groups, each procedure also contained 2 reference groups of *Renibacterium salmoninarum* that aided in interpreting the results of an analysis. They were untreated bacteria, and a corresponding bacterial preparation that had been heated at 80°C for 20 min to represent 100% inactivation. The red fluorescence intensity profile for untreated bacteria was centered near channel 60 on the 1 to 200 scale (log) and represented 100% viability. The corresponding profile for an identical bacterial preparation heated at 80°C for 20 min, typically located near channel 120, represented 100% inactivation.

Final procedure for FC. An Ortho Cytofluorograph 50H with a model 2150 computer (Ortho Diagnostic Systems, Westwood, MA, USA) was used for this study. The FITC and PI were excited at 488 nm with a model 164-05 argon ion laser (Spectra Physics, Mountain View, CA, USA).

For FC, a constant number of the 5.75 µm fluorescent microspheres were added to the control and treatment groups, then the bacterial cells were simultaneously stained with FITC-IgG and PI for 60 min in a 15°C circulating water bath. After incubation with the fluorescent dyes, the bacteria were held on ice until they were analyzed.

During flow cytometric analysis, individual areas from the 2-parameter histograms of forward versus right angle light scatter were selected that represented the microspheres and the bacteria. To standardize the analyses among treatment groups, data were collected for the red fluorescence intensity of bacteria that passed the detector during the period required to

detect a predetermined number of microspheres. Both the microspheres and the bacteria were counted based on FITC emissions detected with a 525/30 nm emission filter, and the red fluorescence of the PI emissions was detected with a 600 nm long-path emission filter. The red fluorescence of each cell was simultaneously measured and included in a profile describing the relative red fluorescence distribution for all of the cells analyzed from a given experimental suspension.

Reference bacterial viability assays. The numbers of viable *Renibacterium salmoninarum* in each control and experimental bacterial preparation were determined by bacteriological culture. Samples were cultured at 15°C for 30 d after inoculation onto plates of a charcoal agar medium (Daly & Stevenson 1985) supplemented with 1.5% (v/v) of the cell-free supernatant of KDM 2 broth which had been used to grow *R. salmoninarum* for 14 d at 15°C (Evelyn et al. 1990). Each inoculum consisted of a 100 µl volume of undiluted bacteria, or of material from one or two 10-fold dilutions of bacteria in peptone-saline, that was evenly dispersed with a glass spreader over the surface of the agar medium in each of 4 petri dishes.

Bacterial inactivation measured by FC. For FC, the relative positions of profiles for groups of bacteria exposed to chlorine under different experimental conditions were analyzed using the locations of the untreated and inactivated control profiles as reference points. Two methods were used to determine the proportion of dead cells:

(1) High red fluorescence intensity (HRFI) method based upon the heat-control group. A bacterial cell was considered dead if it produced a red fluorescence that exceeded channel 100 on a histogram of red fluorescence intensity. The proportion of inactivated bacterial cells in each replicate of a treatment group was then calculated on the basis of the corresponding replicate of the heat-control group.

$$\%\ \text{Inactivation}_{ij} = \frac{\text{Treatment}_i\,\text{Replicate}_j}{\text{Heated (80°C) control replicate}_j} \times 100$$

(2) Curve subtraction (CS) analysis method based upon untreated control group. Each replicate of the heat control group and of the chlorine treatment groups was compared to the corresponding replicate of the untreated control group with the Multiple Option Two Parameter Analysis program (Phoenix Flow Systems, San Diego, CA, USA). The area of the control profile was subtracted from that of the corresponding treatment group and the remaining cells were considered dead. The program compensated for differences in the total number of cells evaluated in each replicate subgroup within a given comparison. The proportion of inactivated bacterial cells in each replicate of a treatment group was then calculated on the basis of the corresponding replicate of the heat-control group according to the formula given above.

For direct comparison of FC data to bacterial plate assays, a means of establishing an accurate measure of concentration (number per unit volume) was required for FC-processed samples. The FC analysis produces counts that can only be interpreted as proportions of live and dead categories. A reduction in organism concentration due to disintegration would not be revealed using such a procedure. It is difficult to verify volumes or flowrates directly from flow cytometer operating data. Accordingly, we added a selected volume of a suspension containing precisely defined numbers of fluorescent microspheres, readily distinguishable from target organisms by size. Counts of target organisms were then controlled by counting a fixed number of fluorescent microspheres.

Data analysis. Dunnett's test was used for the pairwise comparisons of the mean bacterial count for the untreated control to the mean bacterial count for a given treatment group. Pairwise comparisons of the mean red fluorescence intensities for groups of untreated bacteria and those for a given treatment group were done by the Scheffé's *F*-test. To determine if a correlation existed between the results from different measures of bacterial inactivation, a coefficient of determination (r^2) was calculated between the duration of exposure and individual viability estimates, or pairwise among the various viability estimates.

RESULTS

Population selection

Rapid differentiation of live and dead bacterial cells by FC was evaluated by use of suspensions of *Renibacterium salmoninarum* exposed to either a lethal heat treatment, or to various concentrations of chlorine. Initially, 2 light scatter measurements were used to select the bacteria for which the relative viability was assessed by measuring the red fluorescence of PI. Results were encouraging when intact cells killed by heat and stained with PI were tested by fluorescence microscopy and FC. However, the ability of the forward scatter and right angle FC measurements to resolve *R. salmoninarum* from background events was compromised by the relatively small size of the bacterium (0.3–0.5 µm by 0.1–1.0 µm). Machine settings required for these analyses with the Ortho 50H flow cytometer made it difficult to distinguish the bacterium from electronic noise and events recorded presumably because of other material not related to the bacterium (Fig. 1).

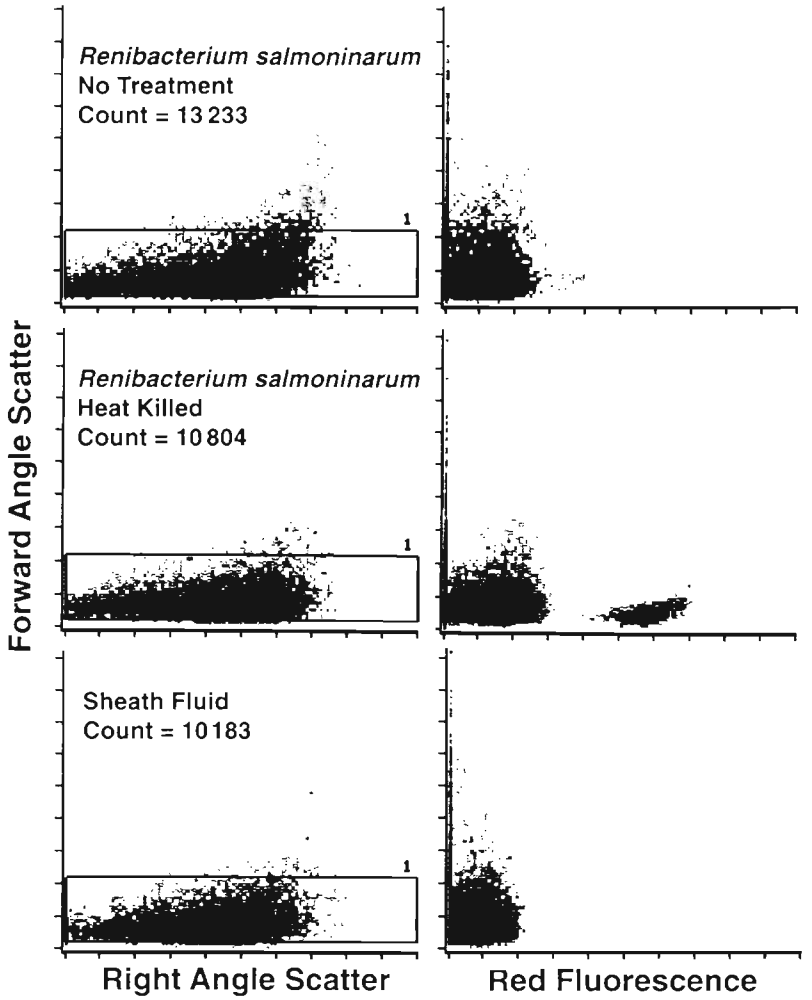

Fig. 1. Cytograms for untreated and heat-killed *Renibacterium salmoninarum* cells, and sheath fluid only. Forward angle versus right angle light scatter and PI red fluorescence intensity. The final concentration of PI was 5 µg ml^{-1} The number of detections represented in each cytogram for the area designated by the box (Region 1) is given with the corresponding treatment designation

Development of a two-color immunofluorescence flow cytometric assay

To more accurately gate the *Renibacterium salmoninarum* cells from background noise when assessing their viability, a 2-color immunofluorescence procedure using FITC-IgG and PI was selected for further testing. The bacteria in each experimental suspension were simultaneously stained with FITC-IgG and exposed to PI. The green fluorescence emitted by the bound conjugate was a signal to the detection system in the flow cytometer that an *R. salmoninarum* cell was passing in the stream of sheath fluid.

Dilution of the FITC-IgG

Analysis by 2-color fluorescence of a suspension containing equal amounts of untreated and heat-killed bacteria showed that the dilution of FITC-IgG affected the accuracy of the red fluorescence measurements, and consequently, discrimination of live from dead bacteria. The predicted red fluorescence profile contained 2 peaks, one representing relatively weak fluorescence intensities or live bacteria, and one comprised of dead cells with very high red fluorescence intensities. The highest tested concentration (1:20 v/v) of the FITC-IgG gave the poorest resolution of the 2 peaks (Fig. 2). The peaks became more distinct as the concentration of FITC-IgG was decreased. A CS analysis based upon red fluorescence profiles of the untreated bacteria stained with PI at 5 µg ml^{-1} and the appropriate dilution of FITC-IgG indicated that a conjugate dilution of at least 1:40 (v/v) was required for adequate separation of the peaks. On the basis of the red fluorescence

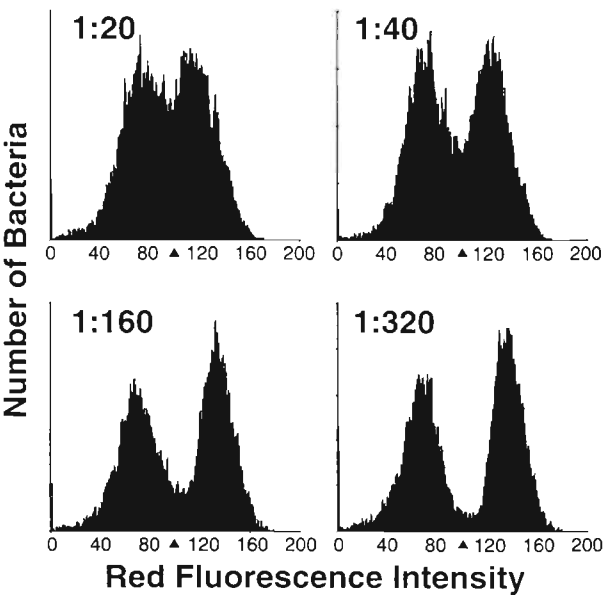

Fig. 2. Effect of the dilution of FITC-IgG on the differentiation of live and dead *Renibacterium salmoninarum* cells by FC. A suspension containing equal numbers of live and dead cells was incubated with 1 dilution of FITC-IgG and PI at a final concentration of 5 µg ml^{-1} for 60 min at 15°C. The red fluorescence intensity profile for the 1:80 (v/v) dilution of the FITC-IgG is not shown. Channel 101 (▲) is the beginning of the region of HFRI, and any bacteria with an HFRI (≥ channel 101) were categorized as dead

histograms for the FITC-IgG at either a 1:160 or a 1:320 (v/v) dilution, the red high intensity region began at channel 100. The proportions of *Renibacterium salmoninarum* cells considered dead in the mixed suspensions based upon their red fluorescence intensity exceeding channel 100 were very similar to those obtained by CS analysis. As the FITC-IgG was serially diluted from 1:20 to 1:320 (v/v), the percentages of dead cells according to CS analysis were 45.6, 47.3, 48.4, 52.3, and 52.0%, respectively.

When the red fluorescence profiles of identically stained individual treatment groups of untreated or heat-killed *Renibacterium salmoninarum* were compared by plotting them on the same histogram, the profiles diverged as the FITC-IgG dilution increased (data not shown). Again, maximum separation of the peaks occurred when the conjugate was diluted 1:160 or 1:320 (v/v). On the basis of CS analysis, the percentages of dead cells in the heat-killed *R. salmoninarum* suspensions approached the expected level of 100% as the FITC-IgG concentration decreased; the proportions of *R. salmoninarum* cells categorized as dead in the heat-killed suspension at each conjugate dilution were 94.1% (at an FITC-IgG dilution of 1:40 [v/v]), 96.5% (1:80 [v/v]), 99.1% (1:160 [v/v]), and 99.3% (1:320 [v/v]). These proportions correlated with the those obtained when the red fluorescence profiles were divided at channel 100 (r^2 = 0.99); the corresponding proportions of *R. salmoninarum* cells considered dead in the heat-killed suspensions were 97.4, 98.6, 99.8, and 99.9%. Dividing the histograms at channel 100 also suggested that some of the bacteria in the untreated preparations were dead (1.35 to 3.51%), and that a percentage of the bacteria in the heat-treated preparations were not stained with propidium iodide (0.12 to 2.56%). These phenomena were represented by the overlap in the red fluorescence intensity profiles for the untreated and heated preparations at each dilution of FITC-IgG; the minimum overlap occurred when the FITC-IgG was diluted 1:320 (v/v). On the basis of the results from testing the untreated and heat-killed *R. salmoninarum* preparations individually or as mixed suspensions, a 1:320 (v/v) final dilution of the FITC-IgG conjugate was selected to be used for FC in conjunction with PI at 5 µg ml^{-1}. Channel 101 was assigned as the beginning of the region of high red fluorescence intensity (HRFI), and any bacteria with an HRFI (≥ channel 101) were categorized as dead.

Standardization with fluorescent carboxylate microspheres

The addition of 5.75 µm fluorescent carboxylate microspheres to each bacterial suspension was investigated as a method to standardize the analyses, and to permit accounting for disintegration (disappearance of organisms) as a product of lethal exposure. These microspheres, which could be distinguished from *Renibacterium salmoninarum* by their size (Fig. 3), contained a dye that has excitation and emission maxima very near that of FITC and is matched to the 525/30 nm emission filter. A constant number of microspheres was added to each control or treatment bacterial suspension, then the FITC-labelled *R. salmoninarum* cells were tabulated during the time required for a preselected number of fluorescent microspheres to pass the detector.

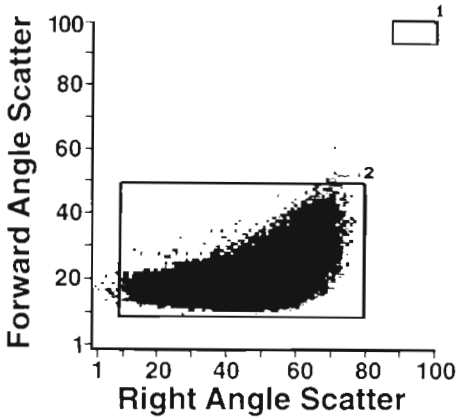

Fig. 3. Cytogram showing differentiation between 5.75 µm fluorescent carboxylate microspheres (Region 1) and *Renibacterium salmoninarum* cells (Region 2)

The experimental step at which the beads were added to an *Renibacterium salmoninarum* suspension affected the variability in the number of bacterial cells counted among replicate samples. Fluorescent microspheres were added to identical suspensions of *R. salmoninarum* at 5×10^6 bacteria ml^{-1} before or after control or chlorine treatments, then the number of bacteria was tabulated during the period necessary for detection of 2000 fluorescent microspheres; the ratio of bacteria to microspheres was 200:1, and the chlorine treatment groups were exposed to a starting concentration of 1 mg l^{-1} free chlorine for 20 min at 15°C. When the beads were added before the *R. salmoninarum* cells were divided into the control and chlorine treatment groups, the variability among the replicates in the control and chlorine-treatment groups was 15.9 and 7.1%, respectively. The variability among the replicates in the same groups was 4.0 and 3.9% when the beads were added after treatment and before being aliquoted into the tubes for staining and replicate analysis by FC. For all subsequent experiments the fluorescent microspheres were added to the *R. salmoninarum* suspensions after treatment, but before being aliquoted into the replicate tubes for addition of the PI and FITC-IgG.

Variation in the red fluorescence intensity profile for untreated *Renibacterium salmoninarum*

The interassay variation of the red fluorescence intensity profile for untreated *Renibacterium salmoninarum* was examined by analyzing bacteria from suspensions prepared on 3 dates with the final 2-color imunofluorescence procedure. The cytogram for forward angle versus right angle light scatter was aligned near channel 60 and the size of region 2 was adjusted to ensure that at least 95% of the bacteria were included in the analysis. Each suspension was adjusted to 5×10^7 bacteria ml^{-1} and the bacteria were analyzed as a predetermined number of fluorescent microspheres passed the detector. Flow cytometry results suggested that the method used in this study to prepare *R. salmoninarum* cells for heat or chlorine treatment yielded a population of bacteria that had a predictable red fluorescence intensity distribution (Fig. 4). A comparison of the mean red fluorescence profiles among the 3 experimental days by the chi-square test or the Kolmogorov-Smirnov test was not possible because of the large number of bacteria analyzed from each suspension (>160 000 *R. salmoninarum*) resulted in significant differences between replicate samples from the same preparation.

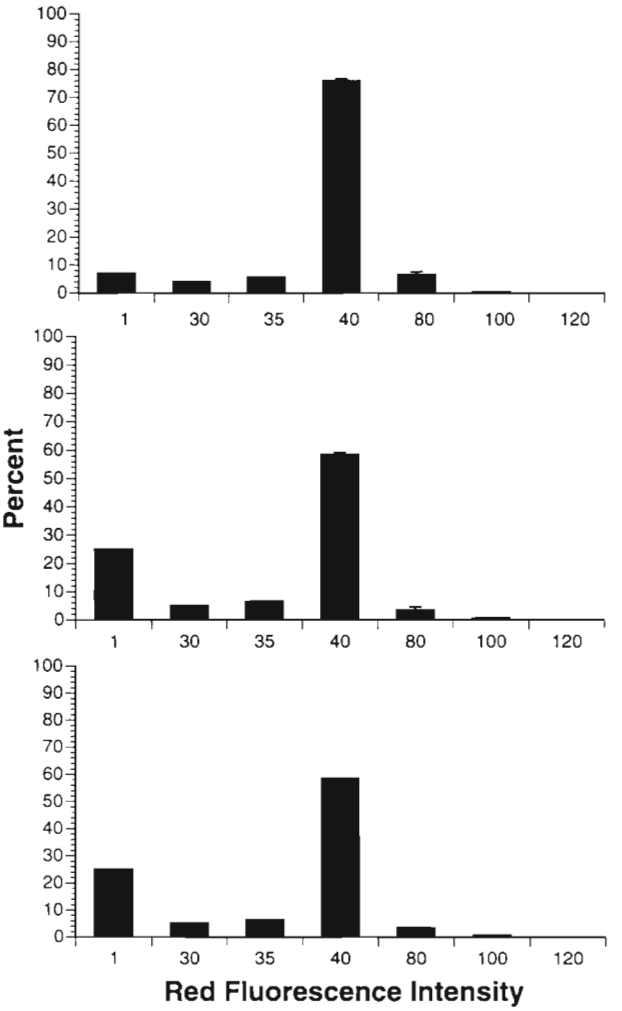

Fig. 4. Histograms of mean red fluorescence intensity distributions for untreated *Renibacterium salmoninarum* prepared on 3 dates. Bars represent 1 standard deviation. Four replicate samples from each preparation were analyzed by FC after the bacteria were stained with PI and FITC-IgG for 60 min at 15°C. The final concentration of PI was 5 µg ml^{-1}, and the final dilution of FITC-IgG was 1:320 (v/v)

FC assessment of the inactivation of *Renibacterium salmoninarum* exposed to chlorine

The final 2-color immunofluorescence assay was then evaluated as a method to describe the inactivation of *Renibacterium salmoninarum* by chlorine. For each evaluation the red fluorescence profiles of control groups of *R. salmoninarum* cells (untreated or heated at 80°C for 20 min) were compared with bacteria from the same suspension that had been exposed to chlorine. The flow cytometric assessments, and the corresponding bacteriological culture data, for 2 representative exposures of *R. salmoninarum* to chlorine are summarized in Table 1. The assessments were done under experimental conditions that differed with regard to the concentration of *R. salmoninarum* cells, initial concentration of chlorine, and duration of chlorine exposure. In one assessment a relatively high concentration of *R. salmoninarum* (8.65×10^6 bacteria ml^{-1}) was exposed to chlorine at an initial level of 1 mg l^{-1} for periods from 1 to 20 min (high-Rs assessment), and in the second assessment the concentration of *R. salmoninarum* was reduced 5-fold to 1.76×10^6 bacteria ml^{-1} and exposed to an initial level of 0.8 mg l^{-1} free chlorine for periods from 20 s to 5 min (reduced-Rs assessment). The mean red fluorescence intensity of the profile for each chlorine-exposure group was significantly higher than that for the untreated control in the high-Rs assessment ($p < 0.0001$; Fig. 5). In the low-Rs assessment, the mean red fluorescence intensities of the exposure groups were significantly higher than that for the untreated control only when the *R. salmoninarum* were exposed to chlorine for at least 1 min ($p \leq 0.01$; Fig. 6). On the basis of the HRFI and CS analyses, the proportion of dead cells generally increased with the duration of chlorine exposure in both assessments. Whereas the rates of inactivation derived from those analyses did not correlate with the duration of exposure in the high-Rs assessment ($r^2 \leq 0.27$), there was a strong correlation in the reduced-Rs assessment between either estimate and the duration of exposure ($r^2 \geq 0.92$). In both Rs assessments, there was an

Table 1. Comparison of FC and bacteriological culture measurements of the inactivation of *Renibacterium salmoninarum* by heat or chlorine at 15°C. There were 4 replicate red fluorescence intensity (RFI) profiles for each treatment

Treatment	Free chlorine (mg l^{-1})[a]	Mean (SD) inactivation (%) — RFI[b] — High RFI (HFRI)	Curve subtraction (CS)	Bacteriological culture[c]
(A) High-Rs assessment. ***R. salmoninarum*** **concentration 8.65 × 10^6 ml^{-1}**				
Controls:				
Heat (80°C)	NA[d]	–	–	100
No treatment	NA	2.15 (0.12)	–	–
Chlorine exposure:				
1 min	0.66	34.96 (1.15)	64.82 (1.20)	99.999
3 min	0.65	82.74 (4.32)	93.00 (1.62)	99.998
5 min	0.61	91.07 (3.93)	96.47 (1.47)	99.999
10 min	0.54	85.45 (3.31)	94.54 (1.58)	100
20 min	0.46	86.14 (2.92)	95.09 (1.45)	100
(B) Reduced-Rs assessment. ***R. salmoninarum*** **concentration 1.76 × 10^6 ml^{-1}**				
Controls:				
Heat (80°C)	NA[d]	–	–	100
No treatment	NA	11.46 (0.82)	–	–
Chlorine exposure:				
20 s	0.66	12.30 (0.73)	2.67 (2.62)	99.957
40 s	0.53	14.15 (0.44)	5.53 (2.43)	99.999
1 min	0.47	16.36 (0.64)	9.94 (1.72)	100
2 min	0.43	50.38 (4.05)	54.01 (6.11)	100
3 min	0.40	79.19 (4.57)	80.47 (3.17)	100
5 min	0.38	97.78 (1.88)	98.40 (1.10)	100

[a]The initial concentrations of free chlorine in the high-Rs and the reduced-Rs assessments were 1.0 and 0.8 mg l^{-1}, respectively

[b]The proportion of inactivated *R. salmoninarum* cells in each replicate of a treatment was calculated on the basis of the corresponding replicate of the heat-control group.

High-Rs assessment:

$$\%\ \text{Inactivation}_{ij} = \frac{\text{Treatment}_i\ \text{Replicate}_j}{\text{Heated (80°C) control replicate}_j} \times 100$$

The mean (SD) percentage of dead bacteria in the heat-control group based upon HRFI analysis was 93.18 (1.48), and 93.75 (0.52) based upon CS analysis.

Reduced-Rs assessment:

$$\%\ \text{Inactivation}_{ij} = \frac{\text{Treatment}_i\ \text{Replicate}_j}{\text{Heated (80°C) control}} \times 100$$

The percentage of dead bacteria in the heat-control group based upon the HRFI analysis was 83.89%, and 73.53% based upon CS analysis; these percentages are based on a single determination

[c]No standard deviation was calculated for these percentages; the numbers of *R. salmoninarum* colonies on each of 4 replicate agar plates at the appropriate dilution were averaged

[d]Not applicable

extremely rapid loss by *R. salmoninarum* of its ability grow in bacteriological culture following exposure to chlorine; neither the duration of exposure nor the HRFI and CS inactivation estimates correlated with the results of bacteriological culture ($r^2 \leq 0.22$). In both assessments, there was a correlation between the estimates of inactivation based upon the HRFI and CS analyses ($r^2 > 0.99$).

For each control and chlorine-treatment group in the high-Rs assessment, the replicate red fluorescence profiles were made from the *Renibacterium salmoninarum* cells that passed the detectors during the period required to count 1000 fluorescent microspheres. Although the time necessary to detect that number of fluorescent microspheres ranged from 28 to 36 s among the 28 analyses in this investigation, there was no correlation between the time necessary to count 1000 fluorescent microspheres and the total number of *R. salmoninarum* cells detected and analyzed ($r^2 = 0.40$). The mean numbers of bacteria detected in the chlorine-treatment groups and the control group that received the lethal heat treatment, however, were significantly lower than that for the untreated control group ($p < 0.01$; Table 2). The changes in the mean numbers of bacteria analyzed did not correlate with the duration of exposure, and there were no significant differences in the mean counts among the chlorine exposure groups ($p > 0.05$).

DISCUSSION

In this report, we have described the development of a flow cytometric technique that uses PI staining to quantify the proportions of living and dead bacterial cells in a *Renibacterium salmoninarum* population.

As a prerequisite to flow cytometric live-dead discrimination, we found that it was necessary to label *Renibacterium salmoninarum* with FITC-IgG to permit the bacterium to be accurately identified for analysis. The relatively small size, and possibly the diplococcoid morphology, of *R. salmoninarum* contributed to errors in gating the bacterium strictly on the basis of right angle and forward light scatter. The accuracy of the light scatter measurements was

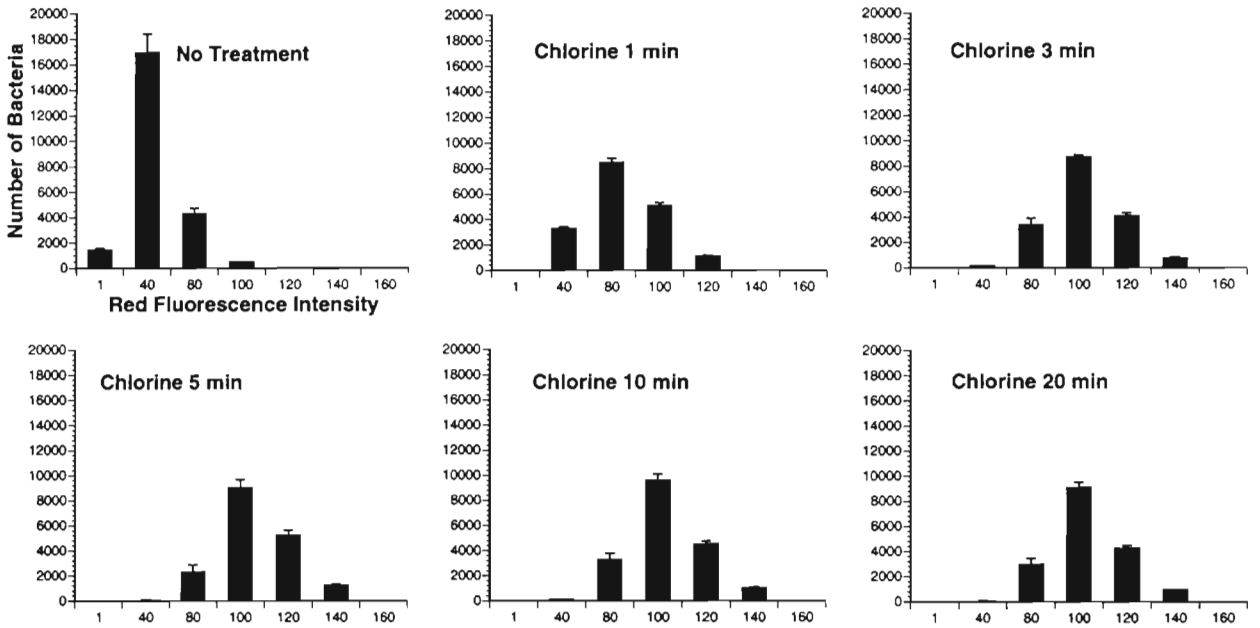

Fig. 5. Red fluorescence intensity histograms for suspensions of *Renibacterium salmoninarum* in the high-Rs assessment groups. Bacteria were analyzed by FC after receiving no treatment, or after exposure to chlorine for 1 to 20 min at 15°C. The concentrations of free chlorine are shown in Table 1A. The bacteria in each preparation were stained with PI and FITC-IgG for 60 min at 15°C. The final concentration of PI was 5 μg ml^{-1}, and the final dilution of FITC-IgG was 1:320 (v/v). The concentration of *R. salmoninarum* in each suspension was 8.65×10^6 bacteria ml^{-1}. The channel at which the mean (SD) red fluorescence intensity occurred in each control or treatment group was as follows: no treatment 66.78 (0.21); chlorine for 1 min 93.70 (0.22); chlorine for 3 min 111.80 (1.19); chlorine for 5 min 115.65 (1.14); chlorine for 10 min 112.98 (0.99); and chlorine for 20 min 113.08 (0.83)

principally affected by the extreme gain settings necessary for resolving such a small bacterium with the Ortho 50H flow cytometer. Other reports have described similar problems associated with bacterial morphology in resolving certain bacteria from electronic noise, or distinguishing between bacterial species, by flow cytometric methods (Barnett et al. 1984, Donnelly & Baigent 1986, Monfort & Baleux 1992).

Table 2. Duration of analysis necessary for 1000 fluorescent microspheres to be tabulated by the flow cytometer, and the number of *Renibacterium salmoninarum* cells detected during that period. There were 4 replicate determinations for each control or treatment group

Group	Duration (s)		*R. salmoninarum* cells
	Range	Mean (SD)	Mean (SD)
Controls:			
Heat (80°C)	29–35	32.25 (2.50)	12502 (1163)
No treatment	30–34	32.25 (1.71)	23245 (1929)
Chlorine exposure:			
1 min	31–36	32.75 (2.22)	18173 (663)
3 min	29–33	31.25 (1.71)	17209 (263)
5 min	29–32	30.25 (1.50)	17970 (1399)
10 min	32–35	33.25 (1.50)	18647 (908)
20 min	29–34	31.50 (2.08)	17428 (854)

Staining the surface antigens of *Renibacterium salmoninarum* with an appropriate amount of FITC-IgG was directly correlated with the resolution of the red fluorescence profiles for untreated and killed bacteria by FC. Because there is some overlap in the red regions of the emission spectra for FITC and PI, the detector for the red fluorescence of PI will also detect some fluorescence that is actually contributed by FITC (Parks et al. 1986). When FITC-IgG was used at high concentrations, this contribution was sufficient to shift the mean red fluorescence profile for the untreated *R. salmoninarum* significantly toward the region of HRFI.

The mean counts of *Renibacterium salmoninarum* labelled with the FITC-IgG decreased among bacterial preparations exposed to heat or chlorine, suggesting that the cellular structure was damaged by these treatments. Severe disruption of the cell wall may have resulted in bacterial fragments still capable of binding the FITC-IgG, but too small to be detected by the flow cytometer. Longer durations of chlorine exposure would probably cause disruption of higher proportions of bacteria, yet the counts did not decrease relative to the length of exposure. These data suggest that a large subpopulation of the *R. salmoninarum* cells (i.e., ca 30% as affected by chlorine; ca 50% as affected by heat) may have been more susceptible to lethal treatment, e.g., oxidation by chlorine, because of their age

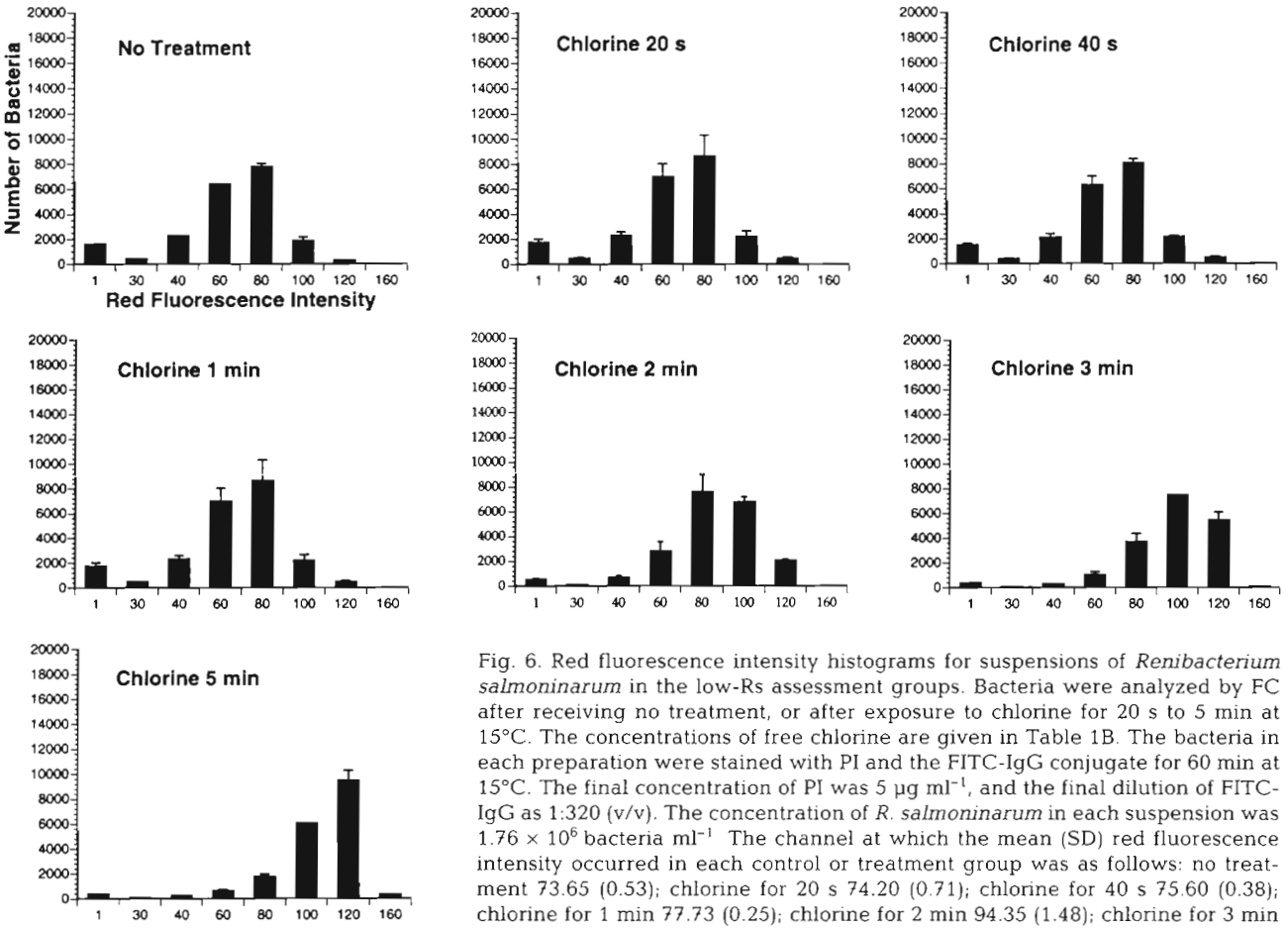

Fig. 6. Red fluorescence intensity histograms for suspensions of *Renibacterium salmoninarum* in the low-Rs assessment groups. Bacteria were analyzed by FC after receiving no treatment, or after exposure to chlorine for 20 s to 5 min at 15°C. The concentrations of free chlorine are given in Table 1B. The bacteria in each preparation were stained with PI and the FITC-IgG conjugate for 60 min at 15°C. The final concentration of PI was 5 µg ml^{-1}, and the final dilution of FITC-IgG as 1:320 (v/v). The concentration of *R. salmoninarum* in each suspension was 1.76×10^6 bacteria ml^{-1} The channel at which the mean (SD) red fluorescence intensity occurred in each control or treatment group was as follows: no treatment 73.65 (0.53); chlorine for 20 s 74.20 (0.71); chlorine for 40 s 75.60 (0.38); chlorine for 1 min 77.73 (0.25); chlorine for 2 min 94.35 (1.48); chlorine for 3 min 105.88 (1.76); and chlorine for 5 min 116.18 (1.01)

or random structural defects, resulting in the loss of approximately equal proportions of cells in each chlorine-exposure group.

Whereas the use of the 2-color fluorescence procedure appeared promising for delineating live from dead cells, neither the PI nor the FITC-IgG allowed direct identification of cells that had been inactivated by heat or chlorine treatments, particularly when the FC protocol was standardized by counting a constant number of bacteria from each preparation. Modifying the FC protocol to analyze bacteria over a uniform period of time was also found to be inappropriate because the bacteria passed the detectors at variable rates. Consequently, the addition of fluorescent carboxylate microspheres to each bacterial suspension was adopted to standardize the analyses.

The detection of intact dead *Renibacterium salmoninarum* cells by fluorescence FC relied upon those cells loosing their membrane integrity, and thus becoming permeable to a fluorescent exclusion dye. PI was selected for this study because it is the recommended exclusion dye for testing membrane integrity (Shapiro 1988); the fluorescence of PI may actually increase several-fold after it intercalates with a double-stranded nucleic acid (Krishan et al. 1978). Under the conditions of this study, the oxidative action of chlorine was contributed by hypochlorous acid, a chlorine species with potent germicidal activity that is related to its ability to penetrate a microorganism's membrane (Haas & Englebrecht 1980). A range of physiological effects on aquatic microorganisms has been correlated with the biocidal effectiveness of chlorine, including action on one or more metabolic pathways and damage to an organism's DNA (Breisch et al. 1984). Haas & Englebrecht (1980) investigated mechanisms that might be responsible for the lethal effects of chlorine on *Escherichia coli*, *Mycobacterium fortuitum*, and *Candida parapsilosis*. These authors found that the effects of aqueous chlorine were dependent upon its action on the cell membrane and passage into the cell, followed by damage to the DNA. The red fluorescence intensity of a particular *R. salmoninarum* will be dependent upon passage of the PI past the cell wall and the intercalating of the dye at sites on the double-stranded nucleic acids. The latter should correlate with the amount of double-stranded nucleic acid present, and

possibly the opening of additional binding sites by the action of chlorine. The amount of double-stranded nucleic acid was not uniform among individual *R. salmoninarum* cells in the control or treatment groups because the nucleic acid content of a bacterium is dependent upon its growth rate (Birge 1988). Because of the uniform conditions used for the preparation of test cultures, approximately equal numbers of cells in the different reproductive states should have been present in each group, and changes in the red fluorescence intensity distributions could be attributed to the action of chlorine.

The mean red fluorescence intensity of the *Renibacterium salmoninarum* chlorine-treatment groups increased with the duration of exposure to chlorine. The results of this study suggested that the concentration of free chlorine or the number of bacteria may have also affected the magnitude of those increases. Only in the reduced-Rs assessment did the proportions of dead cells based upon the HRFI and CS analyses correlate with the duration of chlorine exposure. The PI fluorescence intensity profile of human fibroblast DNA has also been reported to change following a nonlethal oxidative stress by free radicals (Le Goff et al. 1992).

Clearly, when based upon bacteriological culture, the rate of *Renibacterium salmoninarum* inactivation by chlorine was much faster than suggested by either the HRFI or CS analyses. The cellular functions necessary for *R. salmoninarum* to replicate and form colonies on agar plates may be much more sensitive to chlorination than are the biophysical characteristics associated with the entry and binding of PI. The critical issue here is ultimately whether membrane damage as indicated by PI-emitted red fluorescence or culturability by the standard plate assay is more closely related to the potential infectivity of *R. salmoninarum* to salmonids. With only data from a single study available (Pascho et al. 1995), relying on membrane damage would produce a more conservative result and less likelihood of underestimation of disinfection requirements to protect fish from infection.

In an earlier study, Pascho et al. (1995) provided evidence that some *Renibacterium salmoninarum* within bacterial aggregates may be protected from the bactericidal effects of chlorine. It is possible that the discrepancies between the inactivation estimates based upon red fluorescence and bacteriological culture were also caused by the presence of *R. salmoninarum* injured by sublethal chlorine levels within the aggregates. The definition of a dead microbe varies among reports, and this may be a bacterium that has lost all metabolic activity and still possesses an intact cell wall (Mason et al. 1986), or one that has simply lost the ability to multiply under suitable growth conditions (Davis & Dulbecco 1973, Postgate 1976, Kurath & Morita 1983). Some Gram-positive and Gram-negative bacteria that are stressed or injured by chemical treatment can recover and resume growth under suitable conditions (Hurst 1977, Camper & McFeters 1979, Flowers & Ordal 1979, Ray 1979, Kaprelyants & Kell 1992). The Gram-negative fish pathogenic bacterium *Aeromonas salmonicida* has been reported to enter a nonculturable state under low-nutrient conditions (Morgan et al. 1991). Whereas the inactivation of *R. salmoninarum* in this study was presumed to be a result of cell death, the chlorine may have sublethally damaged a proportion of the cells. These bacteria may have been unable to form colonies on agar plates, yet retained enough metabolic activity to remain impermeable to an exclusion dye. Injured bacteria may have also died during the recovery phase. Because such problems create uncertainty in distinguishing dead from live unicellular organisms, Postgate (1976) cautioned that rapid assessment of cell death with vital stains is only appropriate when the microorganism has been subjected to a 'catastrophic' stress. In both assessments of this study, the best agreement between the red fluorescence intensity estimations and bacteriological culture occurred in groups of *R. salmoninarum* that had been exposed to chlorine for relatively long durations (several minutes).

Bacteriological culture is the only quantitative method currently available for routinely determining the proportions of viable *Renibacterium salmoninarum* in such groups, but the slow growth of the bacterium limits the usefulness of culture as a research tool. In contrast, selected attributes of microorganisms can be described by FC with such speed that thousands of cells in a test group can be evaluated in a single analysis. The speed and flexibility of FC potentially make it an ideal alternative to bacteriological culture for any *R. salmoninarum* investigation requiring an immediate viability assessment of a test population. In addition, multicolor analysis of the *R. salmoninarum* preparations with FITC-IgG and PI in this study further simplified the assessment because both dyes can be excited with an argon laser.

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