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COMPUTATIONAL FLUID DYNAMICS AND A QUANTITATIVE POLYMERASE CHAIN REACTION AS TOOLS FOR MEASURING BIOPROCESS CONTAINMENT

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Models derived from a computational fluid dynamics (CFD) package (CF4X) were used to predict the fate of micro-organisms lost in aerosols as an incidental feature of the normal operation of bioprocess equipment. The model predicts the tracks of particles from their assumed source to their point of capture in an Aerojet cyclone impinger. In a set of controlled experiments micro-organisms were sprayed into a small cabinet (volume = 0.36 m^3). The efficiency which CFD predicted for their capture in the cyclone was compared with the actual efficiency measured with a quantitative polymerase chain reaction (QPCR) which was specific for the released organism. The capture efficiency was constant at about 40% over six orders of microbial concentration in the aerosol. This is consistent with the CFD predictions provided the coefficient of restitution of 50% of the particles is about 0.2. This coefficient determines the momentum with which the particles will rebound from the surface and continue their flight. Experiments in a bioprocess pilot plant, where QPCR was used to measure the quantity of process organisms and CFD was used to predict the capture efficiency of the released aerosols, confirm that the release from well-maintained equipment is very low. Only when the primary containment is broken as part of the normal operation of the process is there a significant release. Even this is small, amounting to about 10 μl of fermentation broth, or 0.2 μl of the concentrated process fluid. These quantitative methods for measuring levels of containment should aid the design of equipment and processes in which a proper balance is struck between the releases which are incidental to the normal operation of a process and the much larger losses which would follow an accidental failure in the equipment. In the long term this balance defines the safety of the containment.

Keywords: containment; recombinant micro-organism; aerosol; monitoring; downstream process; safety.

INTRODUCTION

A key problem in the interpretation of legislation governing contained use of recombinant micro-organisms, such as the European Directive 90/219/EEC[1], or the NIH *Guidelines for Research Involving Recombinant DNA Molecules*[2], is the lack of quantitative methods to measure release from bioprocesses. Both the legislation and the guidance suggest that operations should, depending on the level of containment deemed appropriate, 'minimise' or 'prevent' release. These terms are qualitative and they limit the objective interpretation of the legislator's aims. Moreover, as the report from the Asilomar Conference[3] pointed out, it is impossible to 'prevent' the release of micro-organisms from the processes which use them because, in the long run, there is always a finite probability of a credible accident[4]. Ideally, the guidance should contain objective criteria which allow the designers and the operators of manufacturing processes to test their performance. In their absence biochemical engineers have argued over the precise interpretations of the guidance[5] with a consequential effect on cost if not safety as equipment has become more complex in an attempt to reduce the losses during normal operations to undetectable levels.

Previous attempts at detecting release have largely been concerned with the escape of process micro-organisms by the aerosol route since, in such a state, the released organisms are not easily contained[6] and may pose a threat to human health and to the environment. Additionally, many bioprocess operations have the potential to release aerosols containing micro-organisms[6,7]. However, the methods used have not been truly quantitative. The frequent use of cell culture to measure the cells recovered from aerosols underestimates the total viable number present in the aerosol either due to the effect of sampling stress[8,9] or to the presence of viable but non culturable cells[10,11]. Moreover the quantitative relationship between the captured sample and the release is not known.

Detection of airborne micro-organisms by PCR has been shown by some workers[8,12,13] and this technique has the

advantage that sampling stress does not affect detectability[8]. Recently, we have shown how a competitive PCR method[14,15] can be used to measure target *E. coli* K-12 cells released into fermenter exit gas[16]. In that study, the released cells were directed into an air sampling device, an Aerojet General Cyclone impinger[17,18] which samples at a high flow rate, ensuring high recovery of cells from the exit-gas. The method allowed the specific measurement of target cells over six orders of magnitude with a precision of ± 0.11 logs and a limit of detection of 5×10^3 cells per m^3 of sampled air. However, when the techniques are applied to the measurement of release in bioprocess plants, a significant proportion of the cells released will not reach the cyclone impinger and so the quantitative nature of the method is compromised. To measure bioprocess release in such environments, it is necessary to determine what proportion of the released cells actually becomes entrained within the cyclone and is subsequently counted by the quantitative PCR assay (QPCR).

In this paper, we show how this proportion can be established for releases into a small, confined space, and how by the use of air flow models generated with computational fluid dynamics (CFD) we are attempting to determine the proportion of released process cells entering the cyclone when sampling is carried out in the more spacious environment typical of a bioprocess facility. Indeed the data acquired from the small confined spaces are used to validate the CFD models that are applied within the bioprocess environment Furthermore, we use the cyclone-QPCR technique in conjunction with CFD modelling to monitor the containment provided by a series of bioprocess operations in a case study at an industrial pilot plant. This has confirmed the utility of the approach for the assessment of process releases.

MATERIALS AND METHODS

Preparation of *E. coli* Cell Suspensions

E. coli strain JM107 pQR701[19,20] was the target (process) strain used in these studies. The strain was grown at 37°C in 25 g l^{-1} nutrient broth (Oxoid, Unipath Ltd., Basingstoke, UK) with the addition, after autoclaving, of 20 $\mu g\,ml^{-1}$ kanamycin (Sigma, Poole, Dorset, UK). Cultures were grown overnight in 50 ml volumes in a 250 ml shake flask in an orbital shaker at 200 rpm. To prepare cell suspensions for spraying into the Bassaire cabinet, overnight cultures were serially diluted into sterile nutrient broth and were stored at 4°C until use (within 2 hours).

Fermentation

For larger scale experiments the culture was grown at 37°C on broth containing (in g l^{-1}, unless otherwise stated): KH_2PO_4 (2.3); K_2HPO_4 (3.8); Bactotryptone (8); yeast extract (16); glycerol (3 ml l^{-1}); polypropylene glycol (antifoam) (0.2 ml l^{-1}); and kanamycin (20 mg l^{-1}). The pH was controlled at 7.0 by addition of 0.5 M NaOH and DOT was maintained at above 20% by variation of the stirrer speed between 300–1000 rpm, while the fermentation was aerated at 1 (vessel) vol per (vessel) vol per min. Antifoam was added to the broth to control the extent of foaming. The seed culture (6%) was inoculated into two 10 l fermenters and, after 8–12 hours growth, the contents were transferred to 50 l for 8–12 hours and then to a 500 l scale. The contents of the 500 l fermenter were harvested after 12 hours.

Production of Aerosols Using an Atomizer

Aerosols were generated by use of a glass atomizer (Warren Spring Laboratories, now at AEA Technology, Harwell, Didcot, Oxon, UK) consisting of two concentric tubes[21]. The suspension of *E. coli* was pumped up through the inner tube at 1 ml min^{-1}, whilst compressed air at 68 KPa was passed through the outer tube. Liquid reaching the end of the inner tube is subject to rapid air flow causing aerosolization. For each experiment, a volume of 18 ml was aerosolized and then the spray line was flushed by passing 5 ml of sterile thiosulphate ringers solution (TRS) (Oxoid Unipath Ltd., Basingstoke UK) through at the same rate.

Operation of the Cyclone

The air sampling device used was an Aerojet General Cyclone impinger[16,17,18]. During operation airborne micro-organisms are drawn into the cyclone by rapid air flow and are deposited on the walls of the device and subsequently washed off by recirculating liquid. At the end of operation, all circulating liquid is collected, the volume is measured and the liquid sample is used in the PCR. In these experiments, air was drawn into the cyclone by an air pump (Air Control Installations, Chard, Somerset, UK) at 360 l min^{-1}, and the collecting liquid, 80 ml TRS, was recirculated at 20 ml min^{-1} using a peristaltic pump. In order to achieve consistent results, the cyclone has been modified by the incorporation of an attachment cone which introduces the liquid stream into the device (Figure 1). Sampling was carried out in batch-mode for 10–30 minute periods. The cyclone was cleaned by immersion into 1% Tego solution (Th. Goldschmidt Ltd., Milton Keynes, UK) as previously described[16].

Sampling Aerosols from Within the Bassaire Cabinet

The Bassaire cabinet (Bassaire Ltd, Swanwick, Southampton, UK) is a sealed laminar flow cabinet of 0.36 m^3 volume with inlet and outlet fan-assisted HEPA filters[21]. There are ports on the side of the cabinet which allow the connection of a cyclone and an atomizer through drilled bung adaptors (Figure 2). Before spraying, the Bassaire cabinet was washed inside with 1% Tego solution

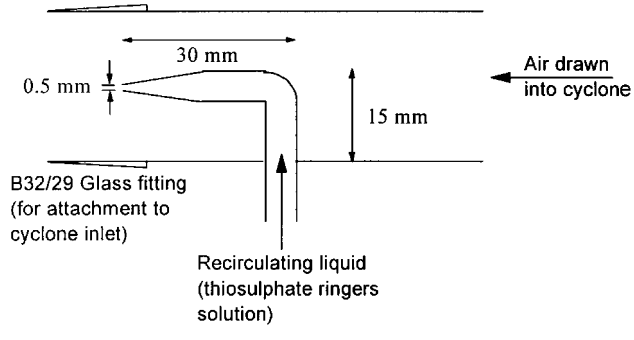

Figure 1. Cross-sectional schematic view of the cyclone attachment cone.

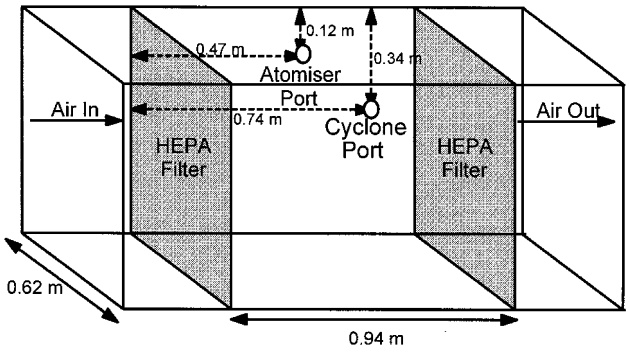

Figure 2. Isometric view of the Bassaire cabinet showing the positions of the atomizer and cyclone ports. The walls of the cabinet are constructed of clear perspex. The rear panel with the two ports can be removed for cleaning.

and then the HEPA filter fans were turned on for 30 minutes to flush out the cabinet. Spraying took place with the fans turned off so that the only active air movement inside the cabinet was caused by the spraying (at 68 Kpa, mass flow = 23 l min^{-1} input), and the cyclone air sampling (at 360 l min^{-1} extracted). The balance of the air was indrawn through the HEPA filters. Throughout the spraying the cyclone was operating and was left to run for a further 7 minutes to bring the total sampling time to 30 minutes. When the experiment was finished, 30 ml of 0.5% Tego was sprayed into the cabinet and the atomizer, cyclone and all tubing were washed out by immersion in 1% Tego. The inside of the cabinet was wiped clean and then the HEPA fans were started in preparation for the next experiment. In order to account for any carryover from previous experiments, 18 ml of sterile TRS was sprayed and collected by the cyclone to give a background reading before any cells were sprayed. Additionally, the lower cell concentrations were always used before the higher ones.

Sampling Around Pilot Scale Downstream Operations

The unit operations which were carried out at pilot scale with *E. coli* JM107 pQR701 cell paste harvested from the 500 l fermentation, and the sampling regimes which monitored the possible release of the cells during those operations, are summarized below. In all the experiments, the cyclone was operated for 15 minute periods.

Tubular bowl centrifugation

The 500 l fermenter was harvested using a Sharples AS 16 VB tubular bowl centrifuge (Alfa-Laval, Camberley, Surrey, UK) operating at 15,000 × g. The entire volume of the fermenter was harvested in two batches yielding a total of approximately 5 kg of cell paste. After centrifugation the bowl was removed and transferred to a safety cabinet for the 'dig-out'. This is the process step during which the cell paste is removed from the centrifuge bowl.

The bowl was removed from the centrifuge and its ends were capped. It was then moved by hand across to the other side of the room and placed in the safety cabinet (Figure 3). The acetate sheet, which lines the inside of the bowl, was then drawn out, and its cover of cell paste was scraped off and packaged before being transferred to a −20°C store. This dig-out is essentially an uncontained operation in which the primary containment is broken. It relies for its safety on good practice and effective secondary containment.

For this reason the Sharples centrifuge is situated within a room (Figure 3) where the air pressure is negative with respect to the surrounding area, thus providing secondary containment. Moreover the safety cabinet provides an additional level of safety while the contents of the bowl are removed.

Air sampling was carried out within the Sharples room before any operation, during centrifugation and during the solids dig-out procedure. The position of the cyclone inlet in relation to the centrifuge and the safety cabinet is shown in Figure 3.

Bead mill disruption

In these experiments 2 kg of *E. coli* JM107 pQR701 cell paste was resuspended at 10% (w/v) in 20 l of 0.1 M sodium/potassium phosphate buffer, pH 7.0. This suspension was fed into a Dyno-Mill KDL bead mill (Glen Creston Ltd, Stanmore, Middlesex, UK) at a rate of 2 l h^{-1}, using a continuous flow chamber (600 ml capacity). The agitation speed was 4200 rpm, 0.2–0.5 mm diameter glass beads were used, and the separator gap was set at 0.05 mm. The chamber and bearings were cooled by recirculating glycol at −5°C.

The bead mill is contained within a flexible film isolator with double HEPA inlet and exhaust filters (MDH Ltd, Andover, Hampshire, UK). It is normally operated at a negative pressure of −75 Pa relative to the surrounding environment (Figure 4), providing secondary containment. The cyclone was attached to the side of the isolator. While the air inside was sampled the isolator's own air extraction was turned off, so that the only air flow (360 l min^{-1}) was created by the air drawn through the cyclone.

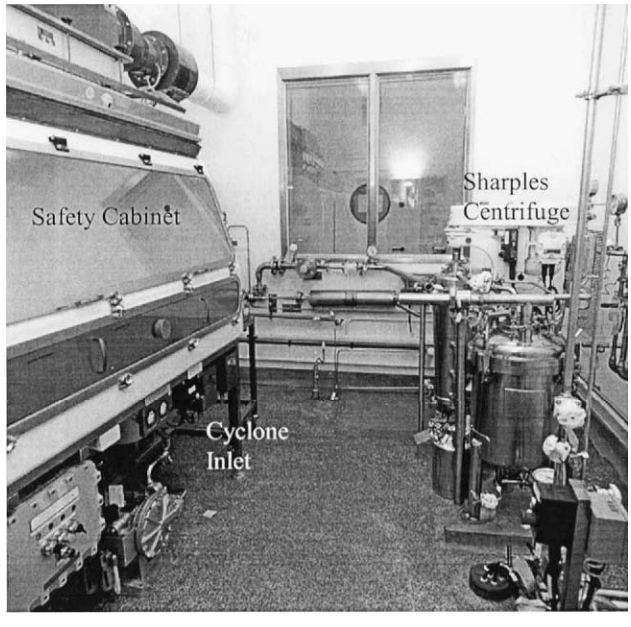

Figure 3. The layout of the Sharples centrifuge room. The safety cabinet is situated on the left hand side and the centrifuge on the right at the rear of the room. The cyclone intake was positioned adjacent to the safety cabinet at the position marked. The arrow indicates the direction of air flow into the cyclone. Note that there is an air outlet duct on the right-hand side wall, adjacent to the centrifuge. The air inlet is on the ceiling at the front left-hand side of the room (above the safety cabinet). Room dimensions: 3.70 m × 3.00 m × 3.97 m (h × w × d). Cyclone inlet: 1.07 m from floor; 1.22 m from left-hand side wall; 2.35 m from back wall.

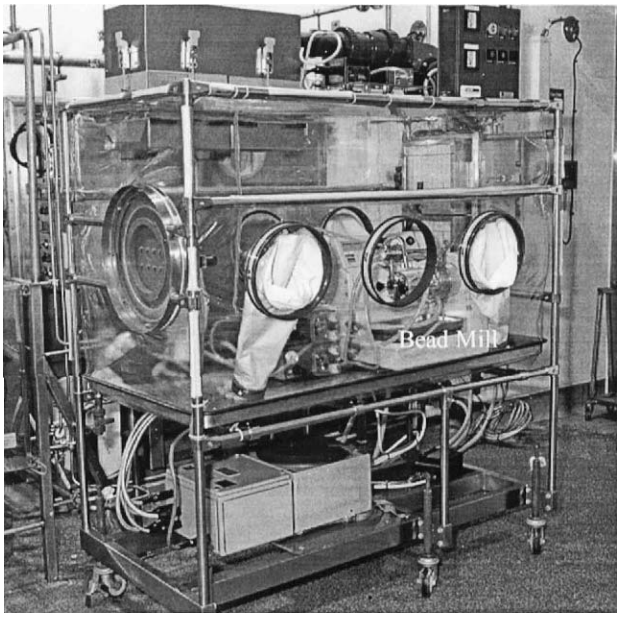

Figure 4. The flexible isolator housing the Dyno-Mill KDL bead mill homogenizer. The isolator (1.6 m × 0.9 m × 0.77 m (w × h × d)) normally operates at a negative pressure relative to the room of – 75 Pa (Materials and Methods) The Dyno-Mill is operated through the sleeve ports of which there are three on each of the near and far sides of the cabinet. For sampling within the isolator, the cyclone was attached with flexible hosing to the central sleeve port on the far side of the isolator.

Background samples

In view of the sensitivity of QPCR in detecting the *E. coli* strain, two controls were employed to estimate the background levels of the target plasmid. In the first the cyclone was occasionally run in process areas where there had been no known use of the plasmid. This is useful in estimating the background level which might remain in the cyclone after cleaning, or might enter by cross-contamination of the samples. In the second control the air outside of the bead-mill cabinet was sampled before and during operation of the bead mill itself. This estimates the general background level of the plasmid in the processing area.

Cell Counting

The number of *E. coli* cells in a sample was counted under a microscope using a Helber Bacteria Counting Chamber with Thoma rulings (Weber Scientific International Ltd, Teddington, Middlesex, UK) as previously described[16].

Quantitive Polymerase Chain Reaction (QPCR)

E. coli JM107 pQR701 has a plasmid encoded transketolase gene (tkt) which is derived from the chromosome of *E. coli*[19]. Primers were chosen such that the amplified section crosses the point of insertion of the tkt gene. The size of the amplified product using the M13R1 (GGACCAAGCTATGACCATG) and CMTA1 (CGTCAAAGAGTGTATTGAGG) primers was 332 base pairs (bp). The PCR was carried out in 25 µl reaction volume (10 µl sample volume) using Taq polymerase (Life Technologies, Uxbridge, UK) as previously described[16].

The internal standard DNA (IS(T)) for quantitative DNA was made according to the method of Forster[22] using primer TKL (GTGTATTGAGGGATCGATCAGGG-CGTCTAT) as the linker primer. The size of the IS(T) amplified product was 247 bp. Isolation and preparation of IS(T), and quantification of the PCR products are described elsewhere[16].

CFD Methods

The computer programme CF4X (CFDS-AEA Technology, Harwell, OX11 0RA, UK) was used to predict the air flows. The software was run on an IBM RS6000 with 128 MB RAM. The operating system was AIX. The air flows were assumed to be three-dimensional, isothermal, Newtonian, incompressible and at a steady state[23]. A hybrid differentiating scheme was used to model the convective terms of the transport equations (CF4X Users Manual, CFDS-AEA Technology). The standard k-ε turbulence model[24] and the SIMPLE C algorithm[25] were used to produce a computationally economic solution.

In modelling the trajectories of particles entrained in the airflows, their density, diameter, and coefficient of restitution were all varied independently.

RESULTS AND DISCUSSION

Measurement of *E. coli* JM107 pQR701 Whole Cells by QPCR

E. coli JM107 carrying the plasmid pQR701 is a convenient strain for the measurement of containment. It is a self-cloned organism in which the plasmid encodes a copy of the organism's own transketolase enzyme. Although recombinant this organism requires only the lowest level of containment formally equivalent to Good Large-Scale Practice. The inevitable small-scale leakage from containment implicit in the experimental plan does not therefore compromise normal working practice. It is also the strain used in some of our previously reported experiments[21].

The fact that the strain is recombinant allows for precise and unambiguous measurement of the number of organisms in the samples taken. The PCR method with a competitive internal standard provides a sensitive measurement of the target organism in the environment[26,27,28,29]. Although other *E. coli* K-12 strains were used in the same environment these do not interfere with the measurement[16].

As used here the method measures the number of copies of the target plasmid pQR701 in a sample. Multiple copies of this plasmid are present within each cell of the target strain, so it is necessary to correct for the number of plasmid copies per cell. This has been achieved by measuring (by QPCR) the number of plasmid copies in a sample derived from the process stream where the cell density is known (from microscopic cell counting). Using this method, the number of plasmids per cell has been determined to be 140 for pilot plant studies (being their concentration at the harvest of the 500 l fermentation) and typically between 100 and 500 for the Bassaire experiments (where cells were grown in shake flasks). The figure, which is determined for each set of experiments, is used to convert the measured plasmid numbers into cell concentrations[16].

Errors in these estimates arise both from the real variation in the number of plasmids per cell, and from variations in the assay method itself. We find[16] that the former varies by

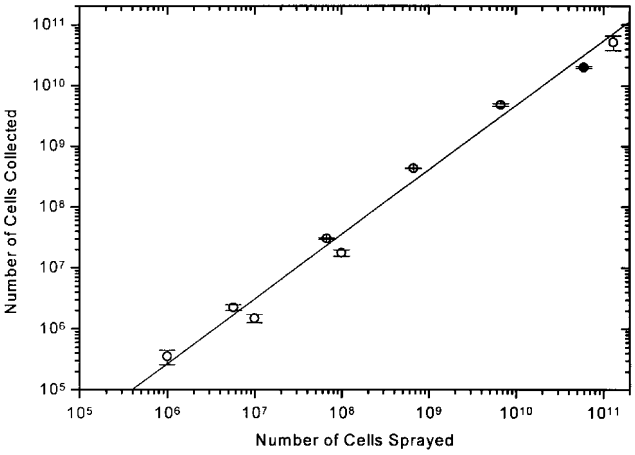

Figure 5. The relationship between cells aerosolized from nutrient broth into the Bassaire cabinet and cells collected and enumerated by QPCR. All data are average values of 2 determinations. Bars represent the standard errors of the means. All data gained using 30 minute boiling lysis step[16]. The single solid data point represents the data of Ferris *et al.*[21]. Gradient = 1.06, $r = 0.999$.

about ±25%, but that between assay variations on the same sample are smaller (±15%) provided that the experimental samples are always compared with the proper controls.

Microfiltration through a 0.2 mm cellulose triacetate filter was used as a routine check to ensure that all of the target plasmid was cell-associated. Any cell-free plasmid passes through the filter and is measured separately as a control[16]. In the experiments reported here it accounts for less than 2% of the total plasmid concentration.

Recovery of Cells Atomized in the Bassaire Cabinet

There is a good correlation between the number of cells sprayed into the 0.36 m^3 Bassaire cabinet and the number estimated by the QPCR method after their collection in the cyclone. The average recovery of sprayed cells in these experiments is 41 ± 7% (Figure 5). This is consistent with our previous data[21] which were derived from similar experiments on the same equipment but where the cells were counted under a microscope. In the experiments reported here the recovery is independent of the cell concentration in the aerosol (Figure 5). We believe this data, which is based on QPCR techniques and extends over 5 orders of magnitude, to be more reliable than the earlier

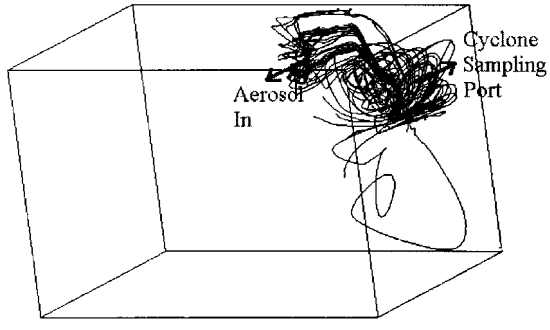

Figure 6. Predicted particle trajectories in the Bassaire cabinet when the aerosols containing the organisms have a coefficient of restitution of zero and a diameter of 5 μm.

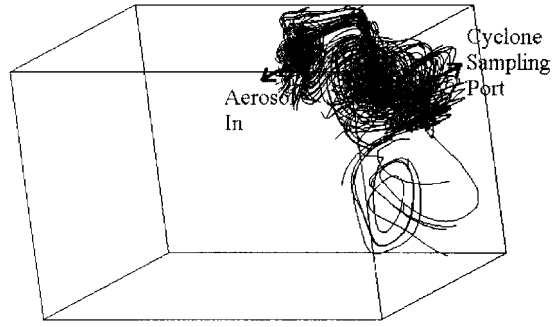

Figure 7. Predicted particle trajectories in the Bassaire cabinet when the aerosols containing the organisms have a coefficient of restitution of 0.2 and a diameter of 5 μm.

data[21] where the efficiency of collection appeared to vary with the concentration.

At the lower end of the concentration range the cell suspensions used for producing aerosols in the cabinet were very dilute and in no way representative of a process stream. However any release from a process is likely to be highly diluted by the surrounding air, and the cyclone may be at some distance in space and time from the point of release. The low aerosol concentrations do therefore represent a realistic air sample which might be encountered in practice.

CFD Analysis of Aerosolized Cells in the Bassaire Cabinet

Upton *et al.*[18] have shown that, for particles with aerodynamic diameters greater than 2 μm, a cyclone of the design used in these experiments has an entry efficiency that is close to 100%. Since the majority of the *E. coli* cells in the aerosols we have studied are themselves about 2 μm in diameter, and the particles which contain them must be somewhat larger, we believe that the measured recovery of 41% is principally governed by the proportion of cells reaching the cyclone inlet. The greater proportion of the cells released into the cabinet must therefore either fall to the floor or stick to the walls or ceiling of the cabinet. In our previously reported experiments we recovered about 11% of the input from the floor[21].

The airflow in the Bassaire cabinet was modelled with CFD and the trajectories of particles released from the aerosol were recorded. In each case 100 particles were followed (Figures 6 and 7) and the number falling into the inlet of the cyclone was used to calculate the theoretical efficiency of their capture. Of the features of the particles which were allowed to vary, only their diameter (range 1–10 μm) and their coefficient of restitution significantly affected their trajectories. This coefficient defines the behaviour of a particle when it hits a surface. It is the ratio of its rebound velocity normal to the surface compared to the same component of its incoming velocity. If the value is zero the particles will stick to the surface but as the value increases to the maximum value of 1 then the particles will tend to rebound with increasing momentum. In practice increasing the coefficient above a value of 0.1 has only a small effect on the number of particles captured in the cyclone, at least not those with a diameter large enough (2 μm or greater) to contain micro-organisms (Figure 8). The small coefficient is sufficient to lift the particle out of the static boundary layer close to the surface so that it is carried back into the moving air.

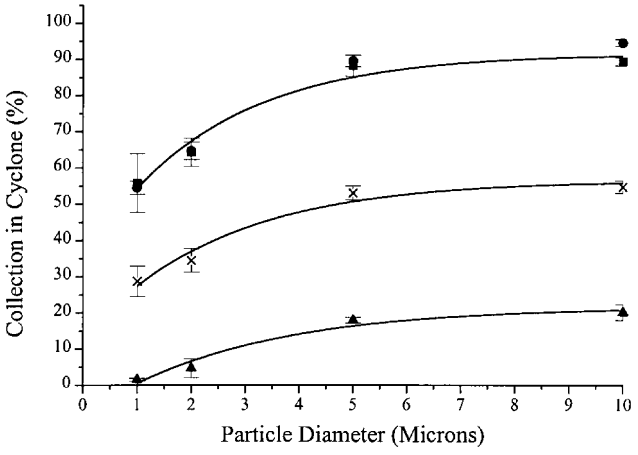

Figure 8. CFD predictions of percentage collection within the Bassaire cabinet as a function of aerosol diameter and coefficient of restitution. Coefficient of restitution = 0 (▲); = 0.1 (■); = 0.2 (●). Mixed population with 50% having a coefficient of 0 and 50% of 0.2 (×) **(see text).**

The CFD analysis predicts a very confined envelope for the particles leaving the atomizer, particularly when the coefficient of restitution is zero. The air flow into the cyclone pulls the aerosol released from the atomizer through 180° preventing its dispersal throughout the cabinet. The proportion falling into the cyclone increases somewhat as the particle size increases, because the larger particles are less likely to be dispersed in turbulent eddies. However the coefficient of restitution has a much greater influence. The capture efficiency for a 10 μm particle is only about 20% when the coefficient of restitution is zero, but it rises to over 90% with even a coefficient as low as 0.1 (Figure 8).

Extension of the CFD Model to More Complex Systems

If, in this simple system, 50% of the particles are assumed to have a coefficient of restitution of zero, and the remainder to have a coefficient of 0.1, then the predicted values for the efficiency of collection in the cyclone (Figure 8) are close to the observed data (Figure 5). This is the model which we have used in this study to determine the incidental release of cells into larger, more complex environments, where the efficiency of their collection would be expected to be much lower. In these cases, where some cells are detected, the CFD model is useful in estimating the actual quantity of cells released during the normal operations of a process. However in open areas such as bioprocess plants—for reasons of hygiene, if for none other—it is not practical to validate the recovery predicted by CFD by spraying a known quantity of process cells into the environment. The extrapolation from a closed model such as the Bassaire cabinet should therefore be used cautiously until we have devised a suitable hygienic and safe method of validating the recovery.

Background Controls (Limit of Detection)

The aim of this study was not only to provide data on the containment of a series of unit operations but also to gain an insight into the issues that arise when this air sampling-QPCR methodology is applied 'in the field'.

During this study, a series of cyclone samples were taken in a room in which the target process organism had not been handled. These samples were used to estimate the level of 'background' that might occur in the absence of the target strain (see Materials and Methods). Although in five cases the background was zero, in three others the plasmid was detected. The average background level found in the cyclone after 15 min sampling of the air was $3.4 \pm 5.5 \times 10^4$ cells (8 samples). The range was skewed from zero ($<10^4$) to 1.5×10^5.

The load of 1.5×10^5 cells in the cyclone is equivalent to about 30 cells per PCR. It is likely to arise from cross-contamination or from ineffective cleaning of the cyclone and other equipment. In subsequent air sampling experiments any sample containing less than twice this number of cells (i.e. 3×10^5 cells) was assumed to be a background signal and this was set as the Limit of Detection (LOD). All subsequent results were regarded as negative if the measured cell concentration was below this figure. The calculated LOD might not have statistical validity, since there are too few background data to be able to determine whether they are normally distributed. Nevertheless the LOD is almost ten-times the average background level and this should be a conservative figure in preventing false positives.

Bead Mill

The bead mill is contained within a flexible film isolator (Figure 4). As a background control the air outside of this isolator was sampled for 15 min periods both before its use to break open the *E. coli* cells and during its continuous operation. No cells were detected by PCR ($<10^4$ cells captured).

While the bead mill was operating its own air supply was turned off, and the air inside the isolator was then sampled. A low PCR signal, equivalent to 1.5×10^5 cells captured, was detected, but at the present state of the development of our methods this should be considered to be below the LOD (3×10^5 cells, see above).

When the airflow inside the isolator is modelled, the circulation of particles (Figure 9) is more complex than it is in the Bassaire cabinet (Figures 6 and 7). This pattern would not be observed if the isolator's own air supply was turned on, but it does represent the flow of particles during the sampling experiment which was arranged to give the best possible chance of detecting a leak from the bead mill. It was assumed that such a leak would occur at the flange where the grinding chamber on the bead mill is pressed up against its face plate.

Given a particle diameter of 5 μm and a coefficient of restitution of 0.2, the cyclone should sample about 10% of any particles released. None are captured if the coefficient is zero. By analogy with the Bassaire cabinet we presume the capture efficiency to be about 5%. If this is correct then the release from the bead mill during the 15 min of sampling could not have been greater than 6×10^6 cells. Assuming that 1 ml of broth at the point of harvest contains 1.7×10^{10} cell ml^{-1} (see Reference 16) this number would be contained in less than 0.4 μl of a typical fermentation broth, or in less than 50 nl of the unbroken cell suspension entering the bead mill.

Centrifuge Room

In the room with the Sharples centrifuge, no release of process cells was detected during centrifugation but a significant release was found during the recovery of the cells.

Release during centrifuge operation

The CFD model predicts that if cells were to be released from the centrifuge during its operation then the cyclone, in the location in which it was sited, would not have captured any aerosolized cells (Figure 10). The design of the room is efficient in so far as any cells which are released while the centrifuge is running will move directly to the air outlet vent and away from the cyclone inlet. This suggests that in order to detect any breach of the primary containment, it would be essential to site the cyclone between the centrifuge and the vent. The presently available equipment, which comprises the cyclone with its air and liquid pumps, precludes this, but it is worth noting that a CFD model of the particle flow in a room would be a useful guide to the proper siting of the cyclone inlet.

Release during recovery of the cell paste

Air sampling in the room containing the Sharples tubular bowl centrifuge operations detected a release in only one instance. This was during the recovery of the cell paste from the centrifuge bowl. The level of release was recorded at 7.0×10^6 cells, over 20 times higher than the LOD value.

There are two potential sources for these cells. One is from the bowl during its transfer from the centrifuge to the safety cabinet; the other is from the safety cabinet during the removal of the cells from the bowl (see Materials and Methods—Tubular bowl centrifugation). We cannot, in retrospect, time the release precisely, but we can model the tracks of particles lost during the bowl's transfer to the safety cabinet (Figure 11). Were they released at this time from the top end of the bowl they would be widely dispersed in the room, provided their coefficient of restitution is significant. Some 10% would be expected to enter the cyclone were they released from the top of the bowl (Table 1). The CFD analysis predicts that about one-third should enter the air vent, while 20 or 30%, depending on the particle size, would be caught up on the centrifuge assembly as they move towards the vent.

Interestingly CFD predicts that if the coefficient of restitution were zero most of the particles would be carried back to the bowl on eddies where they would remain (Table 1). The particle size has a less important effect.

The data from the Bassaire cabinet suggests that we should have detected about 5% of the particles lost, making the total loss the equivalent of about 10 μl of the broth at harvest, or about 0.2 μl the concentrate likely to be present in the bowl itself. This estimate is critically dependent on the site of the release. For example, only some 1.5% of any cells lost from the bottom of the bowl would have been captured in the cyclone, which would raise the loss of concentrate to about 0.7 μl.

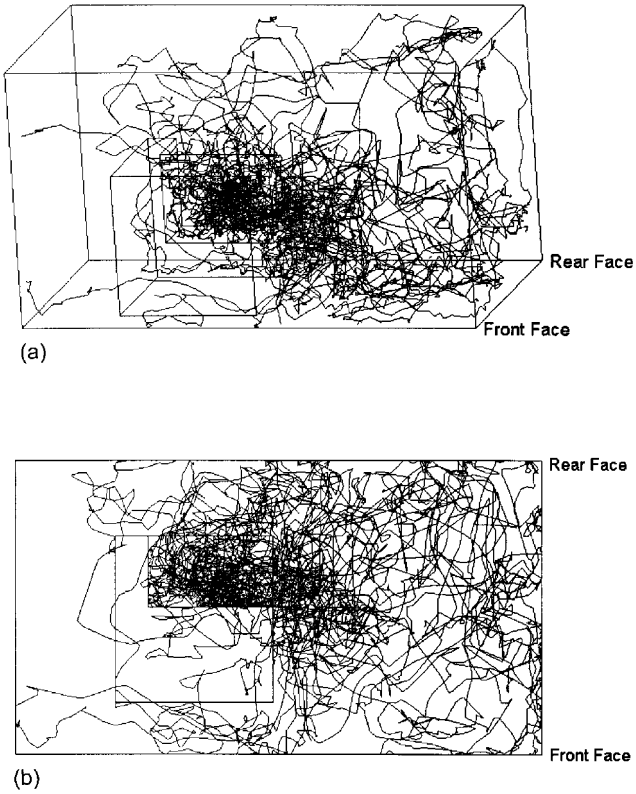

Figure 9. Predicted trajectories of a hypothetical release of aerosol particles having a diameter of 4–5 μm and a velocity of 0.1 m s^{-1}. The particles are assumed to originate from the flange between the grinding chamber and the face plate on the bead mill. The particles had a coefficient of restitution of 0.2. The tracks are shown (a) in an isometric view from the front above right, and (b) in plan view from above. The particles are unable to disperse freely in some directions (see particularly Figure 9(b)) because the grinding chamber of the Dyno Mill KDL sits within a recess. The 'front face' of the cabinet which carries the cyclone sampling port at a central location is on the far side as shown in Figure 4. CFD predicts that the cyclone should sample approximately 10% of the released particles.

CONCLUSIONS

The degree of containment which engineering design provides can only be determined from measurements of the

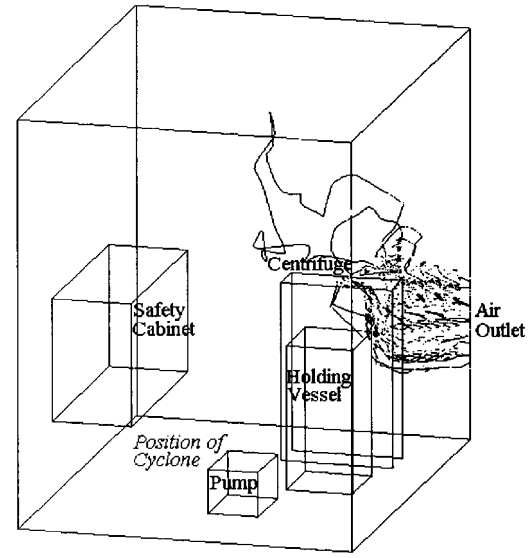

Figure 10. Predicted trajectory of a release of *E. coli* originating from the top of the Sharples tubular bowl centrifuge, when the coefficient of restitution is 0.2. Most of the organisms are drawn towards the air outlet.

Table 1. CFD model of the fate of particles lost from the centrifuge bowl during its transfer from the centrifuge assembly to the safety cabinet. The particles are assumed to leave the bowl in random directions with a velocity of 0.1 m s^{-1}.

Properties of particles			
Diameter, μm	1–10	1–10	5
Coefficient of restitution	0	0.2	0.2
Final location of particles	Predicted percentage at location		
Enter exhaust vent	< 1	36	30
Adhere to walls	< 1	4	5
Adhere to centrifuge	< 1	30	20
Adhere to centrifuge bowl	96	9	5
Enter cyclone	< 1	10	8

actual release itself. As the sampled environment becomes larger and more complex, the linkage between that release and its detected effect, or its concentration at the point of capture, becomes more tenuous. We have gone some way towards creating a firmer link through the use of air flow models which can predict the efficiency with which the released particles are captured.

The accuracy of the models is not only dependent on the volume and complexity of the environment being monitored, but also on the behaviour of the particles themselves. Of particular importance is their coefficient of restitution—roughly speaking, their chance of rebounding from a surface with which they collide. Unless this value is significantly above zero their dispersal is limited. By comparison their size distribution is less important.

Presently we have no direct method of measuring the coefficient, but the relationship between the predicted and observed capture efficiencies in small contained cabinets does allow an indirect estimate to be made. Even if it is considered arbitrary our suggestion that 50% of the particles have a coefficient of zero, and 50% a coefficient of 0.2, fits the data. A distribution of particles with coefficients between the two values might be more reasonable but it is more difficult to model in the CFD analysis. It is a feature which certainly requires further refinement and independent measurement. Given that the efficiency of the cyclone was already known, this effect of the coefficient of restitution is a useful feature to emerge from the small-scale experiments.

The QPCR-air sampling method also requires refinement. We are presently unable to distinguish viable from killed process cells and only the viable cells are a problem for process containment. The occasional cross-contamination of samples is another problem which must be resolved if our methods are to attain their full sensitivity. However even that cross-contamination does indicate how easy it is to detect small numbers of process organisms around a large-scale process which uses them[16].

In this study we have tended to use the CFD analysis retrospectively, but we can now see its value in helping to place the cyclone in the track of the emitted particles. Moreover, CFD identifies the positions at which swab tests should detect surface contamination following some particular release (see Figure 11(b)). Unfortunately in the present study these details emerged too late for a practical analysis to be carried out. It is clear that we must design a more compact cyclone unit which we can place in the path of the particles and find a suitable and safe method of validating the CFD models in an open environment. However these are essentially practical problems which we should be able to overcome given sufficient support.

This study certainly confirms that the scale of the incidental release is small. This is true even where the primary containment is broken to unload a centrifuge. When compared to the potential level of release which might occur in a significant accident, we believe that the propensity for accidents should be a major consideration in containment design. There is now a real need to place the estimates of accidental release on a much firmer basis so that the long-term impact of breaches in containment from this source can be compared with the low levels of incidental release. It raises an important microbiological question as to whether the occasional large release in an accident is more or less hazardous than the very low level of continouous release which is incidental to the normal operation of a well-maintained process even at quite modest levels of process containment.

Bioprocess equipment designed to prevent the release of

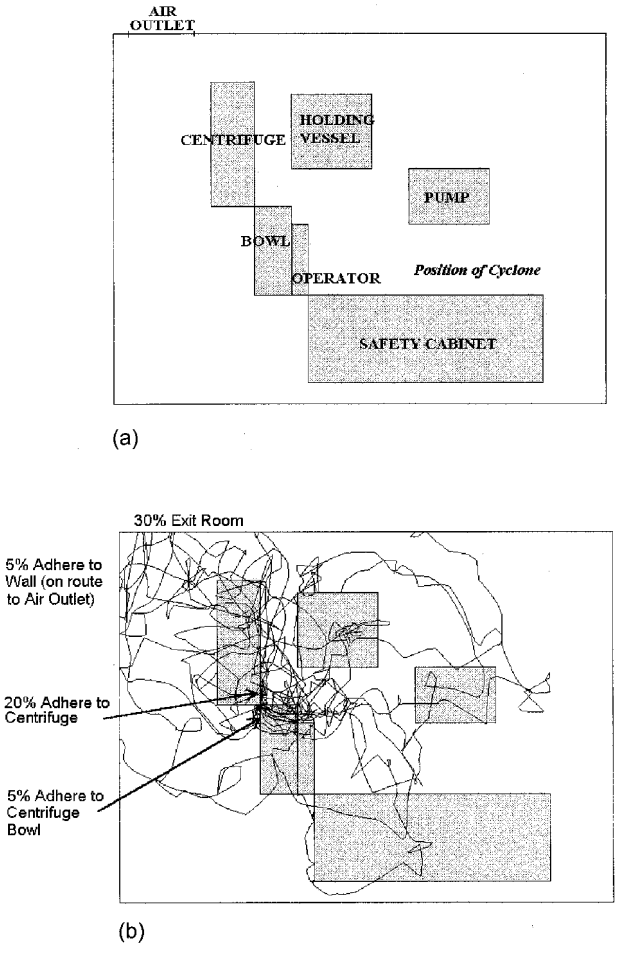

Figure 11. Predicted trajectories of a release of an aerosol from a centrifuge bowl containing an *E. coli* cell paste. The release is presumed to originate from the centrifuge bowl ('top' end nearest centrifuge), during transport of the bowl across the centrifuge room (see Figure 3). The room is shown in plan view from above, and the main features are shown in (a). The fate of the aerosol is shown in (b). The particles were assumed to have a coefficient of restitution of 0.2, and a diameter between 4 and 5 μm. Approximately 10% were expected to be sampled in the cyclone.

micro-organisms is very complex and requires highly skilled operators. In this regard, it is worth noting that in the chemical process industry, where operating conditions are more hazardous than those in bioprocessing, the concept of simple and inherently safe design has taken hold[30]. We believe that an approach based on the two techniques of QPCR and CFD will aid the biochemical engineer in the appropriate design of contained processes. Together they should yield a quantitative framework within which the actual degree of containment and the dispersal of a release can be predicted and validated. With experience this must allow a closer matching of the design to the hazards than is now possible in the containment of bioprocesses.

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