Biotechnology Premanufacture Notices-
Generic Scenario for Estimating Occupational Exposures and Environmental Releases

-Draft-

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ABSTRACT

The purpose of this report is to develop a standardized approach that EPA's Chemical Engineering Branch (CEB) can use to estimate potential occupational exposures and environmental releases during closed-loop fermentation processes that involve the use of genetically engineered microorganisms (GEMs). These estimation techniques may be used by CEB to evaluate future biotechnology premanufacture notices (PMNs). The document also discusses in detail a typical fermentation process, and the control technologies, inactivation procedures and sterilization procedures, that can be expected.

Information and data used to develop the estimation procedures were obtained from a literature search of fermentation processes using recombinant microorganisms, including a review of the 16 biotechnology PMNs that were submitted to EPA between 1987 and 1995.

Based on information and data acquired from the literature search, reasonable worst-case release and exposure estimations can be made using the methodology and calculations that are discussed in detail in Sections 3.0 and 4.0. These calculations are summarized in the following table.

Occupational Exposure Calculations		
Dermal Exposure (CFU/day): E_D	=	$([CFU]_P)(C)$ C = 650 - 1,950 mg/day (incidental, one hand) for lab propagation and sampling = 1,300 - 3,900 mg/day (routine, two hands) for equipment cleaning and product recovery
Inhalation Exposure (CFU/day):		$E_I = (I)(h)([CFU]_{WA})$

Release Calculations		
Medium	**Calculation**	
Air	Total Air Releases (CFU/yr)	= $AR_{FO} + AR_P + AR_S$
	Release from Fermentor Off-Gas (CFU/yr)	= $AR_{FO} = ([CFU]_E)(F)(t)$ <OR> $AR_{FO} = ([CFU]_O)(F)(t)(1-ç_R)$ <OR> $AR_{FO} = ([CFU]_B)(AF)(1-ç_R)(V_B)$
	Fugitive Releases from Large Process Unit Operations Equipment (CFU/yr)	= $AR_P = ([CFU]_B)(V_B)(1-ç_I)(AF)$
	Fugitive Releases from Sampling, Other Process Operations, and Process Tanks	= AR_S = negligible
Water	Total Water Releases (CFU/yr)	= $WR_B + WR_{CW} + WR_{OG} + WR_S$
	Releases from Inactivated Fermentor Broth (CFU/yr)	= $WR_B = ([CFU]_B)(V_B)(1-ç_I)$
	Releases from Cleaning Wastewater (CFU/yr)	= $WR_{CW} = ([CFU]_B)(V_F)(B)(0.01)(1-ç_I)$
	Releases from Fermentor Off-Gas Treatment Wastewater (CFU/yr)	= $WR_{OG} = (AR_{FO})(ç_R)/(1-ç_R)$
	Release from Spent Samples	= WR_S = negligible

Release Calculations	

Medium	Calculation	
Land	Total Land Releases (CFU/yr)	= LR_{FC}+ LR_{SP} + LR_{SS}
	Releases from Filter Cakes and Sludge from Separation Processes	= LR_{FC} + LR_{SP} = $(WR_B)(1-ç_I)(ç_R)/(1-ç_R)$
	Releases from Spent Samples	= LR_{SS} = negligible

where:

AF = Aerosolization factor (default = 1×10^{-9})
AR_{FO} = Air release from fermentor off-gas (CFU/yr)
AR_P = Air release from process equipment (CFU/yr)
AR_S = Air release from sampling
B = Batches per year
C = Typical contact; from Table 3-1 (ml/day)
$[CFU]_B$ = CFU concentration in final fermentation broth (CFU/ml)
$[CFU]_E$ = CFU concentration in exhaust gas, after control technology (CFU/ft^3)
$[CFU]^O$ = CFU concentration in fermentor off-gas, before control technology (CFU/ft^3)
$[CFU]_P$ = CFU concentration in appropriate process stream (CFU/ml)
$[CFU]_{WA}$ = CFU concentration in workspace air (CFU/ml)
E_D = Dermal exposure (CFU/day)
E_I = Inhalation exposure (CFU/day)
F = Volumetric flow rate through fermentor (ft^3/minute)
h = Hours exposed per day
I = Inhalation Rate; from Table 3-2 (m^3/hr; default = 1.25 m^3/hr)
LR_{FL} = Land releases from biomass collected on filter cakes (CFU/yr)
LR_{SP} = Land releases from biomass in sludge from separation processes (CFU/yr)
LR_{SS} = Land releases from spent samples (CFU/yr)
$ç_I$ = Inactivation efficiency
$ç_R$ = Removal efficiency of control technology
t = Yearly operating time (minutes/yr)
V_B = Volume of fermentor broth (ml)
V_F = Volume of fermentor (ml/batch)
WR_B = Water release from inactivated fermentation broth (CFU/yr)
WR_{CW} = Water release from cleaning wastewater (CFU/yr)
WR_{OG} = Water releases from fermentor off-gas (CFU/yr)

Default values for the calculations are presented below:

Default Values	
AF	1×10^{-9}
B	100 batches/yr
$[CFU]_B$	2.05×10^{11} CFU/ml
F	1 volume air per fermentor volume
I	1.25 m^3/hr
PV	7.4×10^{20} CFU/yr
Sporulation Proficiency	1 spore/1×10^{7} CFU
V_F	57,000 L

It should be noted that separate estimates should be made for the release of spores. In general, the calculations for spore estimates are identical to those presented above, except the inactivation efficiency terms should be omitted (because typical inactivation procedures do not affect spores), and final estimates should be multiplied by a sporulation proficiency (default value = 1×10^{-7} spores/CFU).

Appendices A through D present data for control technologies and inactivation procedures as they apply to specific microorganisms. However, the review of past PMNs shows that the specific inactivation and sterilization procedures used vary significantly. Therefore, while a standard estimation methodology has been developed and the appendix tables or default efficiencies can be used, accurate release estimates cannot be made without a thorough knowledge of the specific fermentation process used.

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LIST OF ACRONYMS AND DEFINITIONS

The acronyms and terms presented below are meant to reflect the majority of terms that are typically associated with fermentation processes. These acronyms and definitions are defined in such a way that they may be useful to the reader when discussing or reviewing premanufacture notices (PMNs) for the purpose of estimating or evaluating potential occupational exposures and/or releases to the environment.

Aerosolization Factor	Dimensionless factor indicating the proportion of CFU-containing aerosol particles in the size range of 1 to 10 microns formed per initial number of cells in the liquid volume considered.
Autoclave	Jacketed pressure vessel, used as a batch terminal unit operation. Employs steam sterilization for the inactivation of heat stable processing fluids or solid and liquid biogenic wastes. Typical minimum pressure and temperature is 15 psig and 121°C while in use.
Bacteriophage	Naturally occurring small viruses that can infect bacteria (and other microorganisms).
Biocide	A compound that contains the ability to inactivate microorganisms; disinfectants. Examples include: algicides (inactivate algae), bactericides (inactivate bacteria), vericides (inactivate viruses), fungicides (inactivate fungi).
CDC	Centers for Disease Control.
CFU	Colony forming units. The number of viable vegetative cells that have the potential of propagating if released to the environment.
Chemical Inactivation	Inactivation due to addition of organic or inorganic toxic chemicals (chlorine/chlorine-containing organics are the most common chemicals used).
Chemostat	A continuously operated fermentor.
Containment (primary and secondary)	Primary containment includes design measures that are used to prevent or reduce releases from fundamental process equipment such as valves and seals. Secondary containment prevents microbial releases to the environment in the event of the failure of primary containment. Secondary containment includes the room or facility in which the process unit operations are conducted.
D-value (or decimal reduction time)	Time required to reduce the viable cell concentration by a factor of 10 due to an inactivation procedure ($D = \ln(0.1)/k$).
Decontamination	Physical removal of organisms to a desired level.
Dilution Rate (or	Reciprocal of the mean holding time or mean residence time. The

specific growth rate)	number of tank liquid volumes that pass through the fermentor per unit time.
Disinfection	Destruction of pathogenic agents, biologically active toxins, and viable organisms that synthesize them (generally referring to treatment involving the inactivation of specific organisms to target levels; inactivation of other organisms may occur concurrently, but this is secondary to the main purpose). Spores may not be destroyed. Defined in Florida Rule 100-104, F.A.C., as "A process that destroys or irreversibly inactivates the vegetative cells of infectious microorganisms."
Fermentation	The process by which microorganisms metabolize sugars and other food sources into energy (catabolism) and biomass (anabolism).
GDP	Good Development Practices; a term that was primarily created for use in the manufacturing of GEMs intended to be released into the environment.
GEM	Genetically Engineered Microorganism.
GILSP	Good Industrial Large Scale Practices; a term primarily created for use in manufacturing of GEMs. GILSP generally requires a minimal level of control and containment in established, traditional fermentation processes.
HEPA Filter	High Efficiency Particulate Air filter; depth filters that achieve 99.97% retention of 0.3 micrometer particles; used to decontaminate BL_3/BL_4 clean room air.
Inactivation or Deactivation	Irreversible loss of ability of a population of organisms to reproduce; can involve destruction of the organism or may allow continued metabolic functioning while eliminating the potential for reproduction.
K-value	Efficiency of a terminal unit operation inactivating the GEM; numeric value of rate constant from 1st order, ideal death curve.
Large Scale	Refers to fermentation processes that are greater than 10 liters.
Microfiltration	Membrane filtration using pore sizes ranging between 0.02 and 10 micrometers (typically used for cell harvesting).
NIH Guidelines BL1-4	National Institutes of Health Guidelines (note these are not regulations and are not enforceable) for Biosafety Levels One through Four; levels are determined by CDC, WHO, and other organizations with Level One being the "safest".
NIH	National Institutes of Health.
NIOSH	National Institute of Occupational Safety and Health.
Pathogen	Microorganism(s) that can cause disease.

Plasmid	Circular, autonomous, and self-replicating DNA molecule that is found in many bacterial species. Plasmids contain genetic code for genes that have any number of functions.
Radiation Inactivation	Disinfection due to radiation exposure (including ultraviolet, microwave, and gamma radiation).
Residence Time	Inverse of dilution ratio; (reactor volume)/(flow rate).
Reverse Osmosis Membrane Filtration	Membrane filtration using pore sizes ranging between 0.0001 and 0.001 micrometers.
Size Exclusion Inactivation	Decontamination using filters or cross-flow membranes.
Specific Growth Rate	See "Dilution Rate"
Spore	Dormant forms of bacterial cells that are capable of resisting heat, radiation, and chemicals. Also referred to as "endospores". When spores are returned to surroundings that are suitable for cell function, they can germinate to yield normal, biologically active, functioning ("vegetative") cells.
Sterilization	Destruction or killing of all microorganisms and infectious agents such as viroids.
Thermal Inactivation	Wet or dry heat treatment that causes thermal death.
Treatment	Differs from containment in that it actually destroys or removes microorganisms from process streams or from process areas once the microbes have escaped containment. Defined in Section 381.0098, Florida Statutes as "any process, including steam sterilization, chemical treatment, microwave shredding, and incineration, which changes the character or composition of biomedical waste so as to render it noninfectious".
Ultrafiltration	Membrane filtration using pore sizes ranging between 0.001 and 0.02 micrometers (typically used to filter and retain dissolved macromolecules such as proteins and peptides).
Vector	The vehicle (such as a bacterial plasmid) that is used to carry the donor organism's DNA segment of interest into the host organism by transformation.
WHO	World Health Organization.

1.0 INTRODUCTION

1.1 Background

Under Section 5 of the Toxic Substances Control Act (TSCA), a manufacturer of a new chemical substance is required to submit a Premanufacture Notice (PMN) to EPA. This PMN is reviewed to determine whether manufacture and/or use of the new substance might present an unreasonable risk to human health and the environment.

The role of the Chemical Engineering Branch (CEB) of the Office of Pollution Prevention and Toxics (OPPT) in these reviews is to assess the potential for worker exposure and environmental releases associated with the new substance during its manufacture, processing, and use. These assessments are based on information provided by the PMN submitter, information from readily available databases and literature sources, and standard estimating techniques used by CEB.

CEB has developed a number of "generic scenarios" and modeling approaches for quantifying sources and control efficiencies to use in assessing exposures and releases for various industries. These generic scenarios are a compilation of information from readily available sources and from past CEB assessments. They have helped CEB to standardize its assessments for particular industries.

1.2 Purpose

The purpose of this document is to develop a generic scenario for the assessment of closed-system fermentation uses of genetically engineered microorganisms (GEMs). EPA/OPPT has received several of these "biotechnology" PMNs over the last few years and is seeking to improve its capabilities to assess the risks to human health and the environment for this particular type of application. This biotechnology generic scenario is a compilation of information related to the assessment of occupational exposures and releases of GEMs from closed-system

fermentation processes; it will serve as an aid to CEB in developing a standardized methodology for evaluating biotechnology PMNs.

1.3 Methodology for Developing Estimation Techniques

A literature search was conducted to acquire available data and to research possible theoretical correlations that could be used to develop estimation techniques for this generic scenario. In addition, biotechnology PMNs previously submitted to CEB were reviewed. Based on the results of information acquired during these reviews, a methodology for developing the final estimation technique(s) was developed.

1.3.1 Literature Search

A literature search was conducted to obtain available background information and empirical data for developing correlations to estimate occupational exposures and to develop release estimates. This search included:

- A review of documents and references in the CEB library (see Section 6.0 for a complete list of these references);
- Conversations with CEB contacts within other branches and divisions of EPA, other U.S. government agencies (NIH and FDA), and Canadian agencies (Environment Canada);
- A literature search of medical waste treatment technologies;
- A literature search of the NASA Star (a journal produced by NASA) for inactivation and sterilization techniques; and
- Professional contacts.

1.3.2 Past Premanufacture Notices

Each biotechnology PMN that was submitted to EPA between 1987 and 1995 was reviewed to gather information on typical fermentation processes and inactivation and sterilization procedures that are currently used. The CEB reviews of these PMNs were also analyzed to

determine CEB's past estimation techniques and assumptions. Information from these PMNs and CEB reviews was used to develop some of the estimation methodologies that are presented in this generic scenario.

1.4 Hierarchy for Developing Release and Occupational Exposure Estimates

The goal of this generic scenario is to standardize CEB's approach and methodology to develop accurate release and occupational exposure estimates for biotechnology PMNs. Actual data that are available and the need to make assumptions that are required for individual estimations may vary significantly between PMN reviews. Therefore, the following hierarchy in evaluating PMNs has been developed to provide consistent and accurate assessments.

1. Empirical data: Data obtained from the PMN submission or from contacts with the submitter should be considered first. It is assumed that data from testing will result in the most accurate release estimates. However, these data and the release and exposure estimates that result from their use should be compared to typical and historical release estimates.

2. Analogous data: It is possible that a facility may not have conducted testing on the recombinant microorganism, but did conduct tests on the wild-type microorganism, or other similar microorganisms. It may be appropriate to use results of these tests to estimate releases and exposures. These data and corresponding estimates should also be compared to typical and historical estimates.

3. Generic scenario: In lieu of site-specific testing or analogous data, it may be appropriate to use the methodology described in this generic scenario to develop reasonable worst-case estimates for releases and occupational exposures. The CEB engineer should compare the site-specific information with the assumptions used in the generic scenario and make reasonable adjustments to the methodology based on engineering judgement. The resulting estimates should be compared to historical estimates for consistency.

4. Regulatory limits: If neither site-specific data nor the information needed to develop reasonable estimates using the generic scenario are available, regulatory limits should be considered. It is possible that local, state, or federal agencies may have imposed (or will impose in the future) restrictions on production volumes or GEM concentrations in fermentor broths, facility air, or releases. If such limits exist, they may be used as reasonable worst-case estimates.

5. Modeling: CEB has not developed modeling procedures to estimate releases or occupational exposures from closed-loop fermentation of GEMs. However, Environment Canada is currently considering testing a computer algorithm that quantifies releases and exposures. Tests may be conducted in the near future at Canadian fermentation facilities to verify the accuracy of this modeling. If results prove to be successful, CEB may consider adopting this modeling approach.

2.0 CHARACTERIZATION OF CLOSED FERMENTATION PROCESSES

Fermentation is the process by which microorganisms, typically bacteria or yeast, metabolize food sources. These food sources, substrates such as sugars and other metabolites, are converted into energy and biomass by the microorganism. During this process, the microorganism may produce a desirable byproduct such as an enzyme that the pharmaceutical industry isolates for sale to customers. Process designs, unit operations, and inactivation and sterilization techniques vary significantly between each process, but a standard industrial fermentation process includes laboratory propagation, fermentation, cell removal, concentration, and final formulation. These components are described in the following subsections. A generic process diagram is provided in Figure 2.1.

2.1 Typical Industrial Fermentation Process

The industrial fermentation process can be divided into three main steps: laboratory propagation, fermentation, and recovery (laboratory propagation and fermentation occur in the "Fermentation" subdivision of Figure 2-1 and recovery occurs in the "Downstream Processing" and "Packaging" subdivisions).

2.1.1 Laboratory Propagation

Laboratory propagation consists of preparing a liquid medium that contains a suspension of the GEM and substrates that are required for growth. Initial cultures of the microorganism are aseptically transferred from vials that have been stored in liquid nitrogen or have been lyophilized (freeze-dried) to small shake flasks that contain sterile growth medium. This transfer typically occurs under a laminar flow hood. The shake flasks are incubated until the cell density increases to the desired concentration (typically at the end of the exponential phase of bacterial growth). The medium is then aseptically transferred to larger flasks (manually), and the cell concentration is again increased. Finally, the medium is transferred to a seed fermentor (manually), which has a typical volume ranging between 1 and 20 percent of the main production fermentor.

Figure 2-1. Generic Industrial Fermentation Process Diagram

(Reproduced from Bailey and Ollis, 1986)

This process of continually inoculating a larger and larger volume of fermentation medium with a highly concentrated inoculum has been proven to significantly decrease the time required to complete the fermentation process (due to growth kinetics and microorganism metabolic rates).

2.1.2 Fermentation

After growth to the desired cell concentration in the seed fermentor, the fermentation broth is aseptically transferred to the main production fermentor. This transfer typically occurs through closed, stainless steel piping that has been steam sterilized. Production fermentors are typically submerged, deep tank fermentors that have a variety of sealed ports for sampling, addition of fresh culture medium, sterile air or oxygen sparging (for aerobic processes), addition of antifoam agents, fermentor off-gas vents (with filters to prevent contamination as well as potential release of the GEM), and impellers (to facilitate thorough mixing and aeration). Figure 2-2 presents a typical fermentor.

The fermentation process can last from a few hours to several days, until the desired concentration of the final product is achieved in the broth. Fermentation processes may be batch, semibatch, or continuous depending on the desired product and cell kinetics. Batch processes are typically easier to operate and present fewer contamination concerns. However, products or byproducts of metabolism may be growth inhibitors that are toxic to the GEM. In such cases, semibatch or continuous fermentations may be used to remove fermentation broth at the optimum biomass, product, and byproduct concentrations.

Once fermentation is completed (or the desired volume of broth is removed in the case of semibatch and continuous processes), the GEM is inactivated (rendered incapable of reproducing) or sterilized (killed) for product recovery. Inactivation processes

Figure 2-2. Schematic Diagram of a Typical Industrial Fermentor

(Reproduced from McIntrye, 1993)

are very case-specific. Typically, inactivation procedures include a combination of the following techniques:

- Addition of a germicide or bactericide (e.g., hypochlorite);
- Addition of strong acids or bases to achieve an extreme pH;
- Cessation of aeration and agitation (to cause oxygen depletion in aerobic processes);
- Extreme agitation (to create an extreme shear stress that lyses the cell); and/or
- Heat treatment.

2.1.3 Recovery

The desired product may consist of a metabolic byproduct that is secreted from the microorganism into the fermentation broth, an enzyme that is produced during the exponential or stationary cell growth phase that remains with the cell, or another in vivo cellular component. It may be necessary to lyse the cell to recover the product if it remains within the cell wall of the GEM. Product may be recovered using a number of techniques including, but not limited to:

- Addition of a lysozyme to the fermentation broth (to separate the cells from the desired enzyme);
- Addition of flocculants or filter aid;
- Addition of formulants or other chemical additives to produce the final product;
- Aseptic transfer to a process tank;
- Centrifugation;
- Concentration by vacuum evaporation;
- Filtration;

- pH adjustment; and
- Ultrafiltration.

2.1.4 Sampling

Sampling may occur at a number of points and throughout each step of the fermentation process. Fermentation broth and/or the inoculum may be sampled to determine the concentration of GEMs, contaminants (other, undesired microorganisms), metabolites in the growth medium, toxic byproducts, and the desired product. Typically, the inoculum is sampled prior to introducing it into the next larger volume of growth, medium and the final broth is periodically sampled to ensure that fermentation is progressing satisfactorily.

Sampling activities vary significantly; however, most facilities use procedures and engineering controls that are likely to prevent contamination from outside sources. These precautions in turn reduce the potential for release of the GEM. Below is a typical procedure for sampling fermentation broth:

- The sample line and sample port is steam sterilized (sample ports on fermentation vessels are often equipped with removable parts that can be autoclaved prior to and after sampling, or they have separate casings and valves that can be steam sterilized while in operation).
- The sample port is opened and a quantity of the liquid broth is drawn into a collection vessel. This initial volume of liquid is collected to flush the lines, and is discarded. A sample of the broth is then collected under aseptic conditions to prevent contamination of the fermentor. Aseptic methods include collecting the sample under a biological laminar flow hood or collecting the sample within a few inches of an open flame (which has been shown to kill any potential airborne contaminants).
- The sample port is closed and steam sterilized.

Used sampling equipment, such as flasks, syringes, and gloves, may be chemically treated or autoclaved before disposal. Spent samples may also be treated or autoclaved, or they may be combined with other process wastes.

2.2 Potential Release Points

The success of fermentation processes that involve the use of GEMs depends greatly on the growth kinetics of the specific microorganism. The process must be operated at case-specific optimum physical conditions to ensure that bacterial or fungal growth (and subsequent product formation) is maximized. To ensure this optimum growth, enriched media are used that contain a variety of nutrients required for sustained growth. Unfortunately, these nutrients can be used not only by the GEM, but also by wild-type microorganisms that are dominant in the environment. These wild-type microorganisms will compete with the GEM for the food sources in the growth medium, and cause a drastic reduction in product formation if present. Therefore, most industrial fermentation processes are conducted in closed-loop systems that contain numerous controls to prevent contamination of the system from outside sources. Additionally, most facilities consider GEMs to be extremely proprietary. Therefore, controls are put in place to reduce or theoretically eliminate the release of viable microorganisms to any media. These controls also serve to reduce the potential release of GEMs.

Even with these controls, there is the potential for release of viable colony forming units (CFUs) from several steps of a typical industrial fermentation process.

2.2.1 Air Releases

Air releases of viable CFUs are expected during normal industrial fermentation processes. The potential sources of air releases are:

- Exhaust gases from fermentors;
- Fugitive releases from process tanks that are vented to the atmosphere after inactivation;

- Fugitive releases from process equipment (there is always the potential for fugitive releases from any valve, pump, fitting, etc.; however, process-related fugitive releases are expected from the filtration and centrifugation steps); and

- Sample collection.

2.2.2 Water Releases

The potential exists for water releases from several sources during normal fermentation processes, even with inactivation or sterilization procedures and engineering controls. The potential sources of water releases include:

- Disposal of inactivated fermentation broth;

- Cleaning washwater/rinse water;

- Off-gas treatment system wastewater (many facilities incorporate scrubber systems, cyclone separators with coalescing filters, or mist eliminators to remove aerosolized CFUs from fermentor off-gases; this wastewater has the potential to contain viable CFUs); and

- Spent samples.

2.2.3 Land Releases

Solid releases to landfills or land farming are typically less prevalent than air and water releases. All aerobic fermentation will evolve large quantities of fermentor off-gases and liquid releases from the inactivated fermentation broth, and may or may not result in waste that are disposed to land. However, land releases may still occur. The potential sources of land releases include:

- Biomass collected on filter pads;
- Biomass collected on filter cakes;
- Sludge from separation processes or treatment operations;
- Residue on sampling equipment; and
- Spent samples.

2.3 Control Technologies, Inactivation, and Sterilization Procedures

Each of the potential releases discussed in Section 2.2 should be considered when assessing the PMN. However, as previously stated, most facilities incorporate elaborate engineering controls that concurrently prevent contamination of the fermentation broth with wild-type microorganisms and the release of any GEMs to the environment. In addition, nearly all facilities inactivate the fermentation broth prior to or during product recovery. Facilities also typically sterilize process equipment and process waste prior to disposal. Control technologies, inactivation procedures, and sterilization methods that have been reported by previous submitters of biotechnology PMNs are discussed in Sections 2.4.1 through 2.4.3, respectively. The efficiencies of these methods are discussed in Section 4.5.

An important concern when assessing the environmental impact of GEMs is the potential of the microorganism to form endospores. Spores are essentially dormant forms of the microorganism that are formed when the active, living (or vegetative) cell is subjected to adverse conditions. Microorganisms, including bacteria and fungi, have the capability of producing spores when physical conditions change to threaten the survival of the microorganism, if food sources are depleted, or if growth inhibitors or toxins are detected by the cell.

Spores have been known to survive for hundreds of years in oxygen-deprived environments and are often heat- and pH-resistant. Once conditions surrounding the spore become favorable to growth, it can germinate into a viable, vegetative cell. This phenomenon causes a concern for facilities that attempt to reduce or eliminate the release of any GEMs with inactivation or sterilization techniques. Therefore, many GEMs have been engineered to specifically reduce or eliminate spore production.

A review of the previously submitted biotechnology PMNs shows a typical sporulation proficiency of one spore formed per 1×10^{7} vegetative cells. However, this rate is highly variable among bacteria, and the PMN release assessment should consider potential spore formation on a case-by-case basis (the facility may have conducted testing to determine the sporulation proficiency of the GEM).

Spores, although they may be heat- or pH-resistant, can be killed. For example, one study states that bacterial spores can be killed by heat treatment to 80°C for a minimum of 6 hours or by subjecting them to aqueous iodine preparations, and neutral or slightly alkaline glutaraldehyde solutions (McIntyre, 1993).

2.3.1 Control Technologies

Facilities incorporate a wide variety of control technologies that reduce CFU releases from their fermentation processes. Some of these technologies are based on physical separation and removal of the GEM (such as filtration) while others (such as heat treatment) act to kill the GEM and allow the release of inactivated biomass.

2.3.1.1 Air

A review of past biotechnology PMNs shows that all facilities currently using GEMs incorporate some type of control technology to reduce emissions of CFUs that are entrained in fermentor off-gases. These control technologies include:

- High Efficiency Particulate Air (HEPA) filters;
- Ozone treatment;
- Cyclone separators (usually in conjunction with coalescing filters);
- Mist eliminator systems; and
- Scrubber systems (the scrubber liquid may or may not contain additional chemicals to kill the entrained GEM).

HEPA filters are known to remove 99.97 percent of particles that are greater than 0.3 microns in diameter (Radian, 1986). Microorganisms vary greatly in size and shape, but typical effective spherical diameters are as follows (Bailey and Ollis, 1986):

- Yeast = 5 microns;
- Bacteria = 1-2 microns;
- Mycoplasmas = 0.2 microns; and
- Virus = 0.1 microns.

The bacterium *Escherichia coli*, as an example, is cylindrical with a diameter of approximately 0.2 microns and a length of approximately 2 microns. A 0.01 percent lengthwise passage of *E. coli* through HEPA filters has been estimated (McIntyre, 1993).

HEPA filters and the other methods presented above physically remove viable CFUs from air streams, but may not inactivate the GEM unless they are combined with other methods. For example, water scrubbers may remove 99% of the GEM from the fermentor off-gas; however, the scrubber wastewater is then contaminated and should be considered as a potential water release source.

2.3.1.2 Water

Nearly all facilities incorporate control technologies to terminate the fermentation process and reduce the potential for environmental releases to water. These control technologies include:

- Decreasing agitation (to reduce mixing and the subsequent oxygen diffusion into the fermentation broth for aerobic GEMs; usually combined with stopping aeration);
- Stopping aeration (to reduce oxygen convection and subsequent diffusion into the fermentation broth for aerobic GEMs; usually combined with a reduction or complete stoppage of agitation);
- Filtration, evaporation, or ultrafiltration (to separate the GEM from soluble portions of the fermentor broth);
- Heat treatment (to kill the GEM); and
- Increasing fermentor agitation (to increase shear stress and subsequently lyse the cellular membrane or cell wall for anaerobic GEMs and/or

following a period of decreasing agitation and aeration for aerobic GEMs; usually combined with stopping aeration).

These control technologies are used in conjunction with the inactivation techniques presented in Section 2.3.2 to reduce or eliminate the release of viable CFUs from various process streams. Efficiencies of the individual methods are case-specific and have not been determined; however, CEB and many facilities have estimated the combined efficiency of control technologies and inactivation procedures during assessments of past PMNs. These efficiencies are discussed in Section 4.5, where applicable.

2.3.1.3 Land

Land releases are typically expected to be several orders of magnitude lower than air or water releases. Heat treatment, which may be considered an inactivation procedure, is the primary control technology used to reduce land releases of CFUs. Most solids that have the potential to result in land releases are inactivated as discussed in Section 2.3.2.

2.3.2 Inactivation

The fermentation process is typically allowed to continue until the desired product concentration is achieved. The fermentation is then stopped and product recovery occurs. This process involves inactivating the GEM to prevent it from metabolizing the product, removing unwanted byproducts, and reducing the potential for environmental releases.

Inactivation procedures are extremely site-specific, but may include any combination of the following:

- The engineering controls presented in Section 2.3.1;
- Addition of acids or bases (to result in an extreme pH that will effectively kill the GEM or render it incapable of reproducing);

- Addition of other chemicals or biocides to the fermentation broth (usually after the product has been separated via filtration, ultrafiltration, or centrifugation);

- Caustic addition (typically added to process waste streams such as spent samples, cleaning wash water, or scrubber wastewater rather than to fermentor broth);

- Chemical treatment of waste filter cake and filter pads (typically with hypochlorite, nitric acid, formaldehyde, or calcium hydroxide); and

- Autoclaving spent samples and sampling equipment.

It is difficult to predict the efficiency of an inactivation procedure because the effectiveness of each method is dependent upon the specific death rate kinetics of each GEM. One empirical equation that can be used to determine the final concentration of viable CFUs after inactivation procedures begin is the "Chick Equation" (Bailey and Ollis, 1986).

This equation applies to ideal, first-order, batch reactions:

$$(N/N_o) = e^{-kt}$$

where:

N_o	=	initial CFU concentration
N	=	final CFU concentration
k	=	inactivation rate constant (specific to each GEM)
t	=	time

Unfortunately, the rate constant (k) must be known or experimentally determined to use this equation (and most other death rate equations). Most facilities have not conducted testing to determine empirical rate constants or inactivation rates. Also, a review of past biotechnology PMNs shows that most facilities claim 100% efficiencies of their inactivation or treatment systems, but few conducted verification testing. Inactivation efficiencies are discussed in detail in Section 4-5 and results of inactivation procedures on a variety of microorganisms are presented in Appendices A through D.

2.3.3 Sterilization

Sterilization, for the purpose of this generic scenario, is defined as the procedure by which process equipment is treated to kill the GEM, as opposed to inactivation which does not kill the microorganism, but destroys its viability. Typically, used sample equipment, collection vessels, and process equipment are sterilized before and after each use to reduce or prevent releases of the GEM from the current fermentation and prevent contamination of the subsequent fermentation. Large pieces of production equipment are typically sealed and steam sterilized. They are then washed with a cleaning solution that contains chemicals to kill any remaining viable cells (typically caustic) and are rinsed with water. The wastewater from this process is collected and usually transferred to the inactivation procedure before final release to a POTW. Spent samples, sampling equipment, small volume waste streams, and small pieces of equipment that may contain viable GEMs may be autoclaved and disposed of, or may be transferred to the inactivation process.

The review of past biotechnology PMNs showed that some facilities alternatively sterilize equipment with hypochlorite and/or nitric acid solutions, thermal treatment, or a combination of these methods. Also, some facilities collect and incinerate sampling waste.

3.0 OCCUPATIONAL EXPOSURE

The industrial fermentation processes that apply to this generic scenario are closed systems designed to minimize the release of large quantities of the GEM. However, the potential exists for occupational exposure during several process steps, including:

- Laboratory propagation;
- Sampling;
- Equipment cleaning/maintenance; and
- Product recovery.

Extensive quantitative occupational exposure information is not readily available due to a number of reasons, including: a lack of regulatory requirements, limitations of available sampling equipment, and the highly proprietary nature of GEM products. However, a 1986 NIOSH study at three enzyme fermentation facilities has been reviewed and data from this study may be used to develop an assessment of dermal and inhalation occupational exposures, when site-specific data are not available. Dermal and inhalation exposure assessments are presented in Sections 3.1 and 3.2, respectively. A discussion of the number of workers that may be exposed during a typical industrial fermentation is presented in Section 3.3.

3.1 Dermal Exposure

There is a potential for dermal exposure from each of the four process steps listed above.

Although there is potential for direct contact with the GEM during laboratory propagation, the potential for dermal exposure is low. Workers may handle vials, flasks, or containers of active cells, but these processes are typically conducted under aseptic conditions (laminar hoods and/or closed piping systems) and technicians are expected to wear appropriate protective equipment to prevent contamination from outside sources. Additionally, the time required to transfer or test inoculum is short.

Sampling procedures are also expected to result in a low dermal exposure potential. These activities may not occur under conditions that are as controlled as laboratory propagation, but the quantities handled and the time required for sampling is minimal.

Cleaning and maintenance operations typically occur after sterilization. Therefore, although there may be contact with liquids or solids that contain viable CFUs, the CFU concentration and the corresponding dermal exposure is low.

There is potential for dermal exposure during the product recovery step because workers may contact filter cakes, fermentation broth, or other CFU-containing material before inactivation or sterilization. This step has the highest potential for occupational dermal exposure.

3.1.1 Estimating Dermal Exposure

If no data are available to estimate dermal exposures, a reasonable worst-case estimate can be made by applying current CEB dermal exposure estimation techniques. Table 4-13 of the CEB Engineering Manual presents dermal exposure information for a variety of activities. Portions of the table have been reproduced in Table 3-1. These exposures can be multiplied by the concentration of CFUs expected in each of the fermentation process steps discussed above. The PMN should state these concentrations (each of the biotechnology PMNs that were reviewed either stated the concentrations or provided information to calculate them). It should be noted that workers may be required to wear gloves during some operations, particularly laboratory propagation and sampling. This may significantly reduce the exposure calculated from this method.

Dermal exposure can be calculated by selecting the relevant activity and multiplying the associated contact volume by the CFU concentration as follows:

Table 3-1

Typical Factors for Calculation of Dermal Exposure

Activity	Typical Examples	cm^2	ml/cm^2	Resulting Daily Typical Contact Volume, ml/day
Routine immersion, 2 hands	• Handling wet surfaces	1,300	5 to 14	6,500 to 18,200
	• Filling/dumping containers of powders, flakes, granules			
	• Spray painting			
Routine contact, 2 hands	• Maintenance/manual cleaning of equipment	1,300	1 to 3	1,300 to 3,900
	• Unloading filter cake			
	• Changing filter			
	• Filling drums with liquid			
Incidental contact, 2 hands	• Connecting transfer line	1,300	1 to 3	1,300 to 3,900
	• Weighing powder/scooping mixing (i.e., dye weighing)			
Incidental contact, 1 hand	• Sampling	650	1 to 3	650 to 1,950
	• Loading liquid/bench scale liquid transfer			

Reference: CEB Engineering Manual, Table 4-13.

$$E_D = ([CFU]_P)C$$

where:

E_D	=	Dermal exposure (CFU/day)
$[CFU]_P$	=	CFU concentration in appropriate process stream (CFU/ml)
C	=	Typical contact volume from Table 3-1 (ml/day)

The recommended activity for laboratory propagation and sampling efforts is "incidental contact, one hand", while "routine contact, two hands" is recommended for equipment cleaning/maintenance and product recovery.

3.2 Inhalation Exposure

Bioaerosols consist of individual or clumps of spores or cells with a total diameter of 1 to 10 microns. These cells may be adsorbed on dust particles or enclosed in water droplets, where, under appropriate conditions of humidity and temperature, they are capable of reproducing (McIntyre, 1993). The generation of bioaerosols from fugitive emissions can result in exposures to the GEM throughout the facility, although exposures are expected to be higher in process areas that generate air emissions.

Current CEB techniques can be used to estimate inhalation exposures if data are available. Table 4-1 of the CEB Engineering Manual presents inhalation rates that can be expected for different types of activities. Portions of the table have been reproduced in Table 3-2. The typical (default) inhalation rate is 1.25 m^3/hr. If data are available, case-specific personal monitoring data showing the concentration of CFUs in workspace air should be multiplied by the expected breathing rate and hours of exposure to estimate the daily inhalation exposure. If site-specific personal monitoring data are not available, site-specific area monitoring data may provide the CFU concentration:

Table 3-2

Inhalation Rates

Activity	Inhalation Rates (m^3/hr)
Rest	0.56
Light work	1.18
Medium work	1.75
Medium heavy work	2.63
Heavy work	3.6
Maximum work	7.9
CEB Default Value	1.25

Reference: CEB Engineering Manual, Table 4-1 (source, NIOSH data, 1976).

$$E_I = (I)(h)([CFU]_{WA})$$

where:

E_I	=	Inhalation exposure (CFU/day)
I	=	Inhalation rate (m^3/hr from Table 3-2; default = 1.25 m^3/hr)
h	=	Hours exposed per day
$[CFU]_{WA}$	=	CFU concentration in workspace air (CFU/m^3]

The review of past biotechnology PMNs showed that few submissions contained personal or area monitoring data. If no data are available, the CFU concentration can be estimated based on a study that was conducted by NIOSH in 1986, which evaluated the potential hazards of recombinant DNA bioprocesses. This study focused on conventional enzyme fermentations at three facilities. NIOSH did not conduct personal monitoring testing (which is generally preferred), but did conduct ambient air sampling at or near various unit operations, and of general room and background air. Anderson 2-stage viable samplers were used, typically over two days, to collect samples at locations near:

- Laboratories;
- Inoculum tanks;
- Seed fermentors;
- Fermentor tanks;
- Sample ports;
- Centrifuges;
- Vacuum filters;
- Offices; and
- Various background testing locations.

Table 3-3 presents the geometric mean of CFU concentrations at each facility and the average of the geometric means between the facilities, when applicable. These values may be used to approximate the CFU concentration in lieu of site-specific data.

It should be noted that each facility will use different unit operations, site-specific controls, and inactivation technologies that will significantly affect the release of GEMs and the

subsequent CFU concentration in work space air. Therefore, the PMN assessor should select the facility and corresponding concentration from Table 3-3 that most closely

Table 3-3

CFU Concentrations at Facilities Sampled by NIOSH

Sample Location	CFU Concentration[a] (CFU/m³)						
	Facility One		Facility Two		Facility Three		Average of Geometric Means
	Geometric Mean	Range	Geometric Mean	Range	Geometric Mean	Range	
Fermentor sample port	3.1 - 11.2	0.6 - 80	37 - 1,285	12 - 2,235	120	57 - 167	263
Fermentor agitator shaft	2.6 - 7.7	0.8 - 15	<339[a]	84 - 2,298[b]	0	0	115
Centrifuge	47.6	20 - 329	C	-	C	-	47.6
Rotary vacuum belt filter (knife edge)	<2,461[a]	NA	C	-	C	-	<2,461[a]
Rotary vacuum belt filter (transfer point)	<321[a]	NA	C	-	76	13 - 446	191
Seed fermentor agitator shaft	1.4 - 4.2	0.2 - 22	376	243 - 973	C	-	189
Clean room	0	NA	<4[a]	0 - 73[b]	1	0 - 4	1.7
Scrubber	C	-	131	0 - 647	C	-	131
Incubation room	C	-	103	92 - 118	C	-	103
Laboratory	0	0	50 - 56	32 - 103	C	-	53
Dumpster	C	-	<2,400[a]	1,161 - 4,657[b]	C	-	<2,400[a]
Filter press:						-	
• Open	C	-	<10,599[a]	4,484 - 28,990	C	-	<10,599[a]
• Closing	C	-	<8,758[a]	5,320 - 11,601	C	-	<8,758[a]

Sample Location	CFU Concentration[a] (CFU/m^3)						
	Facility One		Facility Two		Facility Three		Average of Geometric Means
	Geometric Mean	Range	Geometric Mean	Range	Geometric Mean	Range	
• Closed	**C**	-	<3,906[a]	988 - 23,588	**C**	-	<3,906[a]
Cafeteria	**C**	-	113	87 - 132	**C**	-	113
Conference room	**C**	-	358	307 - 399	**C**	-	358
Locker room	**C**	-	<33[a]	7 - 134[b]	**C**	-	<33[a]
Office	**C**	-	<529[a]	372 - 750[b]	**C**	-	<529[a]
Control room	**C**	-	**C**	-	8.1	86 - 548	8.1

Source: NIOSH ambient air monitoring study, 1986.
[a]Values represent the concentration of production organisms (GEMs) unless noted.
[b]Values represent the concentration of CFUs detected. The concentration of production organisms (GEMs) could not be determined.
C Samples were not collected at this facility.
NA = Not available.

approximates the specific process being assessed. If the unit operations are not comparable to any individual facility, the assessor may choose to select the average value to approximate the CFU concentration in workspace air near each unit operation. For example, the assessor may choose to use values from Facility One if the facility utilizes a centrifuge, while values from Facility Two may be more appropriate if a scrubber is used.

The selected concentration in workspace air should then be combined with worker activity profiles (hours per day spent near each unit operation) and the standard breathing rate (1.25 m^3/hr) to determine the average (based on geometric mean) and worst-case (based on the range maximum) inhalation exposure. For example, if it is known that the facility uses a centrifuge and the workers are exposed to this unit for two hours per day, the associated inhalation exposure can be estimated as follows:

Average E_I	=	$(I)(h)([CFU]_{WA})$
	=	119 CFU/day
Worst-Case E_I	=	822 CFU/day

where:

E_I	=	Inhalation exposure (CFU/day)
I	=	Standard breathing rate (1.25 m^3/hr)
h	=	2 hrs/day
Average $[CFU]_{WA}$	=	47.6 CFU/m^3 (from Table 3-3; geometric mean for Facility One)
Worst-Case $[CFU]_{WA}$	=	329 CFU/m^3 (from Table 3-3; range maximum for Facility One)

Note that CEB has not yet established default values for worker activity profiles. Engineering judgement and/or contact with the submitter should be used to estimate the time spent near each unit operation.

The literature search for this generic scenario has shown several potential sources of information that may result in an improved standardized estimation procedure (see Section 5.0).

3.3 Estimating the Number of Workers Exposed

The review of past biotechnology PMNs and their associated assessments showed that most of the PMNs submitted between 1987 and 1995 stated the total number of workers employed at each facility, the number of workers per process area, the number of shifts per day, and the facility operating days per year. The average values are presented in Table 3-4. Note that not all facilities reported this information.

Table 3-4

Average Workers per Facility and Process

	Total Employed	Workers per Process Area (per shift)				Shifts/Day	Days/Year[c]
		Fermentation	Recovery	Lab	Supervisory		
Average[a,b]	107	4	4	3	1	3	160

[a]Some facilities reported values for multiple sites.
[b]Not all facilities reported data.
[c]Reported days per year varied between 33 and 365.

4.0 APPROACHES FOR ESTIMATING RELEASES

Sections 4.1 through 4.3 present methods that can be used to estimate reasonable worst-case emissions to air, water, and land, respectively. The assumptions and basis of estimates are discussed in each section as appropriate; Section 4.4 discusses the potential releases of spores and Section 4.5 discusses the efficiency of various control technologies and inactivation techniques. In general, the estimation methodologies presented are based on the results of the literature search and a comprehensive review of past biotechnology PMNs. Table 4-1, at the end of this section, summarizes the equations needed to develop release estimates. Table 4-2, also at the end of this section, summarizes the standard assumptions and default values that may be used in lieu of facility-specific data. Appendices A through D present tables of the efficiencies of various inactivation technologies on specific microorganisms.

Note that each facility and process will use different control technologies and inactivation techniques that are designed to accommodate site-specific needs. The exact methods used must be determined and evaluated to accurately assess potential releases. PMN submissions often contain sampling data that support inactivation and sterilization efficiency claims. However, submitters typically claimed 100% efficiency based on data showing nondetect CFU levels. In these cases, the detection limit may be used as a method of determining a reasonable, worst-case efficiency.

4.1 Air Releases

As discussed in Section 2.2.1, the potential sources of air releases from standard fermentation processes include:

- Fermentor off-gases;
- Fugitive releases from process equipment unit operations such as rotary drum filters and centrifuges;
- Fugitive releases from process tanks; and

- Fugitive releases from other process equipment.

Potential releases from each of these sources should be considered and calculated as described below. Control technology and inactivation procedure efficiencies should be considered in each calculation, and the releases should be summed to determine the overall release to air.

4.1.1 Fermentor Off-Gases

The primary source of air releases is expected to be from fermentor off-gases. These exhaust gases are a result of sparging air (or in rare cases pure oxygen) through the fermentation broth to provide an oxygen source for the GEM. If the GEM is anaerobic, this process will not be required and no releases from this source will occur.

The number of viable CFUs that are generated from this process is highly case-specific and is dependent upon the air flow rate through the fermentation broth, CFU concentration in the broth, control technologies or inactivation techniques used, and the specific GEM used. Therefore, any sampling data that are provided should be considered to estimate releases from this source. The following equations can be used to estimate these releases:

- If the CFU concentration in the final exhaust vent to the atmosphere is known (after all control technologies):

$$AR_{fo} = ([CFU]_E)(F)(t)$$

- If the final CFU concentration is not known, but the CFU concentration before the control technology or inactivation technique is known:

$$AR_{fo} = ([CFU]_O)(F)(t)(1-ç_R)$$

- If no sampling data or other method to determine the CFU concentration in fermentor off-gas is available:

$$AR_{fo} = ([CFU]_B)(AF)(1-ç_R)(V_B)$$

where:

AF	=	Aerosolization factor (1×10^{-9})
AR_{fo}	=	Air release from fermentor off-gas (CFU/yr)
V_B	=	Volume of fermentation broth (ml)
$[CFU]_B$	=	CFU concentration in final fermentation broth (CFU/ml)
$[CFU]_E$	=	CFU concentration in exhaust gas, after control technology (CFU/ft^3)
$[CFU]_O$	=	CFU concentration in fermentor off-gas, before control technology (CFU/ft^3)
F	=	Volumetric flow rate through fermentor (ft^3/minute)
$ç_R$	=	Removal efficiency of control technology or inactivation technique (see Section 4.5)
t	=	Yearly operating time (minutes/year)

If the volumetric air flow rate is unknown, a reasonable default value is one volume air per fermentor volume per minute. This value was observed in past PMN submissions and is reasonable based on industry experience.

The aerosolization factor is a dimensionless factor that estimates the proportion of CFU containing aerosol particles in the size range of 1 to 10 microns that are formed per initial number of cells in liquid volume considered (1 to 10 microns is the expected size range of particles that will be aerosolized and entrained in fermentor off gases). This factor is case-specific, but 1×10^{-9} has been suggested as a standard use (McIntyre, 1993) (past biotechnology CEB assessments assumed 1×10^{-7}).

Control technologies and inactivation techniques are discussed in Section 4.5. The efficiency of the technique(s) used should be determined and applied to the above calculations on a case-specific basis. If no information on control technologies can be obtained, a default value of 99% is a reasonable assumption for all GEM fermentation processes based on EPA's research for the Biotechnology Tier One Exemption Rule. This research determined that all GEM fermentations are expected to use some sort of technology to reduce exhaust gas emissions by a minimum of 99 percent.

4.1.2 Fugitive Releases From Process Unit Operation Equipment

Process equipment, such as centrifuges and rotary drum filtration, can generate releases. The review of past biotechnology PMNs showed that the fermentation broth is typically inactivated (or partially inactivated) before it reaches this stage of the recovery process. Also, air releases from these sources are often collected and processed through control technologies with fermentor off-gas. Therefore, the releases from these sources are expected to be less than those from fermentor off-gases.

As a worst-case scenario, it is reasonable to estimate air releases from these sources in a similar manner as stated for releases from fermentor off-gas. It may be reasonable to assume that these releases are negligible compared to releases from fermentor off-gas if inactivation has occurred. The calculation for a worst-case estimate is presented below:

$$AR_P = ([CFU]_B)(V_B)(1-ç_I)(AF)$$

where:

AF	=	Aerosolization factor (1×10^{-9})
AR_P	=	Air release from process equipment (CFU/yr)
$[CFU]_B$	=	CFU concentration in fermentation broth (CFU/ml)
V_B	=	Volume of fermentation broth (ml/yr)
$ç_I$	=	Inactivation efficiency (if applicable; see Section 4.5)

Control technologies and inactivation techniques are discussed in Section 4.5. The efficiency of the technique(s) used should be determined and applied to the above calculations on a case-specific basis.

4.1.3 Fugitive Releases from Sampling, Other Process Operations, and Process Tanks

Sampling activities are expected to occur under aseptic conditions and air releases from these activities have been shown to be either undetectable or several orders of magnitude lower than those from fermentor off-gas, centrifugation, and filtration. Releases from process

tanks, valves, pumps, and other small process operations also may exist but are expected to be negligible in comparison to releases from fermentor off-gas. Unless data exist to suggest significant releases, the air releases from these sources should be considered negligible (see Section 5).

4.2 Water Releases

As discussed in Section 2.2.2, the potential sources of water releases from standard fermentation processes include:

- Disposal of inactivated fermentation broth;
- Cleaning wastewater;
- Off-gas treatment system wastewater; and
- Spent samples.

Potential releases from each of these sources should be considered and calculated as discussed below. Inactivation procedures should be considered in each calculation and the releases should be summed to determine the overall release to water.

The potential releases to water are dependent upon the site-specific inactivation and sterilization procedures applied. The effectiveness of these procedures is specific to each GEM; however, reasonable worst-case default values may be applied in lieu of specific data. Efficiencies of specific inactivation procedures are discussed in Section 4.5.

The general methodology for estimating releases to water from each potential source is identical. The CFU concentration in the potential release stream must be determined and multiplied by the stream volume and the efficiency of the inactivation or sterilization procedure applied to the specific stream. The calculations for each potential waste stream are presented below.

4.2.1 Inactivated Fermentation Broth

This is the primary source of potential water releases. The calculation to estimate the release is as follows:

$$WR_B = ([CFU]_B)(V_B)(1-ç_i)$$

where:

WR_B	=	Water release from inactivated fermentor broth (CFU/yr)
$[CFU]_B$	=	CFU concentration in fermentation broth (CFU/ml)
V_B	=	Yearly volume of broth (ml)
$ç_i$	=	Inactivation efficiency (see Section 4.5)

Note that the average CFU concentration in the fermentation broth reported in past biotechnology PMNs is approximately 2.05×10^{11} CFU/ml and the average reported GEM production is approximately 7.4×10^{20} CFU/yr.

4.2.2 Cleaning Wastewater

Cleaning wastewater is derived from process cleanouts during sterilization procedures that occur after every batch (note that equipment is cleaned after every batch to prevent contamination of subsequent batches). Wastewater may contain viable CFUs due to residual GEMs that remain in process equipment. The standard CEB assumption of 1% residual per batch may be used to estimate the volume of CFU-containing wastewater generated. This volume should be multiplied by the CFU concentration in the fermentation broth. This assumption may overestimate the quantity of viable CFUs present in the wastewater if equipment has been steam sterilized or treated with caustic solution prior to rinsing but it is reasonable as a worst-case assumption. The calculation to estimate the release is as follows:

$$WR_{CW} = ([CFU]_B)(V_F)(B)(0.01)(1-ç_i)$$

where:

WR_{CW}	=	Water release from cleaning wastewater (CFU/yr)
$[CFU]_B$	=	CFU concentration in fermentation broth (CFU/ml)
V_F	=	Volume of fermentor (ml/bt) (note that the entire fermentor volume should be used rather than the volume of broth because typical fermentations do not utilize the full fermentor capacity, but the entire surface of the fermentor will be saturated with broth due to agitation and air sparging)
B	=	Batches per year
$ç_i$	=	Inactivation or sterilization efficiency (see Section 4.5)

Releases from this source may be combined with other process wastewater for additional inactivation or treatment. If so, an additional treatment efficiency factor should be applied to this release.

4.2.3 Off-Gas Treatment System Wastewater

There is a potential water release from this source, if such a system is used, because fermentor off-gas treatment water will contain viable CFUs that have been separated from the air stream and transferred to the water.

It can be assumed that the off-gas treatment wastewater will be disposed of once per batch. The total CFUs released from this source can be assumed to be equal to those removed from the fermentor off-gas by the separation system. Therefore, the calculation methodology to determine the release from this source is equivalent to that for the release from fermentor off-gas before the control technology is used, multiplied by the treatment efficiency. The calculations are presented below:

- If the CFU concentration in the fermentor off-gas, before treatment, is known:

$$WR_{OG} = ([CFU]_O)(F)(t)(ç_R)$$

- If the CFU concentration in the fermentor off-gas, before treatment, is not known:

$$WR_{OG} = ([CFU]_B)(AF)(\eta_R)(V_B)$$

- If the air release from fermentor off-gas has already been calculated, it can be used to calculate the identical value:

$$WR_{OG} = (AR_{FO})(\eta_R)/(1-\eta_R)$$

where:

AF	=	Aerosolization factor (default = 1×10^{-9})
AR_{FO}	=	Air releases from fermentor off-gas (CFU/yr; calculated in Section 4.1)
$[CFU]_B$	=	CFU concentration in final fermentation broth (CFU/ml)
$[CFU]_O$	=	CFU concentration in fermentor off-gas, before control technology (CFU/ml)
F	=	Volumetric flow rate through fermentor (ft^3/minute)
η_R	=	Removal efficiency of fermentor off-gas treatment system (see Section 4.5)
t	=	Yearly operating time (minutes/yr)
V_B	=	Yearly volume of broth (ml)
WR_{OG}	=	Water releases from fermentor off-gas treatment wastewater (CFU/yr)

Releases from this source may be combined with other process wastewater for additional inactivation or treatment. If so, an additional treatment efficiency factor should be applied to this release. It should be noted that this estimation represents a worst-case scenario that does not account for any cell death once the GEM is captured in the scrubber system and removed from its nutrient source.

4.2.4 Spent Samples

Spent samples are typically collected and autoclaved, or combined with other process streams for inactivation or sterilization. This procedure and the small volume of this potential release source results in negligible releases in comparison to other potential water releases.

4.3 Land Releases

As discussed in Section 2.2.3, the potential sources of releases to land include:

- Biomass collected in filter cakes;
- Sludge from separation processes or treatment operations;
- Biomass collected on filter pads;
- Residue on sampling equipment; and
- Spent samples.

Potential releases from each of these sources should be considered and calculated as presented below. Inactivation procedures should be considered in each calculation and the releases should be summed to determine the overall release to land.

The potential releases to land are dependent upon the site-specific inactivation and sterilization procedures applied. The effectiveness of these procedures is specific to each GEM; however, reasonable worst-case default values may be applied in lieu of specific data. Efficiencies of specific inactivation procedures are discussed in Section 4.5.

4.3.1 Biomass and Sludge Collected from Filter Cakes and Separation Processes

There is a potential land release from these sources because they may contain viable CFUs that have been separated from the liquid process streams. The total CFUs present in these sources can be estimated to be equal to those removed from the liquid stream by the separation process. It should be noted that this estimation represents a worst-case scenario that does not account for cell death once the GEM is removed from the nutrient source in the fermentation broth. The review of past biotechnology PMNs showed that the solids and sludge from these sources are typically collected and then inactivated. The calculation to estimate the release from this source is equal to that for the water release from fermentation broth, before the separation process is used, multiplied by the separation removal efficiency as follows:

$$LR = ([CFU]_B)(V_B)(1-ç_I)(ç_R)$$

If the water release from fermentation broth has already been calculated, it can be used to calculate the identical value:

$$LR = (WR_B)(1-ç_i)(ç_R)/(1-ç_R)$$

where:

$[CFU]_B$	=	CFU concentration in fermentation broth (CFU/ml)
LR	=	Land release (CFU/yr)
WR_B	=	Water releases from fermentation broth (calculated in Section 4.2; CFU/yr)
$ç_i$	=	Inactivation efficiency (see Section 4.5)
$ç_R$	=	Removal efficiency (see Section 4.5)
V_B	=	Yearly volume of broth (ml)

4.3.2 Filter Pads, Sampling Equipment, and Spent Samples

The review of past biotechnology PMNs showed that spent filter pads, used sampling equipment, and spent samples are typically collected, autoclaved, and then subjected to inactivation procedures before being landfilled. This process, and the small volume of waste expected, results in negligible releases in comparison to releases due to biomass collected in filter cakes and sludge from separation processes. Unless data exist that suggest otherwise, the land releases from these processes should be considered negligible.

4.4 Releases of Spores

As previously discussed, the release of spores presents an additional concern in the evaluation of biotechnology PMNs. Inactivation and sterilization procedures may not be effective in destroying a spore's capability to germinate. Therefore, a separate calculation to estimate the release of spores should be conducted for every potential release source.

The methodology for determining these estimates is identical to those presented in Sections 4.1 through 4.3 except inactivation efficiencies should be removed from the calculations

(unless there is reason to believe the procedure will also inactivate the spore). Additionally, a factor representing the sporulation proficiency (the spores produced per viable CFU) should be applied. This proficiency is highly variable between bacterial and final species and every attempt should be made to determine the case-specific value. The PMN submission may state the sporulation proficiency for the specific GEM based on testing. A typical default value based on past biotechnology PMNs and literature values is 1 spore per 1×10^7 CFUs. It should be noted that many GEMs are specifically engineered to reduce or effectively eliminate the ability of spore formation. Therefore, this methodology has the potential to significantly overestimate the release of spores.

4.5 Efficiency of Control Technologies and Inactivation Techniques

Published literature on control and inactivation of GEMs is very limited. Additionally, the review of past biotechnology PMNs showed that most submitters claimed 100% efficiency of their containment and inactivation procedures, but few submissions provided a basis for this claim. Therefore, if the PMN submission does not supply adequate information to estimate these efficiencies, the assessor should consider published data pertaining to efficiencies for the inactivation of wild-type microorganisms. A comprehensive study of control and inactivation of microorganisms from bioprocesses was conducted by Wickramanyake and presented in a six-part article titled "Decontamination Technologies for Release from Bioprocessing Facilities."

Wickramanyake discussed the known technologies and their respective efficiencies for decontamination of wastewater, air, equipment surfaces, and sludge. A qualitative discussion of each technology for each medium is presented along with all known quantitative test results as they apply to various bacteria, viruses, fungi, and subcellular components. The discussions and results for each medium are summarized below and the detailed, quantitative results are reproduced in tables in Appendices A through D.

As discussed by Wickramanyake, "The data indicate that ozone, chloride, chlorine dioxide, heat, ultraviolet light, and ionizing radiation provide good performance potential for

decontamination of processing wastewater. Treatment processes such as incineration, heat treatment, gamma and electron radiation and chemical decontamination appear to be suitable for treatment of sludge. Prevention of the release of microbial concentrations to air and/or decontamination of the air stream can be effectively achieved by filtration or UV radiation. The decontamination of surfaces may be accomplished by the use of heat, UV radiation, or chemicals such as glutaraldehyde, formaldehyde, ethylene oxide, and alcohols."

The PMN assessor should determine the type of control technology and/or inactivation procedure used for each operation at the facility and apply the associated efficiency to each applicable waste stream, as discussed in Sections 4.1 through 4.4, to determine potential releases. The effectiveness of these technologies is expected to vary for each microorganism. However, a reasonable assumption is that their efficiencies will be comparable to those observed when used on the host microorganism or by similar microorganisms. If tests were conducted on the host of a similar microorganism, it is reasonable to assume similar efficiencies for the GEM (results are presented in Appendices A through D).

If no data is available on the host or similar microorganism, it may be appropriate to assume the following efficiencies that CEB has used in past assessments (the assumption basis is listed in parenthesis):

- Use of HEPA filters: 99.97% (Radian, 1986);
- Scrubber removal efficiencies: 99% (CEB standard assumption for "what-if scenario");
- Ozone treatment: 99% (CEB assumption based on kill efficiencies of various organisms using ozone in demand-free water); and
- Cyclone separators and mist elimination systems: 99% (CEB standard assumption for "what-if scenario").

4.5.1 Decontamination of Wastewater

The efficiency of various inactivation techniques can be determined from the data compiled for inactivation of wastewater that is presented in Appendix A. This data applies to wastewater with the following general characteristics:

- 0 - 50°C;
- pH = 5 - 10;
- Turbidity = 1 - 100 NTU;
- Ammonia concentration = 0 - 1 mg/L;
- Total organic carbon concentration = 100 - 5,000 m/L; and
- Suspended solids concentration = 10 - 20,000 mg/L.

Results from testing on eight forms of chemical treatment and three forms of physical separation technologies are presented. These include:

- Chlorine;
- Chloramines;
- Chlorine dioxide;
- Ozone;
- Bromine or bromine chloride;
- Iodine;
- Hydrogen peroxide;
- Potassium permanganate;
- Heat treatment;
- Ionizing radiation; and
- UV radiation.

For example, if the host microorganism is *E. coli* and chlorine treatment is used to inactivate the fermentor broth, Table 1 of Appendix A shows an inactivation efficiency of 99.98 to 99.99 percent. This inactivation efficiency can be used in the equation of Section 4.2.1 to estimate the water release from inactivated fermentor broth.

It should be noted that the data are typically developed from tests conducted in medium that is "demand free." Complex media may react with some of the chemical agents or

may "shelter" the GEM from contacting the agent. Therefore, a higher dose of the agent may be necessary to achieve the same result.

4.5.2 Decontamination of Air Streams

The data compiled for decontamination of air streams is presented in Appendix B. The PMN assessor should review this data and also consider that EPA finalized the "Microbial Products of Biotechnology; Final Regulations under the Toxic Substance Control Act" (the Biotechnology Tier One Exemption Rule) on April 11, 1997. This rule exempts facilities that will use GEMs in certain closed-loop fermentation processes from submitting a PMN. Background research for this rulemaking effort determined that, in EPA's opinion, all facilities incorporating GEMs into closed-loop fermentation processes use control technologies and inactivation procedures that reduce the concentration of viable CFUs in air streams by a minimum of 99 percent.

The assessor should consider that physical treatment processes such as filtration (depth filtration or membrane filtrations), incineration, and UV radiation are more commonly used than chemical treatment in the decontamination of air (Wickramanyake, 1990). Microporous membrane filters have a uniform rigid structure and have an "absolute" rating, typically for 0.45, 0.22, or 0.1 microns. These filters are generally more effective than depth filters because spores that become embedded in depth filters can germinate at a later time, causing a phenomenon known as "grow-through" to occur.

An example for using data from Appendix B is as follows. If the host microorganism is *B. subtilis* and ultra-high efficiency filters made of glass microfibers are used to filter the fermentor exhaust gases, Table 1 of Appendix B shows a removal efficiency from the exhaust gas of 99.998 to 99.9999 percent for spores (depending on the exact filter rating). This removal efficiency can be used where appropriate in the equations of Section 4.1.1 to estimate the air release from the fermentor.

4.5.3 Decontamination of Surfaces and Sludges (Potential Land Releases)

The data compiled for inactivation of surfaces and sludges are presented in Appendices C and D, respectively. The decontaminated surfaces (such as lab equipment and spent sample containers) and sludges have the potential to be released to the land.

Typically, the decontamination of surfaces is conducted by treatment with heat, UV radiation, or the following chemical disinfectants (presented in order of their relative effectiveness per Wickramanyake):

- Glutaraldehyde;
- Formaldehyde;
- Ethylene oxide; and
- Various alcohols.

Note that treatment with alcohols is generally considered effective against vegetative bacteria, but not against spores.

Inactivation and sterilization data for sludges is almost exclusively based on municipal sludges, and the efficiencies of the technologies used are too low to be considered feasible for genetically engineered waste. Wickramanyake discusses five proposed techniques and their associated efficiencies:

- Incineration;
- Thermal treatment;
- Gamma and electric radiation;
- Microwave radiation; and
- Chemical decontamination.

Wet and/or dry thermal treatment (including autoclaving) was specified as a treatment in several of the past biotechnology PMNs. Wickramanyake and other literature sources state that the standard steam sterilization treatment (for complete inactivation) involves heating in an autoclave for a minimum of 15 minutes at 121°C. This includes aerobic and

anaerobic spore-forming and non-spore-forming microorganisms (Appendix D also presents a set of time-temperature minimum guidelines for complete sterilization of hospital laboratory equipment for comparison).

An example for using data from Appendix C is as follows. If the host microorganism is *S. aureus* and glutaraldehyde is used to inactivate the filter cake, Table 1 of Appendix C shows an inactivation efficiency of 90 to 99.99 percent (dependent upon the treatment time). This efficiency can be used in the equations of Section 4.3.1 to estimate the land release from disposal of the filter cake.

Table 4-1

Summary of Calculations for Release Estimates

Medium	Calculation	
Air	Total Release (CFU/yr)	= (release from fermentor off-gas) + (fugitive release from large process equipment) + (fugitive release from process tanks and process operations) + (fugitive release from sampling)
	Release from Fermentor Off-Gas (CFU/yr)	= (CFU concentration in exhaust after control technology; CFU/ft^3) × (sparge rate; ft^3/min) × (operating time; min/yr) <OR> = (CFU concentration in exhaust before control technology; CFU/ft^3) × (sparge rate; ft^3/min) × (operating time; min/yr) × (1-control removal efficiency) <OR> = (aerosolization factor) × (total yearly broth volume; ml) × (final CFU concentration in fermentation broth; CFU/ml) × (1-control removal efficiency)
	Fugitive Releases from Large Process Unit Operations Equipment (CFU/yr)	= (aerosolization factor) × (total yearly broth volume; ml) × (final CFU concentration in fermentation broth; CFU/ml) × (1-inactivation efficiency)
	Fugitive Release from Process Tanks, Other Process Operations, and Sampling	= negligible
Water	Total Release (CFU/yr)	= (release from inactivated fermentor broth) + (release from cleaning wastewater) + (release from fermentor off-gas treatment wastewater) + (release from spent samples)
	Release from Inactivated	= (CFU concentration in fermentation broth;

Medium	Calculation		
	Fermentor Broth (CFU/yr)		CFU/ml) × (volume of broth; ml) × (1-inactivation efficiency)
Water	Release from Cleaning Wastewater (CFU/yr)	=	(CFU concentration in fermentation broth; CFU/ml) × (fermentor volumetric capacity; ml) × (batches/yr) × (0.01; residual per batch) × (1-inactivation efficiency)
	Release from Fermentor Off-Gas Treatment Wastewater (CFU/yr)	=	(air release from fermentor off-gas; CFU/yr) × (air treatment system removal efficiency) × (1-inactivation efficiency)
	Release from Spent Samples	=	negligible
Land	Total Release (CFU/yr)	=	(release from biomass and sludge collected in filter cakes and separation processes) + (releases from spent filter pads, used sampling equipment, and spent samples)
	Release from Filter Cakes and Sludge from Separation Processes	=	(water releases from inactivated fermentation broth; CFU/yr) × (removal efficiency) × (1-inactivation efficiency)
	Releases from Filter Pads, Sampling Equipment, and Spent Samples	=	negligible

Table 4-2

Summary of Default Assumptions for Release Calculations[a]

General Assumptions	Default Value	Basis
	Average CFU concentration in fermentation broth = 2.05×10^{11} CFU/ml	Submitter data from past biotechnology PMNs
	Average production volume = 7.4×10^{20} CFU/yr	Submitter data from past biotechnology PMNs
	Average fermentor size = 57,000 L	Submitter data from past biotechnology PMNs
	Average batches/year = 100	Submitter data from past biotechnology PMNs
	Sporulation proficiency = 1 spore/1×10^{7} CFU	Past biotechnology PMNs and literature (McIntyre, 1993)
	Typical volumetric air flow rate through fermentor = 1 volume air per fermentor volume	Past biotechnology PMNs and industry experience
Medium	**Default Assumption**	**Basis**
Air	Scrubber removal efficiency = 99%	CEB standard assumption (used in past biotechnology PMNs)
	Ozone treatment efficiency = 99%	Ozone kill efficiency of various organisms (past biotechnology PMNs)
	Removal efficiency of cyclone separators = 99%	CEB standard assumption (used in past biotechnology PMNs)
	Removal efficiency of mist eliminators = 99%	CEB standard assumption (used in past biotechnology PMNs)
	HEPA filter removal efficiency = 99.97%	Literature (Radian, 1986)
	Volumetric flow-rate through fermentor = one volume gas per fermentor volume per minute	Submitter data in past biotechnology PMNs and industry experience

Medium	Default Assumption	Basis
	Aerosolization factor = 1×10^{9} (aerosol CFUs)/(CFU/ml in fermentation broth)	Literature (McIntyre, 1993)
Water and Land	Scrubber removal efficiency = 99%	CEB standard assumption (used in past biotechnology PMNs)
	Removal efficiency of mist eliminators = 99%	CEB standard assumption (used in past biotechnology PMNs)
	Removal efficiency of cyclone separators = 99%	CEB standard assumption (used in past biotechnology PMNs)
	Removal efficiency of rotary drum filtration = 99%	CEB standard assumption (used in past biotechnology PMNs)
	Removal efficiency of plate and frame biological filters with cellulose pads = 100% for retention of particles >0.1 microns	Vendor specifications from past biotechnology PMNs
	Inactivation efficiency of caustic scrubber = 99% (in addition to 99% removal efficiency)	CEB standard assumption (used in past biotechnology PMNs)
	Sterilization efficiency of autoclaving (15 minutes at 121°C) = 99.9999%	Literature (Batelle, 1988)
	Removal efficiency of depth filters = 90%	Literature (McIntyre, 1993)
	Removal efficiency of surface filters = 99%	Literature (McIntyre, 1993)
	Inactivation efficiency of high speed, ball mill or homogenizers = 85-90%	Literature (Radian, 1986)

[a]Note that specific inactivation efficiencies for a variety of wild-type bacteria, fungi, and viruses are presented in Appendices A through D. The PMN assessor should consider the use of these efficiencies before using the default value presented in this table, if applicable.

5.0 DATA GAPS AND FUTURE WORK

The development of this generic scenario was primarily based on a review of materials collected from the literature search and past biotechnology PMNs. The resources available did not permit investigation of all potential information sources. Therefore, some data gaps exist. Further research may yield information and data that may be used to improve the methodology presented here. Specific areas for future work have been identified in Sections 5.1 through 5.3.

5.1 Inhalation Exposure

Sections 3.2 and 3.3 present methodology to estimate occupational inhalation exposures. However, to accurately quantify inhalation exposures with these methods, the CFU air concentration in various areas of the fermentation process combined with worker activity profiles is required. Very little testing has been conducted to determine these concentrations, or to derive correlations to estimate these quantities. This generic scenario bases estimates on results of a NIOSH study of three fermentation facilities. Further research may result in more data and improved methodology. Several studies have been identified that may contain information to approximate typical concentrations. The following excerpt from McIntyre, 1993 summarizes these studies and the information they contain:

> Ashcroft and Pomeroy (1983) simulated accidental releases of *Bacillus subtilis var niger* from bioreactors to determine the potential for aerosol formation. Failures of the reactor headspace air filter, pipework, supply of anti-foam, and the reactor walls were all examined. The latter two types of failure created the greatest potential hazard, and empirical factors [for the aerosolization factor] of 3×10^{-5} to 3×10^{-4} were calculated (Ashcroft and Pomeroy, 1983). Earlier, Dimmick et al. (1973) had developed the concept of empirical factors to model the aerosolization of bacteria in common laboratory procedures such as, cell homogenization and centrifugation. Their estimates of empirical factors of 1×10^{-5} to 1×10^{-4} are in close agreement with the estimates of Ashcroft and Pomeroy (1983), and justifies the use of spray factors to estimate aerosol emission from bioprocess equipment.

The formation of biological aerosols in bioreactors is affected by several factors, including aeration rate, agitation rate, broth viscosity, cell density, and defoamer concentration (Pilacinski et al., 1990; Pan et al., 1991; Szewczyk et al., 1992). As shown elsewhere (Ashcroft and Pomeroy, 1983), these bioreactor aerosols can be contained by bacteriological air filters venting the reactor headspace, provided they do not become wet. Even the failure of bacteriological air filters is not necessarily an irreversible source of emissions, as it is known that pinholes 50-100 μm in diameter, can be sealed by spores (of *Bacillus subtilis*) in 0.3-135 minutes (Leaver, 1990).

Sampling of aerosol emissions in biotechnology facilities has been undertaken in very few instances. The most detailed study is that of Martinez et al. (1988), who conducted a detailed characterization of micro-organisms (CFU counts) present in aerosols within different areas of three full-scale fermentation-based enzyme manufacturing plants. In all three cases only traditional, non-genetically modified micro-organisms were employed (*Bacillus subtilis* or *licheniformis* strains) for the production of proteolytic or carbohydrate enzymes. The authors consistently found the highest concentrations of micro-organisms around process equipment, with significant variations between the three plants. Clean rooms, incubation rooms and analytical laboratories had lower microbial concentrations, without significant variation between plants. Factors which could affect the airborne microbial concentration in a plant included operator work practices, and the robustness of the organism used in the process. They concluded that bioaerosol concentration correlated well with the apparent quality and effectiveness of engineering controls in place, and highlighted specific emission sources of greatest concern.

Palchak et al. (1990) investigated airborne endotoxin levels associated with industrial-scale production using *E.coli*. They measured endotoxin levels around various unit operations, including fermentation, centrifugation, and homogenization. Although endotoxin levels are related to the presence of micro-organisms, they are not directly related to the presence of viable organisms. As a result, this work, provides some guidance as to relative releases near unit operations, but not specific release levels.

Smalla et al. (1991), as part of an extensive risk assessment case study, reported the measurement and characterization of microbial emissions from an industrial pilot plant (30 m^3 fermentor) producing alpha amylase enzyme using a recombinant DNA *Bacillus subtilis* strain. They focused on unintended releases via air, separated biomass solids and wastewater routes. The data reported was for the plant in general, not as it related to specific unit

operations. They concluded from their work that only a limited amount of recombinant micro-organisms were released unintentionally by the enzyme production plant.

The Alberta Research Council (1992), as reported in Kershavarz and Mactaggart (1992), undertook aerosol sampling throughout the facility, both prior to processing and during processing. They demonstrated significant elevation of CFU counts during processing.

This study related CFU counts to specific unit operations as a function of proximity. Operation of the tubular bowl centrifuge, the spray dryer, and the stacked disk centrifuge (which had no filter on the air vent), were found to be associated with relatively high CFU counts.

Another recent study relating to aerosol bioemissions has been completed by Dr. Wilike's group (at the University of Cincinnati) for the EPA, however, this material has not yet been publicly released.

An analysis of airborne yeast and penicillin in the external vicinity of a production plant was presented by Preller et al. (1989). The results showed a decline in the number of colony forming units of the production yeast, *Saccharomyces cerevisiae*, downwind of the plant, while no CFU of this production organism were detected upwind of the plant. The results were, however, not correlated to operating conditions of the plant.
Some additional work has also been undertaken regarding mathematical models of released organisms. Relevant articles include Lincoln (1985), Winkler (1988), and Ferrailol et al (1990).

Unfortunately, none of the data available in the literature has been correlated with the specific engineering conditions associated with active unit operations. In most cases, emissions have been simply quantified as absolute values of colony forming units. While gross conditions have been presented or are understood, such as the type of equipment in proximity and its operational state, the specific engineering data (e.g., flow rates, broth cell concentrations, rotational velocities, numbers of filter plates, centrifuge discharge sequences, etc.) that would allow correlation of emissions to process conditions are not precisely known.

5.2 Inactivation and Control Efficiencies

The literature search and associated review revealed data pertaining to the efficiencies of many inactivation techniques and control technologies. However, specific efficiencies for some of the techniques were not readily available. Further research has the potential to provide empirical data for these procedures.

The efficiency of inactivation procedures is highly variable between microorganisms. Therefore, it is desirable to base general efficiency statements on as much data as possible. One potential source of inactivation studies that was not investigated for this review is the American Water Works Association (AWWA). AWWA may have conducted recent studies that could provide additional information on inactivation procedures and control technologies.

In addition, some of the default values for control technology efficiencies presented in Table 4-2 are based on historical CEB estimates for a worst-case, "what-if" scenario. These estimates are not based on empirical data. Further research is needed to more accurately quantify these estimates.

5.3 Environment Canada

Environment Canada has developed a preliminary computer algorithm to estimate theoretical occupational exposures due to the fermentation of GEMs and the corresponding environmental releases (McIntyre, 1993). The basis for correlations used in the algorithm are similar to those that are presented in this generic scenario.

The Environment Canada methods allow for more precise estimates in general, but require considerably more site-specific knowledge of the facility. This includes the exact number and type of valves, pumps, flanges, filters, and other process equipment. Additionally, the modeling approach has not been verified; however, Environment Canada is currently considering conducting a detailed verification study at a Canadian fermentation facility. A more detailed review of the Environment Canada model and/or a combined effort to verify the modeling

approaches may result in the development of a computerized algorithm to estimate releases and occupational exposures.

6.0 BIBLIOGRAPHY

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